Electronic Patent Application Fee Transmittal

Application Number:	13071105
Filing Date:	24-Mar-2011
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Filer:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00866

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Claims in excess of 20	1202	20	60	1200
Independent claims in excess of 3	1201	1	250	250
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1450**

Electronic Acknowledgement Receipt

EFS ID:	11509270
Application Number:	13071105
International Application Number:	
Confirmation Number:	3361
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Customer Number:	11332
Filer:	Sarah Anne Kagan.
Filer Authorized By:	
Attorney Docket Number:	001107.00866
Receipt Date:	30-NOV-2011
Filing Date:	24-MAR-2011
Time Stamp:	14:01:44
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1450
RAM confirmation Number	3
Deposit Account	190733
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Preliminary Amendment	001107prelim00866amd.pdf	112622 2d05049633ecde45742e71819abd860f17e bd9f5	no	17
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	31434 c6b0ee1754b1d8e29ac3e2d1306b90b2ce2 fa060	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	144056		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
13/071,105	03/24/2011	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY ☐ RATE ($)	SMALL ENTITY ☐ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	11/30/2011	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 68	Minus	** 48	= 20	X $ =		OR	X $60=	1200
	Independent (37 CFR 1.16(h))	* 6	Minus	***5	= 1	X $ =		OR	X $250=	250
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	1450

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/ELMIRA HALL/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	PATENT NUMBER	GROUP ART UNIT	FILE WRAPPER LOCATION
13/071,105		1637	

OC000000030585019

Correspondence Address/Fee Address Change

The following fields have been set to Customer Number 11332 on 10/24/2011

- **Correspondence Address**

The address of record for Customer Number 11332 is:

11332
Banner & Witcoff, Ltd.
Attorneys for client 001107
1100 13th Street N.W.
Suite 1200
Washington, DC 20005-4051

PART 1 - ATTORNEY/APPLICANT COPY
page 1 of 1

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
13/071,105	03/24/2011	Bert VOGELSTEIN	001107.00866

CONFIRMATION NO. 3361

22907
BANNER & WITCOFF, LTD.
1100 13th STREET, N.W.
SUITE 1200
WASHINGTON, DC 20005-4051

PUBLICATION NOTICE

Title: Digital Amplification

Publication No.US-2011-0201004-A1
Publication Date:08/18/2011

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Office of Public Records. The Office of Public Records can be reached by telephone at (703) 308-9726 or (800) 972-6382, by facsimile at (703) 305-8759, by mail addressed to the United States Patent and Trademark Office, Office of Public Records, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently http://pair.uspto.gov/. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
13/071,105	03/24/2011	1634	2986	001107.00866	48	5

CONFIRMATION NO. 3361

UPDATED FILING RECEIPT

22907
BANNER & WITCOFF, LTD.
1100 13th STREET, N.W.
SUITE 1200
WASHINGTON, DC 20005-4051

Date Mailed: 07/15/2011

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

Bert VOGELSTEIN, Baltimore, MD;
Kenneth W. KINZLER, Baltimore, MD;

Assignment For Published Patent Application

THE JOHNS HOPKINS UNIVERSITY, Baltimore, MD

Power of Attorney:

Dale Hoscheit--19090
Joseph Skerpon--29864
William Fisher--32133
Sarah Kagan--32141
Lisa Hemmendinger--42653

Domestic Priority data as claimed by applicant

This application is a CON of 12/617,368 11/12/2009 PAT 7,915,015
which is a CON of 11/709,742 02/23/2007 PAT 7,824,889
which is a CON of 10/828,295 04/21/2004 ABN
which is a DIV of 09/981,356 10/12/2001 PAT 6,753,147
which is a CON of 09/613,826 07/11/2000 PAT 6,440,706
which claims benefit of 60/146,792 08/02/1999

Foreign Applications (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.)

If Required, Foreign Filing License Granted: 04/19/2011

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 13/071,105**

Projected Publication Date: 08/18/2011

Non-Publication Request: No

Early Publication Request: No
Title

Digital Amplification

Preliminary Class

435

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 13/071,105

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	330
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	540
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	220
TOTAL CLAIMS (37 CFR 1.16(i))	48 minus 20=	* 28			OR	x 52 =	1456
INDEPENDENT CLAIMS (37 CFR 1.16(h))	5 minus 3 =	* 2				x 220 =	440
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $270 ($135 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	2986

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)	OR	RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

UTILITY PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Group Art Unit: TBA
	)	
Bert VOGELSTEIN et al.	)	Docket No. 001107.00866
	)	
Serial No. 13/071,105	)	Confirmation No: 3361
	)	
Filed: March 24, 2011	)	Examiner: TBA

For: DIGITAL AMPLIFICATION

RESPONSE TO NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

U.S. Patent and Trademark Office
Customer Service Window
Randolph Building, Mail Stop: Missing Parts
401 Dulany Street
Alexandria, VA 22314

Dear Sir:

In response to the Notice to File Missing Parts of Non-provisional Application under 37 C.F.R. §1.53(b), dated May 9, 2011, applicant submits the fees due. The fees are calculated as follows:

2 Independent claims over 3	$440.00
28 total claims over 20	$1456.00
Total Fees	**$1896.00**

We believe that all Patent and Trademark Office requirements have now been fully met and it we respectfully request that the above-identified patent application be forwarded for examination.

Please charge the filing of this paper and any additional fee, which may be associated to our Deposit Account No. 19-0733.

Respectfully submitted,

By: /Sarah A. Kagan/
Sarah A. Kagan
Registration. No. 32,141

Banner & Witcoff, Ltd.
1100 13th Street, N.W., Suite 1200
Washington, D.C. 20005-4051
(202) 824-3000

Dated: July 8, 2011

Electronic Patent Application Fee Transmittal

Application Number:	13071105
Filing Date:	24-Mar-2011
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Filer:	Sarah Anne Kagan./Daphne Cashion
Attorney Docket Number:	001107.00866

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Claims in excess of 20	1202	28	52	1456
Independent claims in excess of 3	1201	2	220	440
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1896**

Electronic Acknowledgement Receipt

EFS ID:	10480321
Application Number:	13071105
International Application Number:	
Confirmation Number:	3361
Title of Invention:	Digital Amplification
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Customer Number:	22907
Filer:	Sarah Anne Kagan./Daphne Cashion
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00866
Receipt Date:	08-JUL-2011
Filing Date:	24-MAR-2011
Time Stamp:	15:24:33
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1896
RAM confirmation Number	11335
Deposit Account	190733
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	
Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant Response to Pre-Exam Formalities Notice	001107_00866_Response_to_Notice_to_File_Missing_Parts_07_08_2011.pdf	69225 66f3abdce50127e06f04ee09626a2f03c50303b1	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	31711 187f04b1ad78c83f4eb09261dd87d763eee412d8	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	100936		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
13/071,105	03/24/2011		1090	001107.00866	48	5

CONFIRMATION NO. 3361

22907
BANNER & WITCOFF, LTD.
1100 13th STREET, N.W.
SUITE 1200
WASHINGTON, DC 20005-4051

FILING RECEIPT

OC000000047240067

Date Mailed: 05/09/2011

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

Bert VOGELSTEIN, Baltimore, MD;
Kenneth W. KINZLER, Baltimore, MD;

Assignment For Published Patent Application

THE JOHNS HOPKINS UNIVERSITY, Baltimore, MD

Power of Attorney:
Dale Hoscheit--19090
Joseph Skerpon--29864
William Fisher--32133
Sarah Kagan--32141
Lisa Hemmendinger--42653

Domestic Priority data as claimed by applicant

This application is a CON of 12/617,368 11/12/2009 PAT 7,915,015
which is a CON of 11/709,742 02/23/2007 PAT 7,824,889
which is a CON of 10/828,295 04/21/2004 ABN
which is a DIV of 09/981,356 10/12/2001 PAT 6,753,147
which is a CON of 09/613,826 07/11/2000 PAT 6,440,706
which claims benefit of 60/146,792 08/02/1999

Foreign Applications (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.)

If Required, Foreign Filing License Granted: 04/19/2011

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 13/071,105**

Projected Publication Date: 08/18/2011

Non-Publication Request: No

Early Publication Request: No
Title

Digital Amplification

Preliminary Class

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An International (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
13/071,105	03/24/2011	Bert VOGELSTEIN	001107.00866

CONFIRMATION NO. 3361

22907
BANNER & WITCOFF, LTD.
1100 13th STREET, N.W.
SUITE 1200
WASHINGTON, DC 20005-4051

FORMALITIES LETTER

OC000000047240068

Date Mailed: 05/09/2011

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing.

Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- Additional claim fees of **$1896** as a non-small entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.

SUMMARY OF FEES DUE:

Total fee(s) required within **TWO MONTHS** from the date of this Notice is **$1896** for a non-small entity

- Total additional claim fee(s) for this application is **$1896**
  - **$440** for **2** independent claims over 3.
  - **$1456** for **28** total claims over 20.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

/mhteklu/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 13/071,105

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	330
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	540
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	220
TOTAL CLAIMS (37 CFR 1.16(i))	48 minus 20=	* 28			OR	x 52 =	1456
INDEPENDENT CLAIMS (37 CFR 1.16(h))	5 minus 3 =	* 2				x 220 =	440
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $270 ($135 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	2986

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)	OR	RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

RAW SEQUENCE LISTING

Loaded by SCORE, no errors detected.

Application Serial Number: 13071105
Source: OPAP
Date Processed by SCORE: 04/08/11

ENTERED

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Kinzler, Kenneth W.

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PTO/SB/05 (08-08)
Approved for use through 09/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office. U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	001107.00866
First Inventor	Bert VOGELSTEIN et al.
Title	DIGITAL AMPLIFICATION
Express Mail Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☐ **Fee Transmittal Form** (e.g., PTO/SB/17)
2. ☐ **Applicant claims small entity status.** See 37 CFR 1.27.
3. ☑ **Specification** [*Total Pages* 30]
   Both the claims and abstract must start on a new page
   (For information on the preferred arrangement, see MPEP 608.01(a))
4. ☑ **Drawing(s)** (*35 U.S.C. 113*) [*Total Sheets* 7]
5. **Oath or Declaration** [*Total Sheets* 2]
   a. ☐ Newly executed (original or copy)
   b. ☑ A copy from a prior application (37 CFR 1.63(d))
   (for continuation/divisional with Box 18 completed)
   i. ☐ DELETION OF INVENTOR(S)
   Signed statement attached deleting inventor(s) name in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. ☑ **Application Data Sheet.** See 37 CFR 1.76
7. ☐ **CD-ROM or CD-R** in duplicate, large table or Computer Program *(Appendix)*
   ☐ Landscape Table on CD
8. **Nucleotide and/or Amino Acid Sequence Submission**
   (if applicable, items a. – c. are required)
   a. ☐ Computer Readable Form (CRF)
   b. Specification Sequence Listing on:
      i. ☐ CD-ROM or CD-R (2 copies); or
      ii. ☑ Paper
   c. ☑ Statements verifying identity of above copies

ADDRESS TO:

Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

ACCOMPANYING APPLICATION PARTS

9. ☐ **Assignment Papers** (cover sheet & document(s))
   Name of Assignee The Johns Hopkins University
10. ☐ **37 CFR 3.73(b) Statement** *(when there is an assignee)* ☐ **Power of Attorney**
11. ☐ **English Translation Document** *(if applicable)*
12. ☑ **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    ☐ Copies of citations attached
13. ☐ **Preliminary Amendment**
14. ☐ **Return Receipt Postcard** (MPEP 503)
    (Should be specifically itemized)
15. ☐ **Certified Copy of Priority Document(s)**
    (if foreign priority is claimed)
16. ☐ **Nonpublication Request** under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
17. ☑ Other: Please use the CRF from parent 09/981,356 filed on Nov. 14, 2003. Contents are identical.

18. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below and in the first sentence of the specification following the title, or in an Application Data Sheet under 37 CFR 1.76:*

☑ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.: 12/617,368

Prior application information: *Examiner* Samuel C. WOOLWINE *Art Unit:* 1637

19. CORRESPONDENCE ADDRESS

☑ The address associated with Customer Number: 22907 ***OR*** ☐ Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone		Email	

Signature	/Sarah A. Kagan/	Date	March 24, 2011
Name (Print/Type)	Sarah A. Kagan	Registration No. (Attorney/Agent)	32,141

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PTO/SB/14 (11-08)
Approved for use through 09/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	001107.00866
	Application Number	
Title of Invention	Digital Amplification	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Applicant Information:

Applicant 1 Remove

Applicant Authority ◉ Inventor ○ Legal Representative under 35 U.S.C. 117 ○ Party of Interest under 35 U.S.C. 118

Prefix	Given Name	Middle Name	Family Name	Suffix
	Bert		VOGELSTEIN	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Baltimore	State/Province	MD	Country of Residence i	US

Citizenship under 37 CFR 1.41(b) i US

Mailing Address of Applicant:

Address 1	3700 Breton Way		
Address 2			
City	Baltimore	State/Province	MD
Postal Code	21208	Country i	US

Applicant 2 Remove

Applicant Authority ◉ Inventor ○ Legal Representative under 35 U.S.C. 117 ○ Party of Interest under 35 U.S.C. 118

Prefix	Given Name	Middle Name	Family Name	Suffix
	Kenneth	W.	KINZLER	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Baltimore	State/Province	MD	Country of Residence i	US

Citizenship under 37 CFR 1.41(b) i US

Mailing Address of Applicant:

Address 1	1403 Halkirk Way		
Address 2			
City	Baltimore	State/Province	MD
Postal Code	21015	Country i	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence information of this application.**

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	001107.00866
	Application Number	
Title of Invention	Digital Amplification	

Customer Number	22907		
Email Address		Add Email	Remove Email

Application Information:

Title of the Invention	Digital Amplification		
Attorney Docket Number	001107.00866	Small Entity Status Claimed	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Suggested Class (if any)		Sub Class (if any)	
Suggested Technology Center (if any)			
Total Number of Drawing Sheets (if any)	7	Suggested Figure for Publication (if any)	1A

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Enter either Customer Number or complete the Representative Name section below. If both sections are completed the Customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	22907		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78(a)(2) or CFR 1.78(a)(4), and need not otherwise be made part of the specification.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
	Continuation of	12617368	2009-11-12
Prior Application Status	Patented		Remove

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	001107.00866
	Application Number	
Title of Invention	Digital Amplification	

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
12617368	Continuation of	11709742	2007-02-23	7824889	2010-11-02

Prior Application Status	Abandoned	Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
11709742	Continuation of	10828295	2004-04-21

Prior Application Status	Patented	Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
10828295	Division of	09981356	2001-10-12	6753147	2004-06-22

Prior Application Status	Patented	Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
09981356	Continuation of	09613826	2000-07-11	6440706	2002-08-27

Prior Application Status	Expired	Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
09613826	non provisional of	60146792	1999-08-02

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. Add

Foreign Priority Information:

This section allows for the applicant to claim benefit of foreign priority and to identify any prior foreign application for which priority is not claimed. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(a).

Remove

Application Number	Country [i]	Parent Filing Date (YYYY-MM-DD)	Priority Claimed
			◉ Yes ○ No

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. Add

Assignee Information:

Providing this information in the application data sheet does not substitute for compliance with any requirement of part 3 of Title 37 of the CFR to have an assignment recorded in the Office.

Assignee 1 Remove

If the Assignee is an Organization check here. ☒

Organization Name	The Johns Hopkins University

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	001107.00866
	Application Number	
Title of Invention	Digital Amplification	

Mailing Address Information:			
Address 1	3400 N. Charles Street		
Address 2			
City	Baltimore	**State/Province**	MD
Country [i]		Postal Code	21218
Phone Number		Fax Number	
Email Address			
Additional Assignee Data may be generated within this form by selecting the **Add** button.		Add	

Signature:

A signature of the applicant or representative is required in accordance with 37 CFR 1.33 and 10.18. Please see 37 CFR 1.4(d) for the form of the signature.

Signature	/Sarah A. Kagan/			Date (YYYY-MM-DD)	2011-03-24
First Name	Sarah A.	Last Name	Kagan	Registration Number	32141

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

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JOINT DECLARATION FOR PATENT APPLICATION

As the below named inventor, we hereby declare that:

Our residence, post office address and citizenship are as stated below next to our names;

We believe we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled DIGITAL AMPLIFICATION, the specification of which

- ☐ is attached hereto.
- ■ was filed on July 11, 2000 as Application Serial Number 09/613,826 and was amended on (if applicable).
- ☐ was filed under the Patent Cooperation Treaty (PCT) and accorded International Application No. _____, filed _____, and amended on _____ (if any).

We hereby state that we have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

We hereby acknowledge the duty to disclose information which is material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

Prior Foreign Application(s)

We hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application(s) for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Country	Application No.	Date of Filing (day month year)	Date of Issue (day month year)	Priority Claimed Under 35 U.S.C. §119

Prior United States Provisional Application(s)

We hereby claim priority benefits under Title 35, United States Code, §119(e)(1) of any U.S. provisional application listed below:

U.S. Provisional Application No.	Date of Filing (day month year)	Priority Claimed Under 35 U.S.C. §119(e)(1)
60/146,792	02 August 1999	Yes

Prior United States Application(s)

We hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Date of Filing (Day, Month, Year)	Status — Patented, Pending, Abandoned

Power of Attorney

And we hereby appoint, both jointly and severally, as our attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith the following attorneys and agents, their registration numbers being listed after their names:

ALTHERR, Robert F.	31,810	HOSCHEIT, Dale H.	19,090	PATEL, Binal J.	42,065
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BODNER, Jordan	42,338	KLEIN, William J.	43,719	PRATT, Thomas K.	37,210
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COOPERMAN, Marc S.	34,143	McDERMOTT, Peter D.	29,411	SHANAHAN, Michael H.	24,438
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We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature _____ Date 11/28/00

Full Name of First Inventor: Vogelstein (Family Name) Bert (First Given Name) _____ (Second Given Name)

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Prior Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Prior Examiner: Samuel Woolwine
	)	
Continuation Application of Serial No. 12/617,368	)	Confirmation No. TBD
	)	
	)	Atty. Dkt. No. 001107.00866
Filed: Herewith	)	
	)	
For: DIGITAL AMPLIFICATION	)	

RECOGNITION OF PRACTITIONERS OF RECORD UNDER 37 C.F.R. § 1.32(c)(3)

U.S. Patent and Trademark Office
Customer Service Window
Randolph Building
401 Dulany Street
Alexandria, VA 22314

Sir:

Pursuant to 37 C.F.R. § 1.32(c)(3), please recognize the following patent practitioners, originally named in the Power of Attorney from an earlier-filed application, as being of record in the above-identified application:

Name	Registration No.
Sarah A. Kagan	32,141
Dale H. Hoscheit	19,090
Joseph M. Skerpon	29,864
Lisa M. Hemmendinger	42,653
William J. Fisher	32,133

A copy of the Power of Attorney from the earlier-filed application is submitted herewith.

Respectfully submitted,
BANNER & WITCOFF, LTD.

Dated: March 24, 2011

By: */SARAH A. KAGAN/*
Sarah A. Kagan
Registration No. 32,141

Customer No. 22907

DIGITAL AMPLIFICATION

The U.S. government retains certain rights in this invention by virtue of its support of the underlying research, supported by grants CA 43460, CA 57345, and CA 62924 from the National Institutes of Health.

The disclosure of all claimed priority applications is expressly incorporated herein.

TECHNICAL FIELD OF THE INVENTION

This invention is related to diagnostic genetic analyses. In particular it relates to detection of genetic changes and gene expression.

BACKGROUND OF THE INVENTION

In classical genetics, only mutations of the germ-line were considered important for understanding disease. With the realization that somatic mutations are the primary cause of cancer, and may also play a role in aging, new genetic principles have arisen. These discoveries have provided a wealth of new opportunities for patient management as well as for basic research into the pathogenesis of neoplasia. However, many of these opportunities hinge upon detection of a small number of mutant-containing cells among a large excess of normal cells. Examples include the detection of neoplastic cells in urine, stool, and sputum of patients with cancers of the bladder, colorectum, and lung, respectively. Such detection has been shown in some cases to be possible at a stage when the primary tumors are still curable and the patients asymptomatic. Mutant sequences from the DNA of neoplastic cells have also been found in the blood of cancer patients. The detection of residual disease in lymph nodes or surgical margins may be useful in predicting which patients

might benefit most from further therapy. From a basic research standpoint, analysis of the early effects of carcinogens is often dependent on the ability to detect small populations of mutant cells.

Because of the importance of this issue in so many settings, many useful techniques have been developed for the detection of mutations. DNA sequencing is the gold standard for the detection of germ line mutations, but is useful only when the fraction of mutated alleles is greater than ~20%. Mutant-specific oligonucleotides can sometimes be used to detect mutations present in a minor proportion of the cells analyzed, but the signal to noise ratio distinguishing mutant and wild-type (WT) templates is variable. The use of mutant-specific primers or the digestion of polymerase chain reaction (PCR) products with specific restriction endonucleases are extremely sensitive methods for detecting such mutations, but it is difficult to quantitate the fraction of mutant molecules in the starting population with these techniques. Other innovative approaches for the detection of somatic mutations have been reviewed. A general problem with these methods is that it is difficult or impossible to independently confirm the existence of any mutations that are identified.

Thus there is a need in the art for methods for accurately and quantitatively detecting genetic sequences in mixed populations of sequences.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods for determining the presence of a selected genetic sequence in a population of genetic sequences.

It is another object of the present invention to provide molecular beacon probes useful in the method of the invention.

These and other objects of the invention are achieved by providing a method for determining the presence of a selected genetic sequence in a

population of genetic sequences. A biological sample comprising nucleic acid template molecules is diluted to form a set of assay samples. The template molecules within the assay samples are amplified to form a population of amplified molecules in the assay samples of the set. The amplified molecules in the assay samples of the set are then analyzed to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence. The first number is then compared to the second number to ascertain a ratio which reflects the composition of the biological sample.

Another embodiment of the invention is a method for determining the ratio of a selected genetic sequence in a population of genetic sequences. Template molecules within a set comprising a plurality of assay samples are amplified to form a population of amplified molecules in each of the assay samples of the set. The amplified molecules in the assay samples of the set are analyzed to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence. At least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence. The first number is compared to the second number to ascertain a ratio which reflects the composition of the biological sample.

According to another embodiment of the invention, a molecular beacon probe is provided. It comprises an oligonucleotide with a stem-loop structure having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end. The loop consists of 16 base pairs which has a T_m of 50-51 C. The stem consists of 4 base pairs having a sequence 5'-CACG-3'.

A second type of molecular beacon probe is provided in another embodiment. It comprises an oligonucleotide with a stem-loop structure having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end. The loop consists of 19-20 base pairs and has a T_m of 54-56 C. The stem consists of 4 base pairs having a sequence 5'-CACG-3'.

Another embodiment provides the two types of molecular beacon probes, either mixed together or provided in a divided container as a kit.

The invention thus provides the art with the means to obtain quantitative assessments of particular DNA or RNA sequences in mixed populations of sequences using digital (binary) signals.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A, 1B, 1C. Schematic of experimental design. (Fig. 1A) The basic two steps involved: PCR on diluted DNA samples is followed by addition of fluorescent probes which discriminate between WT and mutant alleles and subsequent fluorometry. (Fig. 1B) Principle of molecular beacon analysis. In the stem-loop configuration, fluorescence from a dye at the 5' end of the oligonucleotide probe is quenched by a Dabcyl group at the 3' end. Upon hybridization to a template, the dye is separated from the quencher, resulting in increased fluorescence. Modified from Marras *et al.* (Fig. 1C) Oligonucleotide design. Primers F1 and R1 are used to amplify the genomic region of interest. Primer INT is used to produce single stranded DNA from the original PCR products during a subsequent asymmetric PCR step (see Materials and Methods). MB-RED is a Molecular Beacon which detects any appropriate PCR product, whether it is WT or mutant at the queried codons. MB-GREEN is a Molecular Beacon which preferentially detects the WT PCR product.

FIG. 2. Discrimination between WT and mutant PCR products by Molecular Beacons. Ten separate PCR products, each generated from ~25 genome equivalents of genomic DNA of cells containing the indicated mutations of *c-Ki-Ras*, were analyzed with the Molecular Beacon probes described in the text. Representative examples of the PCR products used for Molecular Beacon analysis were purified and directly sequenced. In the cases with Gly12Cys (SEQ ID NO: 11) and Gly12Arg (SEQ ID NO: 10) mutations, contaminating non-neoplastic cells within the tumor presumably accounted for the relatively low ratios. In the cases with Gly12Ser (SEQ ID NO: 8) and Gly12Asp (SEQ ID NO: 12), there were apparently two or more alleles of mutant *c-Ki-Ras* for every WT allele (SEQ ID NO: 7); both these tumors were aneuploid. Analysis of the Gly13Asp mutation is also shown (SEQ ID NO: 9).

FIG. 3. Detecting Dig-PCR products with MB-RED. Specific Fluorescence Units of representative wells from an experiment employing colorectal cancer cells with Gly12Asp or Gly13Asp mutations of the *c-Ki-Ras* gene. Wells with values >10,000 are shaded yellow. Polyacrylamide gel electrophoretic analyses of the PCR products from selected wells are shown. Wells with fluorescence values <3500 had no PCR product of the correct size while wells with fluorescence values >10,000 SFU always contained PCR products of 129 bp. Non-specific products generated during the large number of cycles required for Dig-PCR did not affect the fluorescence analysis. M1 and M2 are molecular weight markers used to determine the size of fragments indicated on the left (in base pairs).

FIG. 4. Discriminating WT from mutant PCR products obtained in Dig-PCR. RED/GREEN ratios were determined from the fluorescence of MB-RED and MB-GREEN as described in Materials and Methods. The wells shown are the same as those illustrated in Fig. 3. The sequences of PCR products from the

indicated wells were determined as described in Materials and Methods. The wells with RED/GREEN ratios >3.0 each contained mutant sequences while those with RED/GREEN ratios of ~1.0 contained WT sequences. WT c-Ki-Ras (SEQ ID NO: 7), Gly12Asp (SEQ ID NO: 13), and Gly13Asp (SEQ ID NO: 9) were analyzed.

FIG. 5. Dig-PCR of DNA from a stool sample. The 384 wells used in the experiment are displayed. Those colored blue contained 25 genome equivalents of DNA from normal cells. Each of these registered positive with MB-RED and the RED/GREEN ratios were 1.0 +/- 0.1 (mean +/- 1 standard deviation). The wells colored yellow contained no template DNA and each was negative with MB-RED (i.e., fluorescence <3500 fluorescence units.). The other wells contained diluted DNA from the stool sample. Those registering as positive with MB-RED were colored either red or green, depending on their RED/GREEN ratios. Those registering negative with MB-RED were colored white. PCR products from the indicated wells were used for automated sequence analysis. The sequence of WT *c-Ki-Ras* in well (SEQ ID NO: 7), and mutant *c-Ki-Ras* in wells C10, E11, M10, and L12 (SEQ ID NO: 14), and well F21 (SEQ ID NO: 15) were analyzed.

DETAILED DESCRIPTION OF THE INVENTION

The method devised by the present inventors involves separately amplifying small numbers of template molecules so that the resultant products have a proportion of the analyte sequence which is detectable by the detection means chosen. At its limit, single template molecules can be amplified so that the products are completely mutant or completely wild-type (WT). The homogeneity of these amplification products makes them trivial to distinguish through existing techniques.

The method requires analyzing a large number of amplified products simply and reliably. Techniques for such assessments were developed, with the output providing a digital readout of the fraction of mutant alleles in the analyzed population.

The biological sample is diluted to a point at which a practically usable number of the diluted samples contain a proportion of the selected genetic sequence (analyte) relative to total template molecules such that the analyzing technique being used can detect the analyte. A practically usable number of diluted samples will depend on cost of the analysis method. Typically it would be desirable that at least 1/50 of the diluted samples have a detectable proportion of analyte. At least 1/10, 1/5, 3/10, 2/5, 1/2, 3/5, 7/10, 4/5, or 9/10 of the diluted samples may have a detectable proportion of analyte. The higher the fraction of samples which will provide useful information, the more economical will be the overall assay. Over-dilution will also lead to a loss of economy, as many samples will be analyzed and provide no signal. A particularly preferred degree of dilution is to a point where each of the assay samples has on average one-half of a template. The dilution can be performed from more concentrated samples. Alternatively, dilute sources of template nucleic acids can be used. All of the samples may contain amplifiable template molecules. Desirably each assay sample prior to amplification will contain less than a hundred or less than ten template molecules.

Digital amplification can be used to detect mutations present at relatively low levels in the samples to be analyzed. The limit of detection is defined by the number of wells that can be analyzed and the intrinsic mutation rate of the polymerase used for amplification. 384 well PCR plates are commercially available and 1536 well plates are on the horizon, theoretically allowing sensitivities for mutation detection at the ~0.1% level. It is also possible that Digital Amplification can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude. This

sensitivity may ultimately be limited by polymerase errors. The effective error rate in PCR as performed under our conditions was 1.1%, i.e., four out of 351 PCR products derived from WT DNA sequence appeared to contain a mutation by RED/GREEN ratio criteria. However, any individual mutation (such as a G to T transversion at the second position of codon 12 of *c-Ki-Ras*), are expected to occur in < 1 in 50 of these polymerase-generated mutants (there are at least 50 base substitutions within or surrounding codons 12 and 13 that should yield high RED/GREEN ratios). Determining the sequence of the putative mutants in the positive wells, by direct sequencing as performed here or by any of the other techniques, provides unequivocal validation of a prospective mutation: a significant fraction of the mutations found in individual wells should be identical if the mutation occurred *in vivo*. Significance can be established through rigorous statistical analysis, as positive signals should be distributed according to Poisson probabilities. Moreover, the error rate in particular Digital Amplification experiments can be precisely determined through performance of Digital Amplification on DNA templates from normal cells.

Digital Amplification is as easily applied to RT-PCR products generated from RNA templates as it is to genomic DNA. For example, the fraction of alternatively spliced or mutant transcripts from a gene can be easily determined using photoluminescent probes specific for each of the PCR products generated. Similarly, Digital Amplification can be used to quantitate relative levels of gene expression within an RNA population. For this amplification, each well would contain primers which are used to amplify a reference transcript expressed constitutively as well as primers specific for the experimental transcript. One photoluminescent probe would then be used to detect PCR products from the reference transcript and a second photoluminescent probe used for the test transcript. The number of wells in which the test transcript is amplified divided by the number of wells in which

the reference transcript is amplified provides a quantitative measure of gene expression. Another group of examples involves the investigations of allelic status when two mutations are observed upon sequence analysis of a standard DNA sample. To distinguish whether one variant is present in each allele (*vs.* both occurring in one allele), cloning of PCR products is generally performed. The approach described here would simplify the analysis by eliminating the need for cloning. Other potential applications of Digital Amplification are listed in Table 1. When the goal is the quantitation of the proportion of two relatively common alleles or transcripts rather than the detection of rare alleles, techniques such as those employing TaqMan and real time PCR provide an excellent alternative to use of molecular beacons. Advantages of real time PCR methods include their simplicity and the ability to analyze multiple samples simultaneously. However, Digital Amplification may prove useful for these applications when the expected differences are small, (*e.g.*, only ~2-fold, such as occurs with allelic imbalances.)

Application	Example	Probe 1 Detects:	Probe 2 Detects:
Base substitution mutations	Cancer gene mutations in stool, blood, lymph nodes	mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	normal or translocated alleles	translocated allele
Gene amplifications	Determine presence or extent of amplification	sequence within amplicon	sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	minor exons	common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	first transcript	reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	first mutation	second mutation
Allelic Imbalance	Quantitative analysis with non-polymorphic markers	marker sequence	marker from another chromosome

The ultimate utility of Digital Amplification lies in its ability to convert the intrinsically exponential nature of PCR to a linear one. It should thereby prove useful for experiments requiring the investigation of individual alleles, rare variants/mutations, or quantitative analysis of PCR products.

In one preferred embodiment each diluted sample has on average one half a template molecule. This is the same as one half of the diluted samples having one template molecule. This can be empirically determined by amplification. Either the analyte (selected genetic sequence) or the reference genetic sequence can be used for this determination. If the analysis method being used can detect analyte when present at a level of 20%, then one must dilute such that a significant number of diluted assay samples contain more than 20% of analyte. If the analysis method being used requires 100% analyte to detect, then dilution down to the single template molecule level will be required.

To achieve a dilution to approximately a single template molecule level, one can dilute such that between 0.1 and 0.9 of the assay samples yield an amplification product. More preferably the dilution will be to between 0.1 and 0.6, more preferably to between 0.3 and 0.5 of the assay samples yielding an amplification product.

The digital amplification method requires analysis of a large number of samples to get meaningful results. Preferably at least ten diluted assay samples are amplified and analyzed. More preferably at least 15, 20, 25, 30, 40, 50, 75, 100, 500, or 1000 diluted assay samples are amplified and analyzed. As in any method, the accuracy of the determination will improve as the number of samples increases, up to a point. Because a large number of samples must be analyzed, it is desirable to reduce the manipulative steps, especially sample transfer steps. Thus it is preferred that the steps of amplifying and analyzing are performed in the same receptacle. This makes the method an *in situ,* or "one-pot" method.

The number of different situations in which the digital amplification method will find application is large. Some of these are listed in Table 1. As shown in the examples, the method can be used to find a tumor mutation in a population of cells which is not purely tumor cells. As described in the

examples, a probe for a particular mutation need not be used, but diminution in binding to a wild-type probe can be used as an indicator of the presence of one or more mutations. Chromosomal translocations which are characteristic of leukemias or lymphomas can be detected as a measure of the efficacy of therapy. Gene amplifications are characteristic of certain disease states. These can be measured using digital amplification. Alternatively spliced forms of a transcript can be detected and quantitated relative to other forms of the transcript using digital amplification on cDNA made from mRNA. Similarly, using cDNA made from mRNA one can determine relative levels of transcription of two different genes. One can use digital amplification to distinguish between a situation where one allele carries two mutations and one mutation is carried on each of two alleles in an individual. Allelic imbalances often result from a disease state. These can be detected using digital amplification.

Biological samples which can be used as the starting material for the analyses may be from any tissue or body sample from which DNA or mRNA can be isolated. Preferred sources include stool, blood, and lymph nodes. Preferably the biological sample is a cell-free lysate.

Molecular beacon probes according to the present invention can utilize any photoluminescent moiety as a detectable moiety. Typically these are dyes. Often these are fluorescent dyes. Photoluminescence is any process in which a material is excited by radiation such as light, is raised to an excited electronic or vibronic state, and subsequently re-emits that excitation energy as a photon of light. Such processes include fluorescence, which denotes emission accompanying descent from an excited state with paired electrons (a "singlet" state) or unpaired electrons (a "triplet" state) to a lower state with the same multiplicity, *i.e.,* a quantum-mechanically "allowed" transition. Photoluminescence also includes phosphorescence which denotes emission accompanying descent from an excited triplet or singlet state to a lower state of

different multiplicity, *i.e.,* a quantum mechanically "forbidden" transition. Compared to "allowed" transitions, "forbidden" transitions are associated with relatively longer excited state lifetimes.

The quenching of photoluminescence may be analyzed by a variety of methods which vary primarily in terms of signal transduction. Quenching may be transduced as changes in the intensity of photoluminescence or as changes in the ratio of photoluminescence intensities at two different wavelengths, or as changes in photoluminescence lifetimes, or even as changes in the polarization (anisotropy) of photoluminescence. Skilled practitioners will recognize that instrumentation for the measurement of these varied photoluminescent responses are known. The particular ratiometric methods for the analysis of quenching in the instant examples should not be construed as limiting the invention to any particular form of signal transduction. Ratiometric measurements of photoluminescence intensity can include the measurement of changes in intensity, photoluminescence lifetimes, or even polarization (anisotropy).

Although the working examples demonstrate the use of molecular beacon probes as the means of analysis of the amplified dilution samples, other techniques can be used as well. These include sequencing, gel electrophoresis, hybridization with other types of probes, including TaqMan™ (dual-labeled fluorogenic) probes (Perkin Elmer Corp./Applied Biosystems, Foster City, Calif), pyrene-labeled probes, and other biochemical assays.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

Step 1: PCR amplifications. The optimal conditions for PCR described in this section were determined by varying the parameters described in the Results. PCR was performed in 7 ul volumes in 96 well polypropylene PCR plates (RPI). The composition of the reactions was: 67 mM Tris, pH 8.8, 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM TTP, 6% DMSO, 1 uM primer F1, 1 uM primer R1, 0.05 units/ul Platinum Taq polymerase (Life Technologies, Inc.), and "one-half genome equivalent" of DNA. To determine the amount of DNA corresponding to one-half genome equivalent, DNA samples were serially diluted and tested via PCR. The amount that yielded amplification products in half the wells, usually ~1 pg of total DNA, was defined as "one-half genome equivalent" and used in each well of subsequent Digital Amplification experiments. Fifty ul light mineral oil (Sigma M-3516) was added to each well and reactions performed in a HybAid Thermal cycler at the following temperatures: denaturation at 94° for one min; 60 cycles of 94° for 15 sec, 55° for 15 sec., 70° for 15 seconds; 70° for five minutes. Reactions were read immediately or stored at room temperature for up to 36 hours before fluorescence analysis.

EXAMPLE 2

Step 2: Fluorescence analysis. 3.5 ul of a solution with the following composition was added to each well: 67 mM Tris, pH 8.8, 16.6 mM NH_4SO_4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM TTP, 6% DMSO, 5 uM primer INT, 1 uM MB-GREEN, 1 uM MB-RED, 0.1 units/ul Platinum Taq polymerase. The plates were centrifuged for 20 seconds at 6000 g and fluorescence read at excitation/emission wavelengths of 485 nm/530 nm for MB-GREEN and 530 nm/590 nm for MB-RED. This fluorescence in wells without template was typically 10,000 to

20,000 fluorescence "units", with about 75% emanating from the fluorometer background and the remainder from the MB probes. The plates were then placed in a thermal cycler for asymmetric amplification at the following temperatures: 94° for one minute; 10 - 15 cycles of 94° for 15 sec, 55° for 15 sec., 70° for 15 seconds; 60° for five minutes. The plates were then incubated at room temperature for at least 20 minutes and fluorescence measured as described above. The fluorescence readings obtained were stable for several hours. Specific fluorescence was defined as the difference in fluorescence before and after the asymmetric amplification. RED/GREEN ratios were defined as the specific fluorescence of MB-RED divided by that of MB-GREEN. RED/GREEN ratios were normalized to the ratio exhibited by the positive controls (25 genome equivalents of DNA from normal cells, as defined in Materials and Methods). We found that the ability of MB probes to discriminate between WT and mutant sequences under our conditions could not be reliably determined from experiments in which they were tested by hybridization to relatively short complementary single stranded oligonucleotides, and that actual PCR products had to be used for validation.

EXAMPLE 3

Oligonucleotides and DNA sequencing. Primer F1: 5'-CATGTTCTAATATAGTCACATTTTCA-3' (SEQ ID NO: 1); Primer R1: 5'-TCTGAATTAGCTGTATCGTCAAGG-3' (SEQ ID NO: 2); Primer INT: 5'-TAGCTGTATCGTCAAGGCAC-3' (SEQ ID NO: 3); MB-RED: 5'-Cy3-CACGGGCCTGCTGAAAATGACTGCGTG-Dabcyl-3' (SEQ ID NO: 4); MB-GREEN: 5'-Fluorescein-CACGGGAGCTGGTGGCGTAGCGTG-Dabcyl-3'. (SEQ ID NO: 5).

Molecular Beacons were synthesized by Midland Scientific and other oligonucleotides were synthesized by Gene Link. All were dissolved at 50 uM

in TE (10 mM Tris, pH 8.0/ 1 mM EDTA) and kept frozen and in the dark until use. PCR products were purified using QIAquick PCR purification kits (Qiagen). In the relevant experiments described in the text, 20% of the product from single wells was used for gel electrophoresis and 40% was used for each sequencing reaction. The primer used for sequencing was 5'-CATTATTTTATTATAAGGCCTGC-3' (SEQ ID NO: 6). Sequencing was performed using fluorescently-labeled ABI Big Dye terminators and an ABI 377 automated sequencer.

EXAMPLE 4

Principles underlying experiment. The experiment is outlined in Fig. 1A. First, the DNA is diluted into multiwell plates so that there is, on average, one template molecule per two wells, and PCR is performed. Second, the individual wells are analyzed for the presence of PCR products of mutant and WT sequence using fluorescent probes.

As the PCR products resulting from the amplification of single template molecules should be homogeneous in sequence, a variety of standard techniques could be used to assess their presence. Fluorescent probe-based technologies, which can be performed on the PCR products "*in situ*" (i.e., in the same wells) are particularly well-suited for this application. We chose to explore the utility of one such technology, involving Molecular Beacons (MB), for this purpose. MB probes are oligonucleotides with stem-loop structures that contain a fluorescent dye at the 5' end and a quenching agent (Dabcyl) at the 3' end (Fig. 1B). The degree of quenching via fluorescence energy resonance transfer is inversely proportional to the 6^{th} power of the distance between the Dabcyl group and the fluorescent dye. After heating and cooling, MB probes reform a stem-loop structure which quenches the fluorescent signal from the dye. If a PCR product whose sequence is complementary to the loop sequence is present during the heating/cooling cycle, hybridization of the MB

to one strand of the PCR product will increase the distance between the Dabcyl and the dye, resulting in increased fluorescence.

A schematic of the oligonucleotides used for Digital Amplifications shown in Fig. 1C. Two unmodified oligonucleotides are used as primers for the PCR reaction. Two MB probes, each labeled with a different fluorophore, are used to detect the PCR products. MB-GREEN has a loop region that is complementary to the portion of the WT PCR product that is queried for mutations. Mutations within the corresponding sequence of the PCR product should significantly impede the hybridization of it to the MB probe. MB-RED has a loop region that is complementary to a different portion of the PCR product, one not expected to be mutant. It thus should produce a signal whenever a well contains a PCR product, whether that product is WT or mutant in the region queried by MB-GREEN. Both MB probes are used together to simultaneously detect the presence of a PCR product and its mutational status.

Practical Considerations.

Numerous conditions were optimized to define conditions that could be reproducibly and generally applied. As outlined in Fig. 1A, the first step involves amplification from single template molecules. Most protocols for amplification from small numbers of template molecules use a nesting procedure, wherein a product resulting from one set of primers is used as template in a second reaction employing internal primers. As many applications of digital amplification are expected to require hundreds or thousands of separate amplifications, such nesting would be inconvenient and could lead to contamination problems. Hence, conditions were sought that would achieve robust amplification without nesting. The most important of these conditions involved the use of a polymerase that was activated only after heating and optimized concentrations of dNTP's, primers, buffer components,

and temperature. The conditions specified in Examples 1-3 were defined after individually optimizing each of these components and proved suitable for amplification of several different human genomic DNA sequences. Though the time required for PCR was not particularly long (~2.5 hr), the number of cycles used was high and excessive compared to the number of cycles required to amplify the "average" single template molecule. The large cycle number was necessary because the template in some wells might not begin to be amplified until several PCR cycles had been completed. The large number of cycles ensured that every well (not simply the average well) would generate a substantial and roughly equal amount of PCR product if a template molecule were present within it.

The second step in Fig 1A involves the detection of these PCR products. It was necessary to considerably modify the standard MB probe approach in order for it to function efficiently in Digital Amplification applications. Theoretically, one separate MB probe could be used to detect each specific mutation that might occur within the queried sequence. By inclusion of one MB corresponding to WT sequence and another corresponding to mutant sequence, the nature of the PCR product would be revealed. Though this strategy could obviously be used effectively in some situations, it becomes complex when several different mutations are expected to occur within the same queried sequence. For example, in the *c-Ki-Ras* gene example explored here, twelve different base substitutions resulting in missense mutations could theoretically occur within codons 12 and 13, and at least seven of these are observed in naturally-occurring human cancers. To detect all twelve mutations as well as the WT sequence with individual Molecular Beacons would require 13 different probes. Inclusion of such a large number of MB probes would not only raise the background fluorescence but would be expensive. We therefore attempted to develop a single probe that would react with WT sequences better than any mutant sequence within the

queried sequence. We found that the length of the loop sequence, its melting temperature, and the length and sequence of the stem were each important in determining the efficacy of such probes. Loops ranging from 14 to 26 bases and stems ranging from 4 to 6 bases, as well as numerous sequence variations of both stems and loops, were tested during the optimization procedure. For discrimination between WT and mutant sequences (MB-GREEN probe), we found that a 16 base pair loop, of melting temperature (Tm) 50-51°, and a 4 bp stem, of sequence 5'-CACG-3', were optimal. For MB-RED probes, the same stem, with a 19-20 bp loop of Tm 54-56°, proved optimal. The differences in the loop sizes and melting temperatures between MB-GREEN and MB-RED probes reflected the fact that only the GREEN probe is designed to discriminate between closely related sequences, with a shorter region of homology facilitating such discrimination.

Examples of the ratios obtained in replicate wells containing DNA templates from colorectal tumor cells with mutations of *c-Ki-Ras* are shown in Fig. 2. In this experiment, fifty copies of genomic DNA equivalents were diluted into each well prior to amplification. Each of six tested mutants yielded ratios of RED/GREEN fluorescence that were significantly in excess of the ratio obtained with DNA from normal cells (1.5 to 3.4 in the mutants compared to 1.0 in normal DNA; $p < 0.0001$ in each case, Student's t-Test). The reproducibility of the ratios can be observed in this figure. Direct DNA sequencing of the PCR products used for fluorescence analysis showed that the RED/GREEN ratios were dependent on the relative fraction of mutant genes within the template population (Fig. 2). Thus, the DNA from cells containing one mutant *C-Ki-Ras* allele per every two WT *c-Ki-Ras* allele yielded a RED/GREEN ratio of 1.5 (Gly12Arg mutation) while the cells containing three mutant *c-Ki-Ras* alleles per WT allele exhibited a ratio of 3.4 (Gly12Asp). These data suggested that wells containing only mutant alleles

(no WT) would yield ratios in excess of 3.0, with the exact value dependent on the specific mutation.

Though this mode is the most convenient for many applications, we found it useful to add the MB probes after the PCR-amplification was complete (Fig. 1). This allowed us to use a standard multiwell plate fluorometer to sequentially analyze a large number of multiwell plates containing pre-formed PCR products and bypassed the requirement for multiple real time PCR instruments. Additionally, we found that the fluorescent signals obtained could be considerably enhanced if several cycles of asymmetric, linear amplification were performed in the presence of the MB probes. Asymmetric amplification was achieved by including an excess of a single internal primer (primer INT in Fig. 1C) at the time of addition of the MB probes.

EXAMPLE 5

Analysis of DNA from tumor cells. The principles and practical considerations described above was demonstrated with DNA from two colorectal cancer cell lines, one with a mutation in *c-Ki-Ras* codon 12 and the other in codon 13. Representative examples of the MB-RED fluorescence values obtained are shown in Fig. 3. There was a clear biphasic distribution, with "positive" wells yielding values in excess of 10,000 specific fluorescence units (SFU, as defined in Materials and Methods) and "negative" wells yielding values less than 3500 SFU. Gel electrophoreses of 127 such wells demonstrated that all positive wells, but no negative wells, contained PCR products of the expected size (Fig. 3). The RED/GREEN fluorescence ratios of the positive wells are shown in Fig. 4. Again, a biphasic distribution was observed. In the experiment with the tumor containing a Gly12Asp mutation, 64% of the positive wells exhibited RED/GREEN ratios in excess of 3.0 while the other 36% of the positive wells exhibited ratios ranging from 0.8 to 1.1. In the case of the tumor with the Gly13Asp mutation, 54% of the positive wells exhibited RED/GREEN ratios >3.0 while the other positive wells yielded ratios ranging from 0.9 to 1.1. The PCR products from 16 positive wells were used as sequencing templates (Fig. 4). All the wells yielding a ratio in excess of 3.0 were found to contain mutant c-Ki-Ras fragments of the expected sequence, while WT sequence was found in the other PCR products. The presence of homogeneous WT or mutant sequence confirmed that the amplification products were usually derived from single template molecules. The ratios of WT to mutant PCR products determined from the Digital Amplification assay was also consistent with the fraction of mutant alleles inferred from direct sequence analysis of genomic DNA from the two tumor lines (Fig. 2).

Digital Analysis of DNA from stool. As a more practical example, we analyzed the DNA from stool specimens from colorectal cancer patients. A representative result of such an experiment is illustrated in Fig. 5. From previous analyses of stool specimens from patients whose tumors contained *c-Ki-Ras* gene mutations, we expected that 1% to 10% of the *c-Ki-Ras* genes purified from stool would be mutant. We therefore set up a 384 well Digital Amplification experiment. As positive controls, 48 of the wells contained 25 genome equivalents of DNA (defined in Materials and Methods) from normal cells. Another 48 wells served as negative controls (no DNA template added). The other 288 wells contained an appropriate dilution of stool DNA. MB-RED fluorescence indicated that 102 of these 288 experimental wells contained PCR products (mean +/- s.d. of 47,000 +/- 18,000 SFU) while the other 186 wells did not (2600 +/- 1500 SFU). The RED/GREEN ratios of the 102 positive wells suggested that five contained mutant c-Ki-Ras genes, with ratios ranging from 2.1 to 5.1. The other 97 wells exhibited ratios ranging from 0.7 to 1.2, identical to those observed in the positive control wells. To determine the nature of the mutant *c-Ki-Ras* genes in the five positive wells from stool, the PCR products were directly sequenced. The four wells exhibiting RED/GREEN ratios in excess of 3.0 were completely composed of mutant c-Ki-Ras sequence (Fig. 5B). The sequence of three of these PCR products revealed Gly12Ala mutations (GGT to GCT at codon 12), while the sequence of the fourth indicated a silent C to T transition at the third position of codon 13. This transition presumably resulted from a PCR error during the first productive cycle of amplification from a WT template. The well with a ratio of 2.1 contained a ~1:1 mix of WT and Gly12Ala mutant sequences. Thus 3.9% (4/102) of the *c-Ki-Ras* alleles present in this stool sample contained a Gly12Ala mutation. The mutant alleles in the stool presumably arose from the colorectal cancer of the patient, as direct sequencing of PCR products

generated from DNA of the cancer revealed the identical Gly12Ala mutation (not shown).

CLAIMS

1. A method for detecting a cancer-associated mutant nucleic acid that is present in a patient sample at a low level relative to a corresponding wild-type nucleic acid, the method comprising:

diluting nucleic acids in a biological sample to form a set comprising a plurality of assay samples;

amplifying the nucleic acids in the assay samples to form a population of amplified molecules;

performing an assay on the amplified molecules in each assay sample to determine whether a cancer-associated mutation is present in at least one of the assay samples;

wherein the step of diluting in performed until at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than a ratio of the mutant nucleic acid to the wild-type nucleic acid required to detect the mutant nucleic acid if it is present in the assay sample.

2. The method of claim 1 wherein the step of diluting is performed until between 0.1 and 0.9 of the assay samples yield an amplification product when subjected to a polymerase chain reaction.
3. The method of claim 1 wherein the step of diluting is performed until all of the assay samples yield an amplification product when subjected to a polymerase chain reaction and each assay sample contains less than 10 nucleic acid template molecules containing a reference genetic sequence.
4. The method of claim 1 wherein the step of diluting is performed until all of the assay samples yield an amplification product when subjected to a polymerase chain reaction and each assay sample contains less than

100 nucleic acid template molecules containing a reference genetic sequence.

5. The method of claim 1 wherein the biological sample is cell-free.
6. The method of claim 1 wherein the number of assay samples within the set is greater than 10.
7. The method of claim 1 wherein the number of assay samples within the set is greater than 50.
8. The method of claim 1 wherein the number of assay samples within the set is greater than 100.
9. The method of claim 1 wherein the number of assay samples within the set is greater than 500.
10. The method of claim 1 wherein the number of assay samples within the set is greater than 1000.
11. The method of claim 1 wherein the step of amplifying and the step of analyzing are performed on assay samples in the same receptacle.
12. The method of claim 1 wherein a molecular beacon probe is used in the step of analyzing, wherein a molecular beacon probe is an oligonucleotide with a stem-loop structure having a photoluminescent dye at one of the 5' or 3' ends and a quenching agent at the opposite 5' or 3' end.
13. The method of claim 1 wherein the step of analyzing employs gel electrophoresis.
14. The method of claim 1 wherein the step of analyzing employs hybridization to at least one nucleic acid probe.
15. The method of claim 1 wherein the step of analyzing employs hybridization to at least two nucleic acid probe.
16. The method of claim 12 wherein two molecular beacon probes are used, each having a different photoluminescent dye.

17. The method of claim 12 wherein the molecular beacon probe detects a wild-type nucleic acid better than a mutant nucleic acid.
18. The method of claim 1 wherein the step of amplifying employs a single pair of primers.
19. The method of claim 1 wherein the step of amplifying employs a polymerase which is activated only after heating.
20. . The method of claim 1 wherein the step of amplifying employs at least 40 cycles of heating and cooling.
21. The method of claim 1 wherein the step of amplifying employs at least 50 cycles of heating and cooling.
22. The method of claim 1 wherein the step of amplifying employs at least 60 cycles of heating and cooling.
23. The method of claim 1 wherein the biological sample is selected from the group consisting of stool, blood, and lymph nodes.
24. The method of claim 1 wherein the biological sample is blood or bone marrow of a leukemia or lymphoma patient who has received anti-cancer therapy.
25. The method of claim 1 wherein the mutant nucleic acid is a translocated allele
26. The method of claim 1 wherein the mutant nucleic acid is within an amplicon which is amplified during neoplastic development.
27. The method of claim 1 wherein the mutant nucleic acid is a rare exon sequence
28. The method of claim 1 wherein the nucleic acids being analyzed comprise cDNA of RNA transcripts.
29. A method for determining the ratio of a selected genetic sequence in a population of genetic sequences from a **blood** sample, comprising the steps of:

diluting nucleic acid template molecules from a **blood** sample to form

a set comprising a plurality of assay samples;

amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the **blood** sample.

30. The method of claim 29 wherein the step of amplifying employs **real-time polymerase chain reactions**.

31. The method of claim 30 wherein the real-time polymerase chain reactions comprise a **dual-labeled fluorogenic probe**.

32. The method of claim 29 further comprising the step of :
identifying **an allelic imbalance** based on the ratio ascertained.

33. The method of claim 29 wherein the selected genetic sequences and the reference genetic sequence are **non-polymorphic markers**.

34. The method of claim 29 wherein the selected genetic sequence and the reference genetic sequence are **on distinct chromosomes**.

35. A method for determining the ratio of a selected **non-polymorphic marker** in a population of genetic sequences in a **biological** sample, comprising the steps of:
diluting nucleic acid template molecules in a biological sample to form

a set comprising a plurality of assay samples;

amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected **non-polymorphic marker** and a second number of assay samples which contain a reference **non-polymorphic marker, wherein the selected and reference non-polymorphic markers are on distinct chromosomes**;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the **biological** sample; and

identifying **an allelic imbalance** based on the ratio ascertained.

36. The method of claim 35 wherein the biological sample is a blood sample.

37. The method of claim 35 wherein the step of amplifying employs real-time polymerase chain reactions.

38. The method of claim 37 wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe.

39. A method for determining the ratio of a selected genetic sequence in a population of genetic sequences from a **blood** sample, comprising the steps of:

amplifying template molecules within a set comprising a plurality of assay samples to form a population of amplified molecules in each of the assay samples of the set, wherein the template molecules are obtained from a **blood** sample;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence, wherein at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the **blood** sample.

40. The method of claim 39 wherein the step of amplifying employs **real-time polymerase chain reactions**.

41. The method of claim 40 wherein the real-time polymerase chain reactions comprise a **dual-labeled fluorogenic probe**.

42. The method of claim 39 further comprising the step of :
identifying **an allelic imbalance** based on the ratio ascertained.

43. The method of claim 39 wherein the selected genetic sequences and the reference genetic sequence are **non-polymorphic markers**.

44. The method of claim 39 wherein the selected genetic sequence and the reference genetic sequence are **on distinct chromosomes**.

45. A method for determining the ratio of a selected **non-polymorphic marker** in a population of **non-polymorphic markers** from a **biological** sample, comprising the steps of:
amplifying template molecules within a set comprising a plurality of

assay samples to form a population of amplified molecules in each of the assay samples of the set , wherein the template molecules are obtained from a biological sample;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected **non-polymorphic marker** and a second number of assay samples which contain a reference **non-polymorphic marker,** wherein at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected **non-polymorphic marker** to total **non-polymorphic markers** required to determine the presence of the selected **non-polymorphic marker,** wherein the selected genetic sequence and the reference genetic sequence are **on distinct chromosomes;**

comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample; and

identifying **an allelic imbalance based on the ratio ascertained.**

46. The method of claim 45 wherein the step of amplifying employs real-time polymerase chain reactions.

47. The method of claim 46 wherein the real-time polymerase chain reactions comprise a dual-labeled fluorogenic probe.

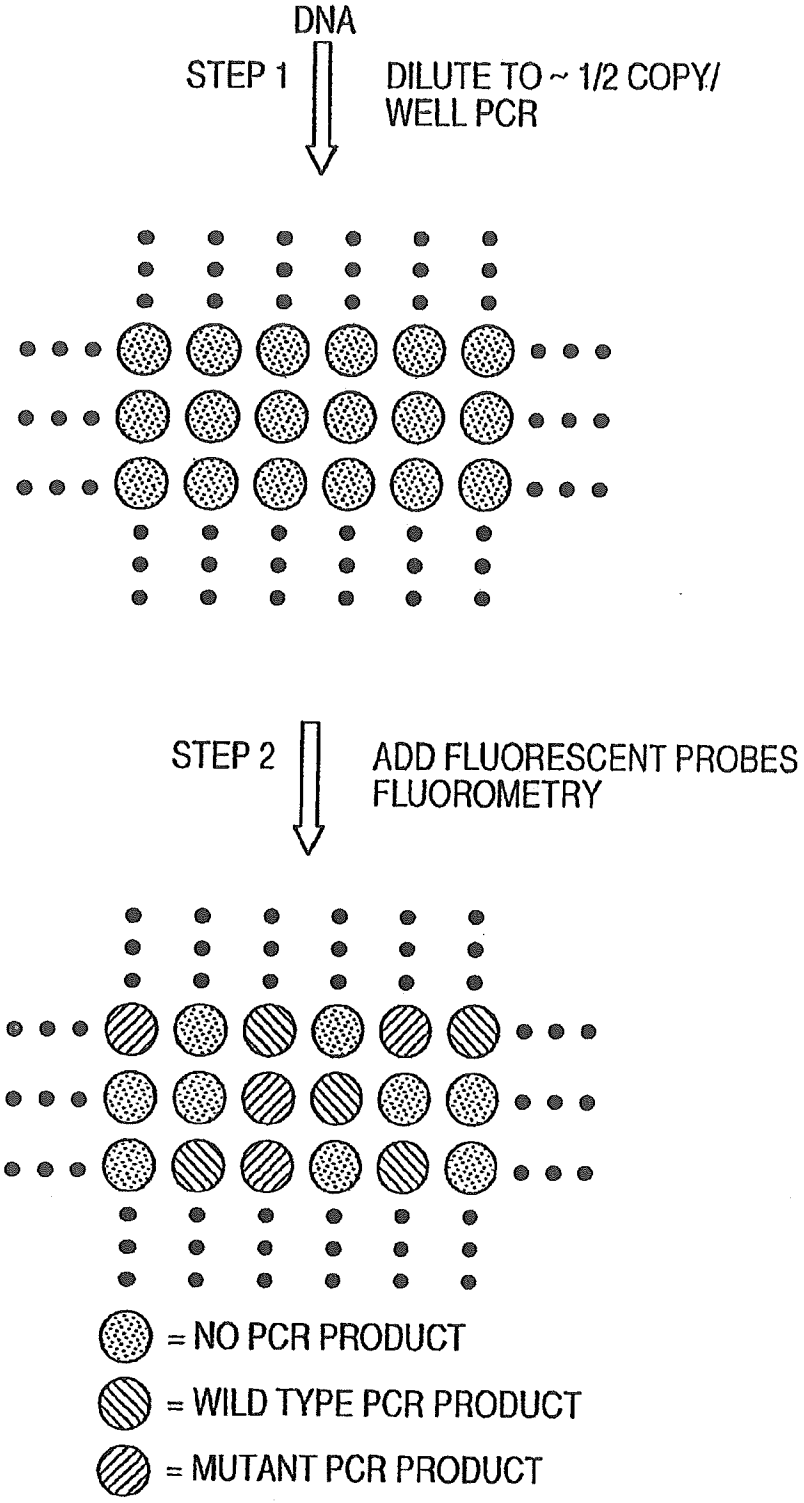
48. The method of claim 45 wherein the biological sample is from blood.

DIGITAL AMPLIFICATION

ABSTRACT

The identification of pre-defined mutations expected to be present in a minor fraction of a cell population is important for a variety of basic research and clinical applications. The exponential, analog nature of the polymerase chain reaction is transformed into a linear, digital signal suitable for this purpose. Single molecules can be isolated by dilution and individually amplified; each product is then separately analyzed for the presence of pre-defined mutations. The process provides a reliable and quantitative measure of the proportion of variant sequences within a DNA sample.

FIG. 1A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DILUTE TO ~ 1/2 COPY/ WELL PCR
STEP 2
ADD FLUORESCENT PROBES FLUOROMETRY
= NO PCR PRODUCT
= WILD TYPE PCR PRODUCT
= MUTANT PCR PRODUCT

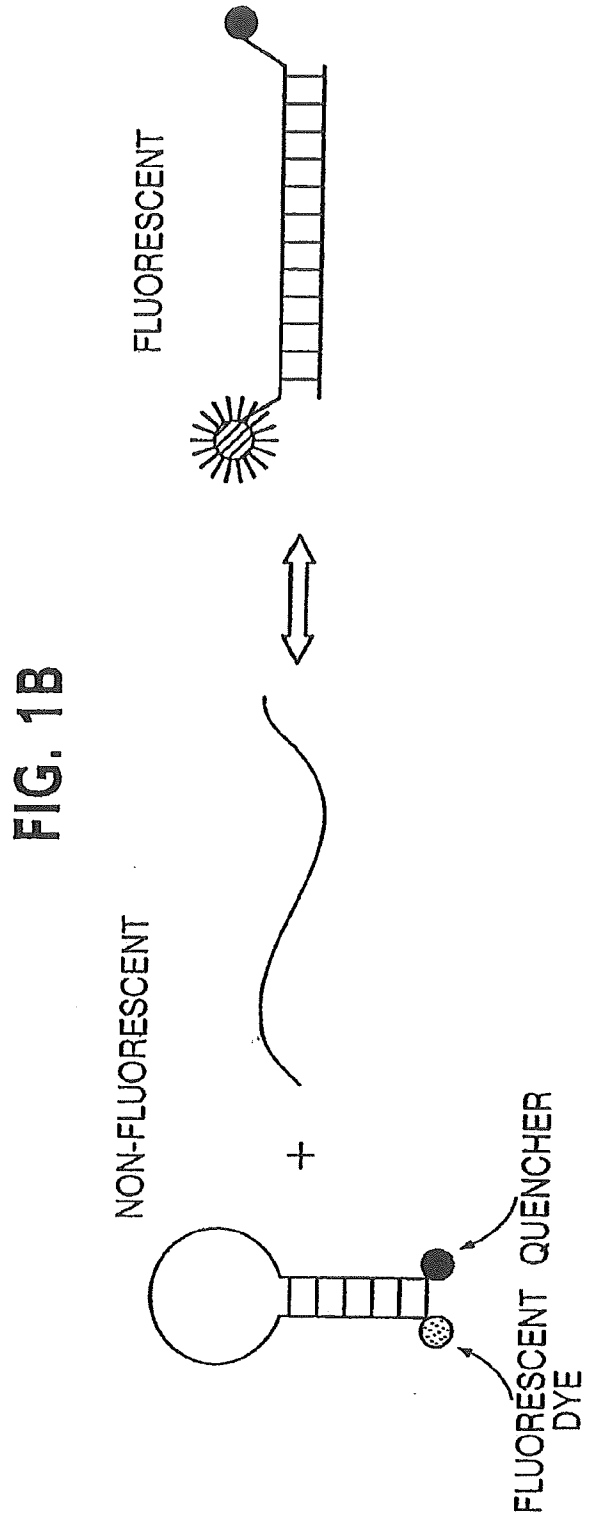


FIG. 1B

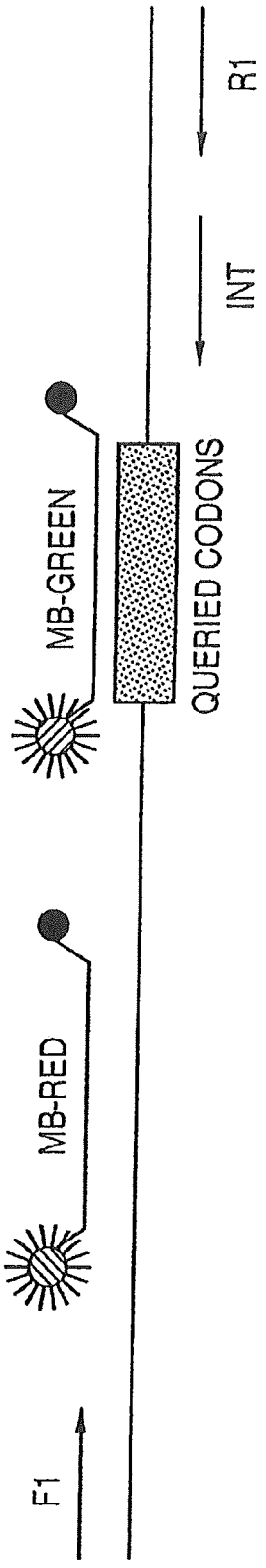
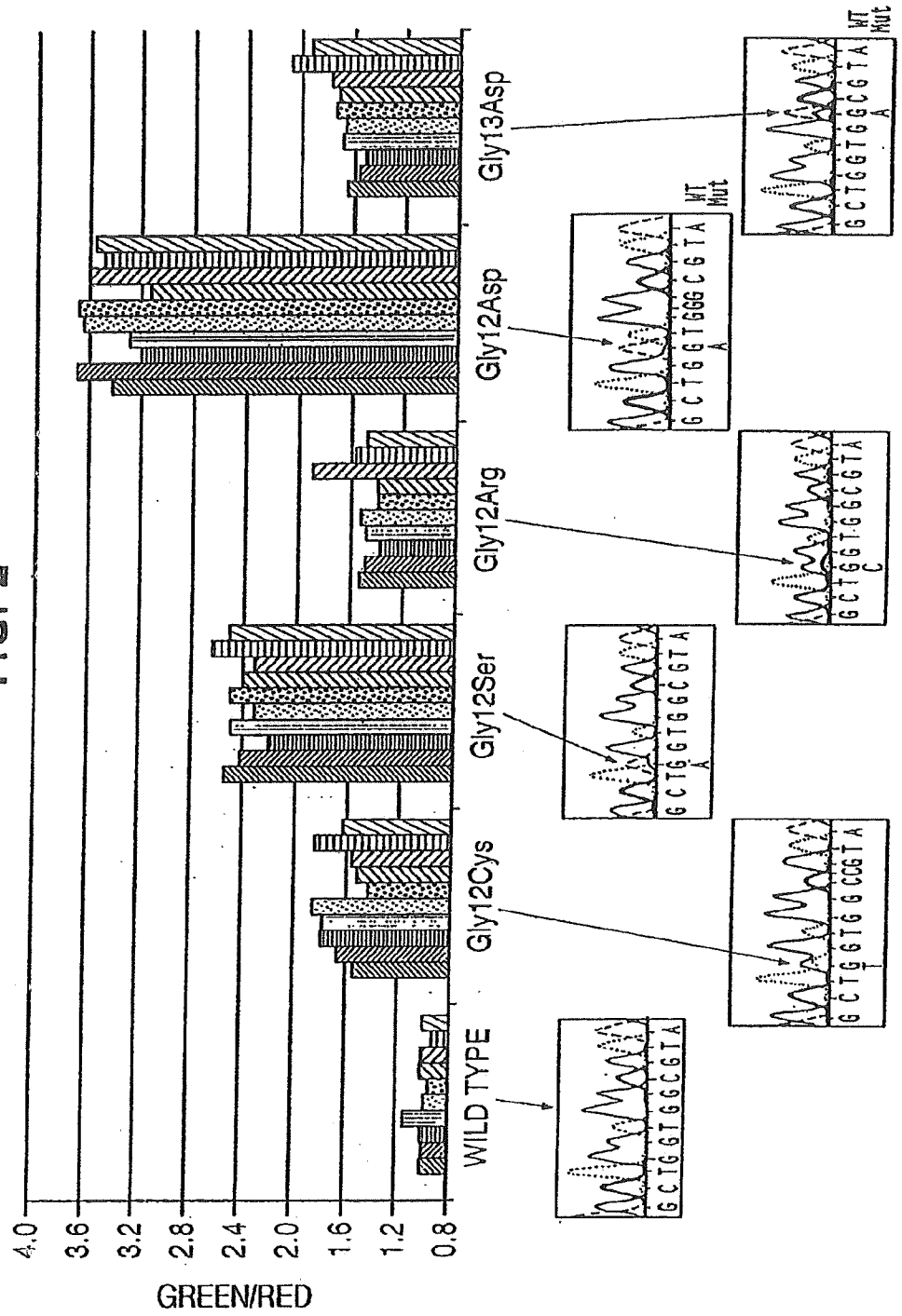


FIG. 1C

FIG. 2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2.4
2.0
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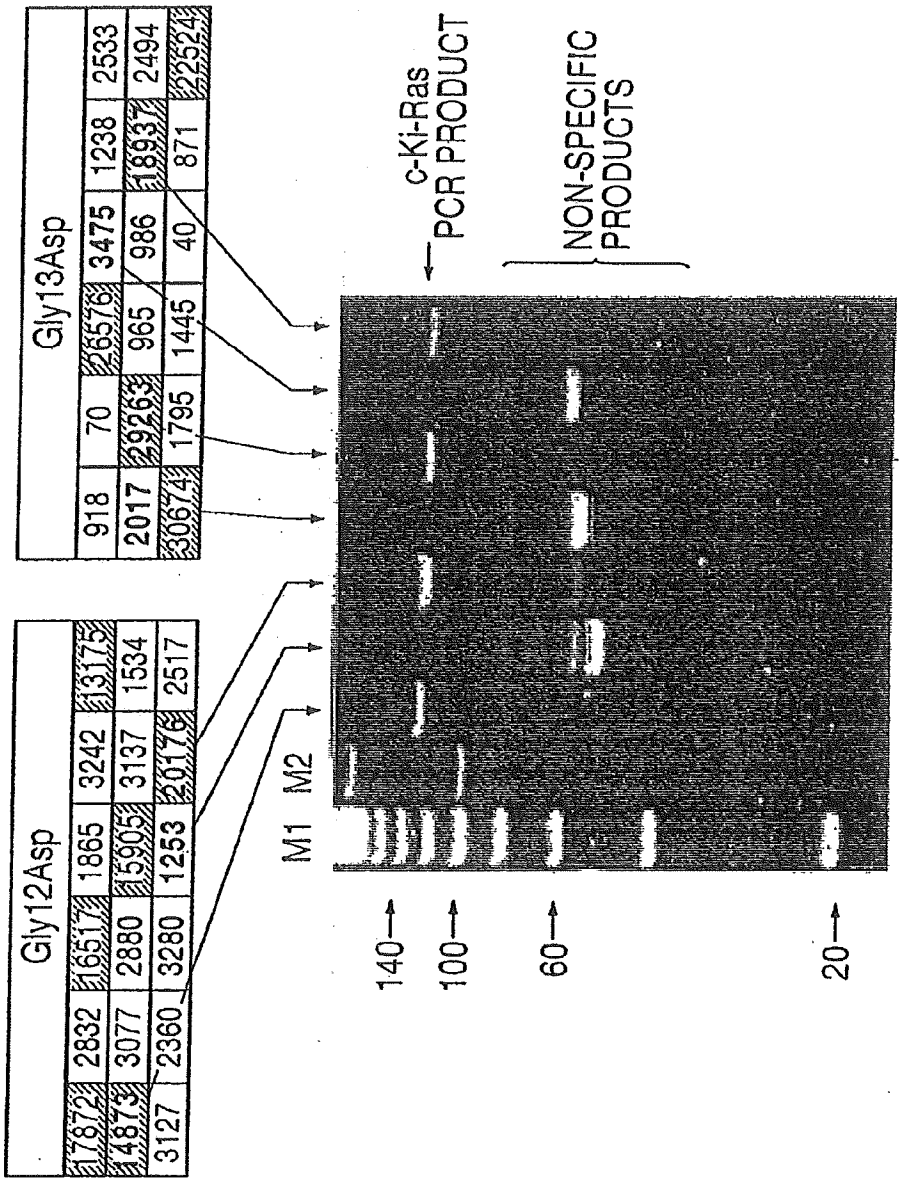
Gly12Arg
Gly12Asp
Gly13Asp
WT
Mut

FIG. 3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29263
965
986
18937
2494
30674
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PRODUCTS
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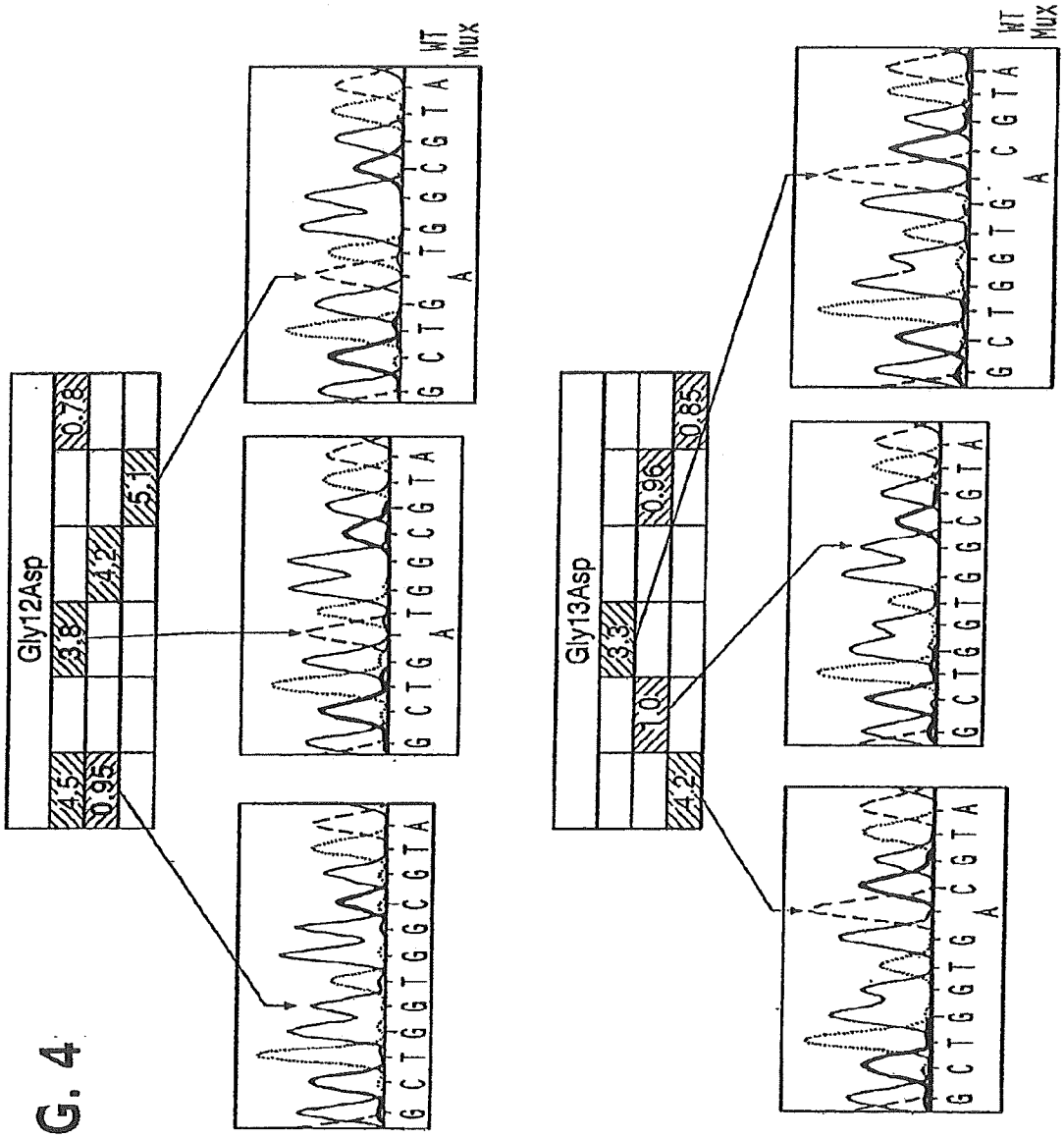
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0.95
3.8
4.2
5.1
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WT
Mux
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4.2
0.85
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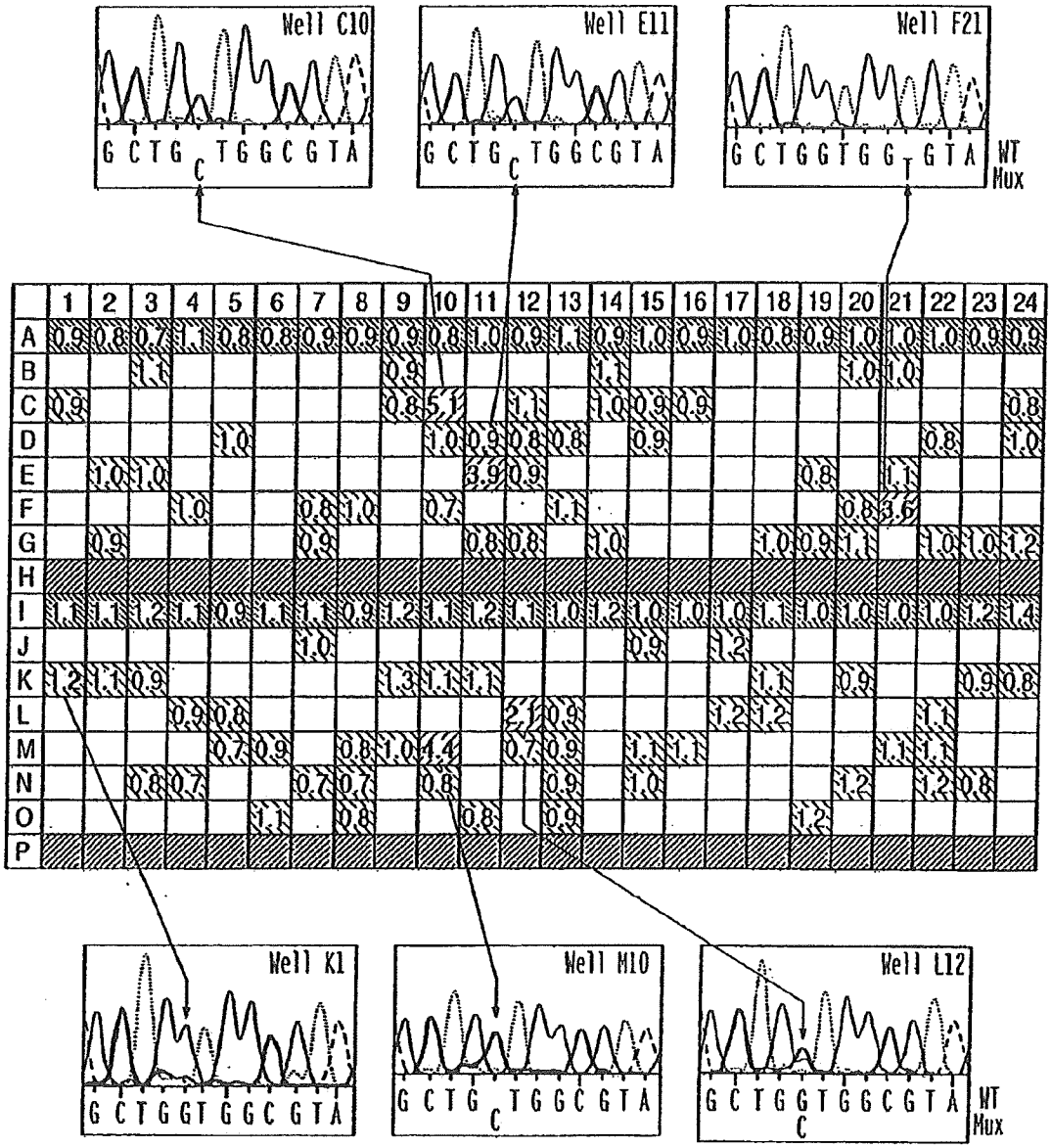
WT
Mux

FIG. 4

FIG. 5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WT
Mux
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A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
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WT
Mux

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Prior Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Prior Examiner: S. Woolwine
	)	
Serial No. TBD	)	Confirmation No. TBD
	)	
Filed: Herewith	)	Atty. Dkt. No. 001107.00866
	)	
For: DIGITAL AMPLIFICATION	)	

SEQUENCE STATEMENT

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401 Dulany Street
Alexandria, VA 22314

Sir:

Applicants respectfully request that the Patent Office use the computer readable form of the sequence listing submitted on November 14, 2003 in parent Application Serial Number 09/981,356 for examination of the instant application. I believe the contents of the referenced computer readable form and the paper copy of the sequence listing submitted herewith are identical. No new matter is added.

Respectfully submitted,

Date: March 24, 2011

By: /Sarah A. Kagan/
Sarah A. Kagan
Registration No. 32,141

Banner & Witcoff, Ltd.
Customer No. 22907

SEQUENCE LISTING

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Kinzler, Kenneth W.

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Prior Group Art Unit: 1637
	)	
Bert VOGELSTEIN et al	)	Prior Examiner: Samuel Woolwine
	)	
Continuation Application of	)	Confirmation No. TBD
Serial No. 12/617,368	)	
	)	Atty. Dkt. No. 001107.00866
Filed: Herewith	)	
	)	
For: DIGITAL AMPLIFICATION	)	

INFORMATION DISCLOSURE STATEMENT

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In accordance with 37 C.F.R. § 1.97, enclosed is a PTO Form 1449 listing documents for consideration by the Examiner in the subject application. Copies of the cited references were submitted in parent Application No. 12/617,368 or were provided by the Examiner attached to an office action. No fee is believed to be due to ensure consideration and entry of the cited documents by the Examiner. However, if a fee is deemed necessary, the Commissioner is authorized to charge our Deposit Account No. 19-0733.

Respectfully submitted,

By: /Sarah A. Kagan/
Sarah A. Kagan
Registration No. 32,141

Date: March 24, 2011

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PTO/SB/08a (01-10)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	
Filing Date	2011-03-16
First Named Inventor	Bert Vogelstein et al.
Art Unit	
Examiner Name	
Attorney Docket Number	001107.00866

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	5213961	A	1993-05-15	Bunn et al.	
	2	5736333	A	1998-04-07	Livak et al.	
	3	5518901	A	1996-05-21	Murtagh	
	4	5804383	A	1998-09-08	Gruenert et al.	
	5	5858663	A	1999-01-12	Nisson et al.	
	6	5670325	A	1997-09-23	Lapidus et al.	
	7	6037130	A	2000-03-14	Tyagi et al.	
	8	5925517	A	1999-07-20	Tyagi et al.	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2011-03-16
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	
	Examiner Name	
	Attorney Docket Number	001107.00866

	9	5928870	A	1999-07-27	Lapidus et al.	
	10	6020137	A	2000-02-01	Lapidus et al.	
	11	6143496	A	2000-11-07	Brown et al.	
	12	6291163	B1	2001-09-18	Sidransky	

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	1	95/13399	WO	A1	1995-05-18			☐
	2	99/13113	WO	A1	1999-03-18			☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2011-03-16
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	
	Examiner Name	
	Attorney Docket Number	001107.00866

	3	0643140	EP	A1	1995-03-15	Canon Kabushiki Kaisha		☐

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	1	LOUGHLIN ET AL., "Association of the Interleukin-1 Gene Cluster on Chromosome 2q13 With Knee Osteoarthritis," Arthritis & Rheumatism, June 2002, 46(6):1519-1527	☐
	2	P. J. SYKES, "Quantitation of Targets for PCR by Use of Limiting Dilution," BioTechniques, 1992, Vol. 13, No. 3, pp. 444-449	☐
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	6	S. TYAGI ET AL., "Molecular Beacons: probes that Fluoresce Upon Hybridization," Nature Biotechnology, 1996, Vol. 14, No. 3, pp. 303-308	☐
	7	W. P. HALFORD ET AL., "The Inherent Quantitative Capacity of the Reverse Transcription-Polymerase Chain Reaction," Analytical Biochemistry, January 15, 1999, Vol. 266, No. 2, pp. 181-191	☐
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	Application Number	
	Filing Date	2011-03-16
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	
	Examiner Name	
	Attorney Docket Number	001107.00866

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	12	W. NAVIDI ET AL., "Using PCR in Preimplantation Genetic Disease Diagnosis," Human Reproduction, 1991, Vol. 6, No. 6, pp. 836-849	☐
	13	H. LI ET AL., "Amplification and Analysis of DNA Sequences in Single Human Sperm and Diploid Cells," Nature, September 29, 1988, Vol. 335, pp. 414-417	☐
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	19	R. PARSONS ET AL., "Mismatch Repair Deficiency in Phenotypically Normal Human Cells," Science, May 5, 1995, Vol. 268, pp. 738-740	☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	2011-03-16
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	
	Examiner Name	
	Attorney Docket Number	001107.00866

	20	MARRAS ET AL., "Multiplex Detection of Single-Nucleotide Variations Using Molecular Beacons," Genetic Analysis: Biomolecular Engineering, Feb. 1999, Vo. 14, pp. 151-156	☐
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	22	M.J. BRISCO ET AL., "Detection and Quantitation of Neoplastic Cells in Acute Lymphoblastic Leukemia, by Use of the Polymerase Chain Reaction," British Journal of Haematology, 1991, Vol. 79, pp. 211-217	☐
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	24	Notice of Reasons for Rejection dispatched April 28, 2010 in Japanese Application No. 2001-513641 and English translation thereof.	☐
	25	Stephens, J. Clairborne, et al. "Theoretical underpinning of the Single-Molecular-Dilution (SMD) Method of Direct Haplotype Resolution," Am. J. Hum. Gen., Vol. 46, pp. 1149-1155 (1990).	☐
	26	NEWTON, PCR Essential Data, pages 51-52, 1995	☐
	27	Office Action dated June 11, 2010, in co-pending application 11/709,742	☐
	28	Office Action dated December 29, 2009 in co-pending application 11/709,742	☐
	29	Office Action dated September 18, 2009 in co-pending application 11/709,742	☐
	30	Office Action dated June 5, 2009 in co-pending application 11/709,742	☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	2011-03-16
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	
	Examiner Name	
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	Application Number	
	Filing Date	2011-03-16
	First Named Inventor	Bert Vogelstein et al.
	Art Unit	
	Examiner Name	
	Attorney Docket Number	001107.00866

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ None

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Sarah A. Kagan/	Date (YYYY-MM-DD)	2011-03-24
Name/Print	Sarah A. Kagan	Registration Number	32141

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Application Number:	
Filing Date:	
Title of Invention:	DIGITAL AMPLIFICATION
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Filer:	Sarah Anne Kagan./Daphne Cashion
Attorney Docket Number:	001107.00866

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Utility application filing	1011	1	330	330
Utility Search Fee	1111	1	540	540
Utility Examination Fee	1311	1	220	220
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1090**

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EFS ID:	9733948
Application Number:	13071105
International Application Number:	
Confirmation Number:	3361
Title of Invention:	DIGITAL AMPLIFICATION
First Named Inventor/Applicant Name:	Bert VOGELSTEIN
Customer Number:	22907
Filer:	Sarah Anne Kagan./Daphne Cashion
Filer Authorized By:	Sarah Anne Kagan.
Attorney Docket Number:	001107.00866
Receipt Date:	24-MAR-2011
Filing Date:	
Time Stamp:	16:36:41
Application Type:	Utility under 35 USC 111(a)

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Payment was successfully received in RAM	$1090
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If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

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EXHIBIT 6

The EMBO Journal vol.12 no.13 pp.4955 - 4967, 1993

Tracing B cell development in human germinal centres by molecular analysis of single cells picked from histological sections

Ralf Küppers[1], Min Zhao[2], Martin-L.Hansmann[2] and Klaus Rajewsky

Institute for Genetics, University of Cologne, Weyertal 121, D-50931 Cologne and [2]Pathologisches Institut, University of Cologne, Josef-Stelzmannstrasse 9, D-50924 Cologne, Germany

Communicated by K.Rajewsky

[1]Corresponding author

Germinal centres are areas of intense B lymphocyte proliferation inside primary B cell follicles in spleen and lymph nodes. Rearranged V genes from single human B cells, isolated from histological sections of two such structures by micromanipulation, were amplified and sequenced. Cells from the follicular mantle were clonally diverse and largely expressed germline V genes. Germinal centres were dominated by a few large B cell clones dispersed throughout these structures and exhibiting intraclonal diversity by ongoing somatic hypermutation. Pronounced counterselection of replacement mutations seen in one of the germinal centres may indicate a late phase of the germinal centre reaction. A polyclonal population of activated B cells expressing unmutated antibodies in the dark zone of the other germinal centre may represent the initial founder cells.

Key words: B cell development/germinal centre/somatic hypermutation/V gene rearrangement/single cell PCR

Introduction

Germinal centres are histologically defined structures in peripheral lymphoid organs. They represent accumulations of predominantly proliferating B lymphocytes and are surrounded by a mantle zone of phenotypically distinct small resting B cells (reviewed in Kroese *et al.*, 1990; Liu *et al.*, 1992). On the basis of histological staining reactions germinal centres can be subdivided into a dark zone with rapidly dividing B cell blasts (centroblasts) and a light zone of non-dividing B cells (centrocytes). Experiments in mice and rats show an oligoclonal development of germinal centres with one to six founder B cells (Kroese *et al.*, 1987; Jacob *et al.*, 1991b; Liu *et al.*, 1991a). Other components of the germinal centres are T helper cells, follicular dendritic cells (FDC) and macrophages (reviewed by Kroese *et al.*, 1990).

Germinal centres arise after antigenic stimulation in T cell dependent immune responses and it has long been suspected that they may be the sites where affinity maturation of antibodies and the generation of memory B cells takes place (see MacLennan and Gray, 1986). Somatic mutation is the hallmark of affinity maturation and the generation of B cell memory, a process by which rearranged antibody variable (V) region genes are modified to give rise to mutant antibodies which are selected for binding of the immunizing antigen with higher affinity (reviewed by Kocks and Rajewsky, 1989). The analysis of splenic germinal centre B cells from immunized mice has indeed shown that in the course of the response these cells not only carry an increasing load of somatic point mutations in rearranged V region genes (Berek *et al.*, 1991), but also that the process of somatic hypermutation is ongoing within the germinal centre (Jacob *et al.*, 1991a). This strongly supports the concept that B cells expressing high affinity mutant antibodies arise in the course of clonal proliferation in the germinal centre microenvironment to become long-lived memory cells. The proliferation kinetics of memory B cells generated upon immunization is consistent with this view (Schittek and Rajewsky, 1990).

The efficiency by which rare high affinity somatic variants are selected at the expense of all other mutants already at early time points of the response (Berek *et al.*, 1991; Weiss *et al.*, 1992) suggests that these B cells are not only generated but also selected within the germinal centre. B cells which through mutations either have lost the ability to produce functional antibodies or whose antibodies have lost the capacity to bind antigen appear to die by apoptosis and to be taken up by macrophages (Liu *et al.*, 1989).

The antigen-mediated signal which rescues germinal centre B cells from apoptosis is not fully understood, but appears to involve surface receptors other than the immunoglobulin receptor complex. Isolated germinal centre B cells undergo rapid apoptosis in tissue culture. However, in the presence of CD23 (the low affinity receptor for IgE) and interleukin 1α or antibodies against the B cell antigen CD40 and surface immunoglobulin, they survive and differentiate into plasmablasts or small, resting (memory?) B cells, respectively (Liu *et al.*, 1989, 1991b). This may indicate that different ligands drive germinal centre B cells into different differentiation pathways, namely either plasma cell or memory cell generation. That germinal centre B cells can indeed differentiate in either direction is supported by evidence from *in vivo* experiments (Coico *et al.*, 1983; Tew *et al.*, 1992).

Interestingly, a subpopulation of FDC in the light zone of the germinal centre expresses CD23 (Johnson *et al.*, 1989; Liu *et al.*, 1992), and recent experiments suggest that a CD40-mediated signal is involved in the interaction between T helper cells which are mainly found in the light zone (Stein *et al.*, 1980), and B cells in human germinal centres (Lederman *et al.*, 1992). Therefore, the selection processes mediated by interaction of B cells with FDC and T cells may be compartmentalized within the germinal centre.

In collaboration with G.Kelsoe's group we have recently initiated an approach by which B cell differentiation is assessed at the molecular level within the histological structures in which the cells reside. For this purpose we isolated B cell populations from individual germinal centres by micromanipulation from histological sections of mouse spleen, and sequenced rearranged V region genes amplified from those cells by the polymerase chain reaction (PCR)

(Jacob *et al.*, 1991a). This allows the identification of clonally related rearrangements, because each rearrangement is common to and unique for all cell members of a given B cell clone. On the basis of shared and unique point mutations in the sequences, clonal genealogies can then be established (McKean *et al.*, 1984). However, the approach was limited in that a selected set of V genes was amplified and cell populations instead of single cells were analysed. Only the latter would allow the assignment of more than one V gene rearrangement (e.g. both H and L chain gene rearrangements) and thus a given antibody binding site to a given cell. Furthermore, the analysis of single cells is required for the formal demonstration that the clonally related but distinct V region sequences which can be isolated from germinal centres (Jacob *et al.*, 1991a) indeed originate from separate cells and thus reflect somatic hypermutation in the course of clonal expansion. Most importantly, however, the assignment of individual sequences to individual cells picked from the various histologically distinct areas of the germinal centre would allow the determination of the extent and the timing of clonal expansion within this structure and whether clonal expansion, intraclonal diversification and cellular selection are compartmentalized. We have therefore extended our previous approach to the analysis of H and L chain V region gene rearrangements in individual cells picked from various positions in histological sections of human germinal centres. The human system was chosen because human germinal centres are particularly well structured histologically and it also seemed important to demonstrate that somatic hypermutation takes place in germinal centres of the human as it does in the mouse.

Results

PCR analysis of single micromanipulated cells and their histological origin

Frozen sections (8–10 μm thick) derived from human lymph nodes were stained (see below) and single cells were micromanipulated with the help of two hydraulic micromanipulators. Rearranged V_H and V_κ region genes from individual cells were amplified in a semi-nested PCR approach using V gene family specific primers, and the amplification products (defined as bands of ~350 bp in length visible on an ethidium bromide stained agarose gel) were directly sequenced as described in Materials and methods. By direct sequencing of PCR products from both strands somatic mutations can be clearly identified as misincorpration of nucleotides by Taq polymerase is negligible (McHeyzer-Williams *et al.*, 1991).

A double blind control experiment performed with micromanipulated mantle zone cells (>90% of which are B cells) and T cells (see Materials and methods) demonstrated the efficiency and reliability of the method. 25 PCR products representing rearranged V region genes (12 $V_HD_HJ_H$ and 13 $V_\kappa J_\kappa$ rearrangements) were obtained from 20 B cells. At first glance, this is less than what one might expect. In the case of heavy chains, for example, all cells should harbour an in-frame rearrangement and about one-third should have an additional non-functional $V_HD_HJ_H$ rearrangement (Yamada *et al.*, 1991). However, there are many reasons why the yield of PCR bands must be significantly below 100%, including the fact that many cells in the section lack part of their nucleus (see Discussion). Only two V region genes were amplified from 19 T cells. These two products could be due to cellular or other contamination, or represent true immunoglobulin V gene rearrangements in T cells, although so far only D_HJ_H cross-lineage rearrangements have been described for these cells (Waldmann, 1987). Nonetheless, the PCR approach chosen appears feasible from a technical point of view as it allows the characterization of V gene rearrangements in a large fraction of B cells at a high level of confidence in terms of the assignment of a given rearrangement to a given cell.

Frozen sections of two germinal centres derived from human lymph nodes (GC2 and GC3; Figures 1a and 2a) were stained with the Ki-67 antibody which can be used to discriminate between dark and light zones of the germinal centre since it exclusively stains cells in cycle (Gerdes *et al.*, 1984). Therefore, most of the cells in the dark zone are stained by this antibody whereas the majority of the cells in the light zone are Ki-67$^-$. The latter is also true for the mantle zone surrounding the GC. Cells from dark, light and mantle zones were isolated for analysis. A total of 157 cells were analysed by PCR amplification, 90 derived from GC2 and 67 from GC3 (Table I). The efficiency of amplification, defined as the percentage of cells with at least one PCR product, was different for cells from different regions of the GC, ranging from 55% for mantle zone cells to 24% for centroblasts of GC3 (Table I; see Discussion). Sequences were determined for 58 PCR products (Table I). Counting clonally related sequences only once, 15 of 20 $V_HD_HJ_H$ sequences represented in-frame rearrangements whereas only 10 of 20 $V_\kappa J_\kappa$ rearrangements were in-frame. In the case of the heavy chain genes, the observed frequency of in-frame $V_HD_HJ_H$ rearrangements (75%) is close to values obtained by others for murine B cells and human peripheral blood B cells (Alt *et al.*, 1984; Yamada *et al.*, 1991). The higher fraction of out-of-frame rearrangements among the V_κ sequences is expected considering that 30–40% of human B cells express λ light chains with most of these cells

Table I. *Summary of PCR amplification results from two germinal centres*

GC	Region	No. of cells analysed	No. of cells positive	% positive	Number of PCR bands	PCR bands sequenced
2	mantle zone	27	15	55	27	8
	dark zone	26	12	46	22	14
	light zone	37	9	24	15	14
3	mantle zone	11	6	54	9	3
	dark zone	25	6	24	7	7
	light zone	31	10	32	12	12

The number of positive cells indicates the number of cells with at least one PCR product for the V_H or V_κ gene amplification.

harbouring one or two non-functional V_xJ_x rearrangements (Graninger *et al.*, 1988).

Nomenclature

Micromanipulated cells were named as follows: a number (2 or 3) indicating the germinal centre from which the cell originates is followed by one or two letters describing the histological origin (M: mantle zone, Ki-67$^-$; MR: mantle zone, Ki-67$^+$; D: dark zone, Ki-67$^+$; L: light zone, Ki-67$^-$; C: light zone, Ki-67$^+$) and a number indicating the numerical order of picking. A V gene rearrangement is described by the designation of the cell from which it originates, followed by 'H' or 'K' to indicate heavy or κ light chain, respectively, and the number of the V gene family.

Mantle zone B cells

V gene rearrangements from a total of 10 mantle zone B cells were analysed by sequencing, seven from GC2 and three from GC3 (Figures 1 and 2 and Table II). Three of

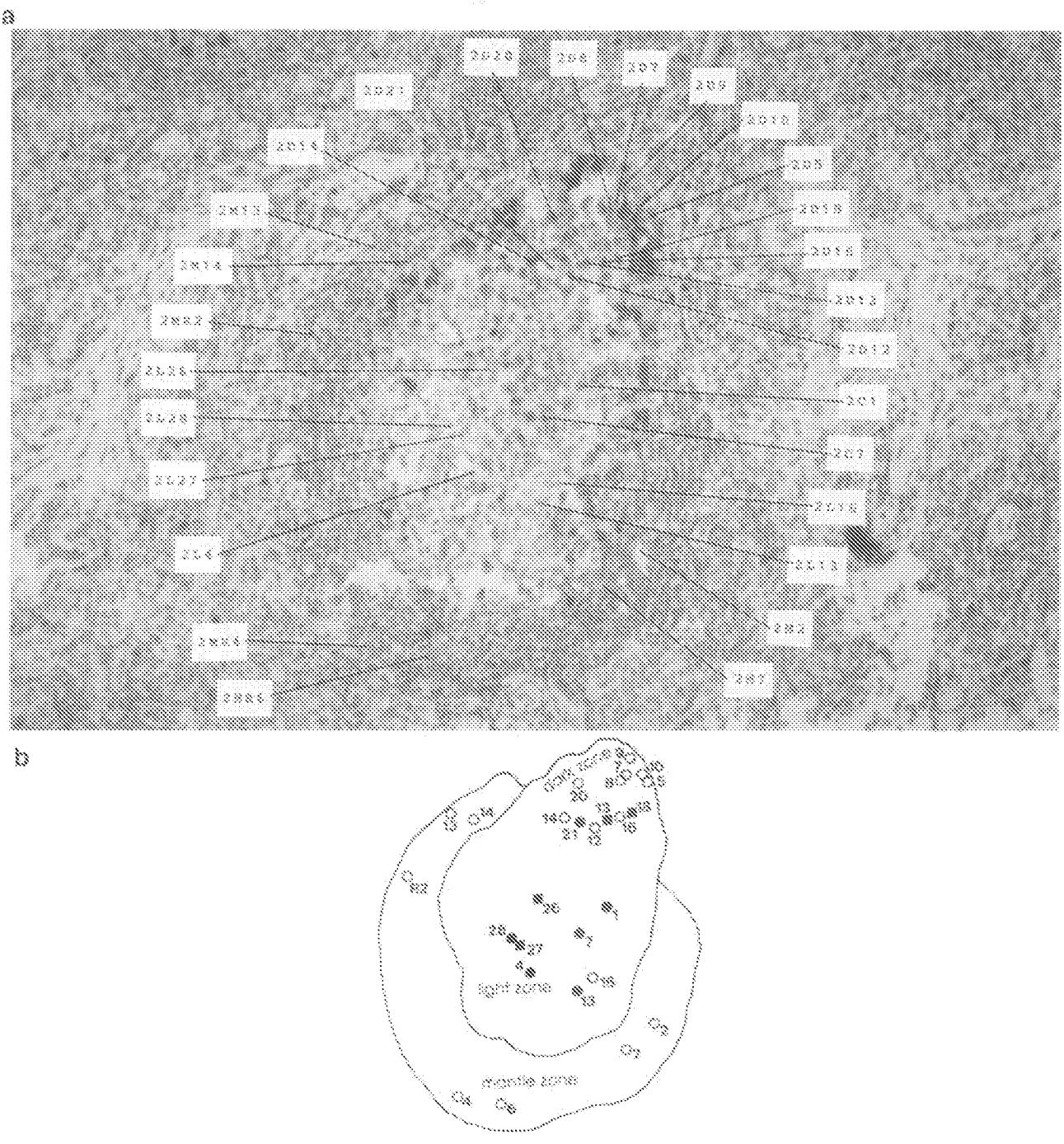

Fig. 1. Histology of germinal centre 2. (a) Frozen section of an inguinal lymph node stained with antibody Ki-67 (red) and haematoxylin with a germinal centre (GC2) in the centre. The locations of the cells for which sequence data were obtained are shown. The picture was taken after micromanipulation of cells 2M2 and 2M7. Therefore, for these cells an empty spot is seen. (b) Diagrammatic representation of GC2 indicating the distribution of the cells for which sequence information on rearranged V region genes was obtained. Clonally related cells are marked with the same closed symbol (a circle for clone 1 and a square for clone 2, see Table III) and clonally unrelated cells with an open circle. Cell numbers appear next to the cells.

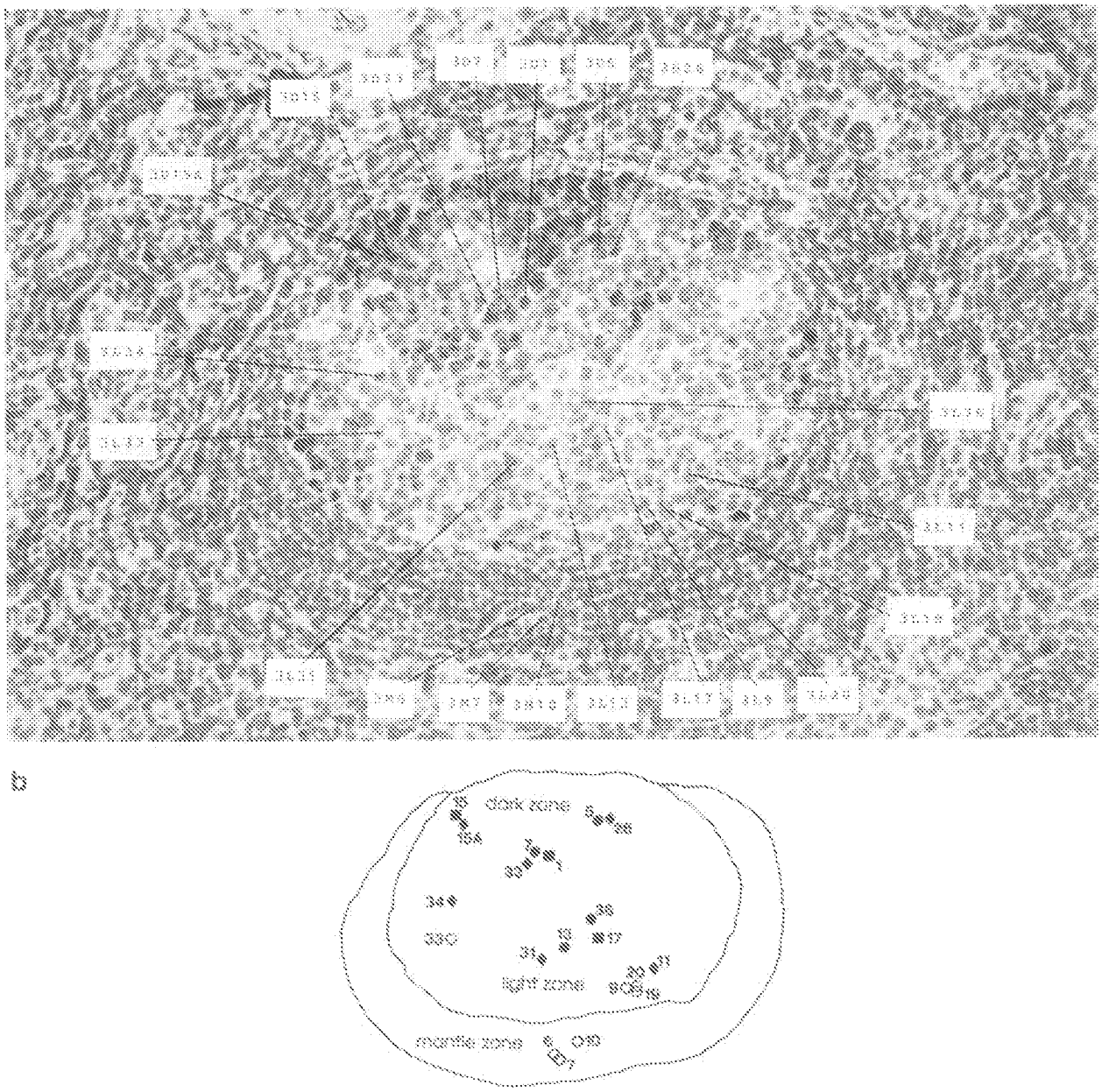

Fig. 2. Histology of germinal centre 3. (a) Frozen section of a cervical lymph node stained with antibody Ki-67 (red) and haematoxylin, with a germinal centre in the centre. The locations of the cells for which sequence data were obtained are shown. The picture was taken before micromanipulation. (b) Diagrammatic representation of GC3 indicating the distribution of the cells for which sequence information on rearranged V region genes was obtained. Clonally related cells are marked with the same closed symbol (a square for clone 1, a circle for clone 2 and a diamond for clone 3; see Table IV) and cells for which no clonal relationship is evident (although one or the other of these cells—with the exception of 3L19—might belong to clone 1 or 2 for which only a functional V_H rearrangement was obtained) with an open circle. Cell numbers appear next to the cells.

these cells (2MR2, 2MR4 and 2MR6) were Ki-67$^+$ and therefore belong to the very small fraction of proliferating cells in the mantle zone. V genes from different V_H and V_κ families were found to be rearranged in the mantle zone cells. Of the 11 V genes sequenced, nine are unmutated by comparison with published germline genes and—in one case—with an expressed V_H gene (VH4-Wil). 2MR6K4 shows one nucleotide difference from the published V_κ4 germline gene whereas 3M10K3 has five nucleotide differences from the V_κ3 gene A27 (Table II). These differences could be due to polymorphism, somatic mutation or to the expression of a previously undescribed V_κ3 gene in the case of 3M10K3. Clonally related cells could not be identified in the mantle zone of either germinal centre.

Germinal centre 2, derived from an inguinal lymph node of a 6 year old child

Sequence data were obtained for 28 V gene rearrangements from a total of 20 cells (Figure 1 and Table III). 14 sequences originated from 12 dark zone B cells. Six V region genes of those cells use unmutated gene segments and four further sequences have only two nucleotide differences relative to published germline genes (Table III). The remaining four V_H4 rearrangements (2D9H4, 2D10H4,

Table II. V region sequence analysis of mantle zone B cells derived from GC2 and GC3

Cell	V gene family	Germline gene	Base pairs difference	In-frame?	Ref.
2M2	VH4	VH4-Wil[a]	0	+	1
2M7	VK4	VK4	0	–	2
2M13	VK2	A3	0	+	3
2M14	VH4	VH4-Wil[a]	0	+	1
2MR2	VK1	O18	0	–	4
2MR4	VK3	A27	0	+	5
2MR6	VH3	VH26	0	–	6
	VK4	VK4	1	+	2
3M6	VK1	L4	0	–	7
3M7	VK3	A27	0	–	5
3M10	VK3	A27	5	+	5

The sequences are compared with those of the most homologous known germline gene. In-frame rearrangements are marked by '+', non-functional (out-of-frame) ones by '–'. References for germline genes: 1: Pratt *et al.* (1991); 2: Klobeck *et al.* (1985); 3: Straubinger *et al.* (1988); 4: Scott *et al.* (1991); 5 (VK325): Radoux *et al.* (1986); 6: Chen (1990); 7 (Va): Pech *et al.* (1984).
[a]In this case the V_H sequence is compared with that of a rearranged V_H4 gene.

Table III. V region sequence analysis of germinal centres cells derived from GC2

Cell	V gene family	Germline gene	Base pairs difference	Clone	In-frame?	Ref.
Dark zone						
2D5	VH4	VH4-Wil[a]	0		+	1
2D7	VH3	DP58	2		+	2
2D8	VK2	A17	0		–	3
2D9	VH4	VH416	5		+	4
	VK3	A27	2		+	5
2D10	VH4	VH421	17		+	4
2D12	VH4	VH421	13		+	4
2D13	VH4	VH411	18	2	+	4
2D14	VK4	VK4	0		–	6
2D16	VK4	VK4	0		–	6
2D18	VK4	VK4	0	2	–	6
2D20	VH4	VH421	2		–	4
	VK2	A3	0		–	7
2D21	VK3	L6	2	1	+	8
Light zone						
2C1	VH4	VH411	14	1	–	4
	VH3	VH26	8		+	9
	VK2	A3	2		+	7
2C7	VH4	VH411	14	1	–	4
	VK3	L6	2		+	8
2L4	VH3	VH26	11	1	+	9
2L13	VH3	VH26	11	1	+	9
2L16	VH4	VH4-Wil[a]	4		+	1
2L26	VH4	VH411	17	2	+	4
	VK1	L9	18		+	10
2L27	VH4	VH411	16	2	+	4
	VK1	L9	15		+	10
	VK4	VK4	0		–	6
2L28	VK3	L6	2	1	+	8

Cells belonging to one of the two clones are indicated. The sequences are compared with those of the most homologous germline genes. '+' means in-frame, '–' a non-functional (out-of-frame) rearrangement. References for germline genes: 1: Pratt *et al.* (1991); 2: Tomlinson *et al.* (1992); 3: Lautner-Rieske *et al.* (1992); 4: Sanz *et al.* (1989); 5 (VK325): Radoux *et al.* (1986); 6: Klobeck *et al.* (1985); 7: Straubinger *et al.* (1988); 8 (Vg): Pech and Zachau (1984); 9: Chen *et al.* (1990); 10 (Ve): Pech *et al.* (1984).
[a]In this case the V_H sequence is compared with a rearranged V_H4 gene.

2D12H4 and 2D13H4) have 5–18 base pair differences from their closest germline homologue. Whether any or all of these four V_H4 sequences are somatically mutated cannot be determined as there is considerable polymorphism in the V_H4 family, and it is not known whether all members of this family have yet been cloned (Weng *et al.*, 1992). Within

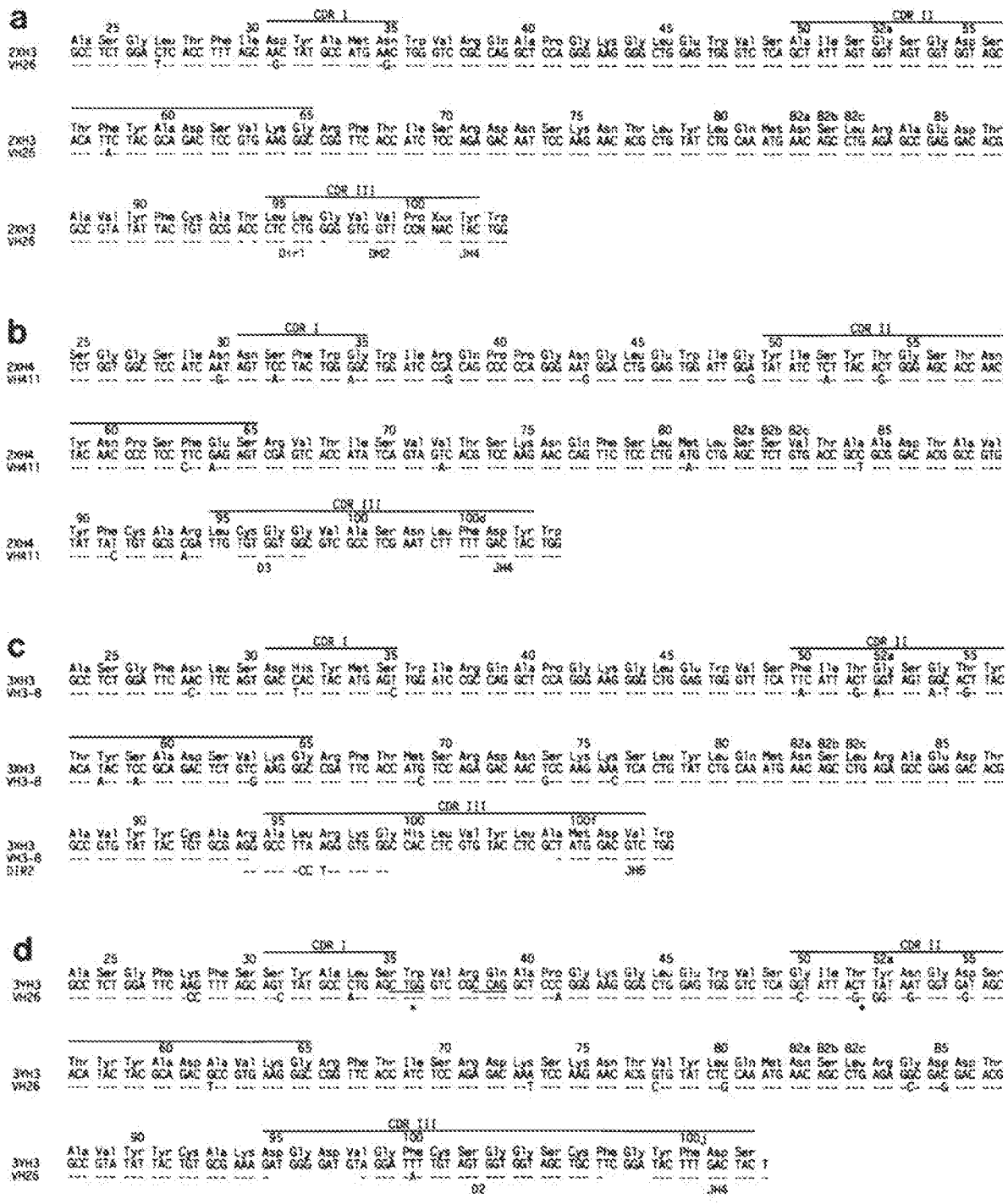

Fig. 3. Sequence analysis of clonally related V_H rearrangements. Dashes indicate sequence identity. Codons are numbered according to Kabat *et al.* (1987). Complementarity determining regions (CDRs) 1–III are marked. (**a**) The sequence of the hypothetical intermediate 2XH3 of cells 2C1, 2L13 and 2L4 (Figure 4a) is compared with that of the most homologous V_H3 germline gene VH26 (Chen, 1990), the D_H gene DM2 (Ichihara *et al.*, 1988), the DIR1 gene (Ichihara *et al.*, 1988) and the J_H4 germline gene (Ravetch *et al.*, 1981). (**b**) Sequence comparison of the hypothetical intermediate in the genealogical tree of the V_H4 rearrangement of cells 2D13, 2L26 and 2L27 (2XH4, see Figure 4b). The sequence is compared with that of the V_H4 germline gene VH411 (Sanz *et al.*, 1990), the D3 D_H gene (Siebenlist *et al.*, 1981) and the J_H4 gene (Ravetch *et al.*, 1981). (**c**) Sequence analysis of the hypothetical intermediate (3XH3, see Figure 4c) in the genealogical tree of the V_H3 rearrangement of cells 3D5, 3D7, 3L13 and 3L36. The sequence is compared with that of the VH3-8 V_H3 germline gene (Winkler *et al.*, 1992), the DIR2 segment (Ichihara *et al.*, 1988) and the J_H6 germline gene (Ravetch *et al.*, 1981). (**d**) Sequence analysis of the hypothetical intermediate 3YH3 (see Figure 4d) in the genealogical tree of the V_H3 rearrangements of cells 3D1, 3D15 and 3L17. The sequence is compared with that of the VH26 V_H3 germline gene (Chen, 1990), the D2 D_H gene (Siebenlist *et al.*, 1981) and the J_H4 germline gene (Ravetch *et al.*, 1981). '*' indicates the beginning and end of the deletion in 3D15H3. A common sequence motif found near non-homologous recombination breakpoints (Chou and Morrison, 1993) is underlined. The accession numbers for the sequences reported here have been deposited in the EMBL data library under accession numbers X73160–X73213.

the dark zone no clonally related sequences were identified, but three of the 12 cells turned out to be clonally related to cells in the light zone (see below).

From the light zone of GC2, eight V_H and six V_κ rearrangements from eight individual cells were sequenced (Table III). Five of these cells (2C1, 2C7, 2L4, 2L13 and

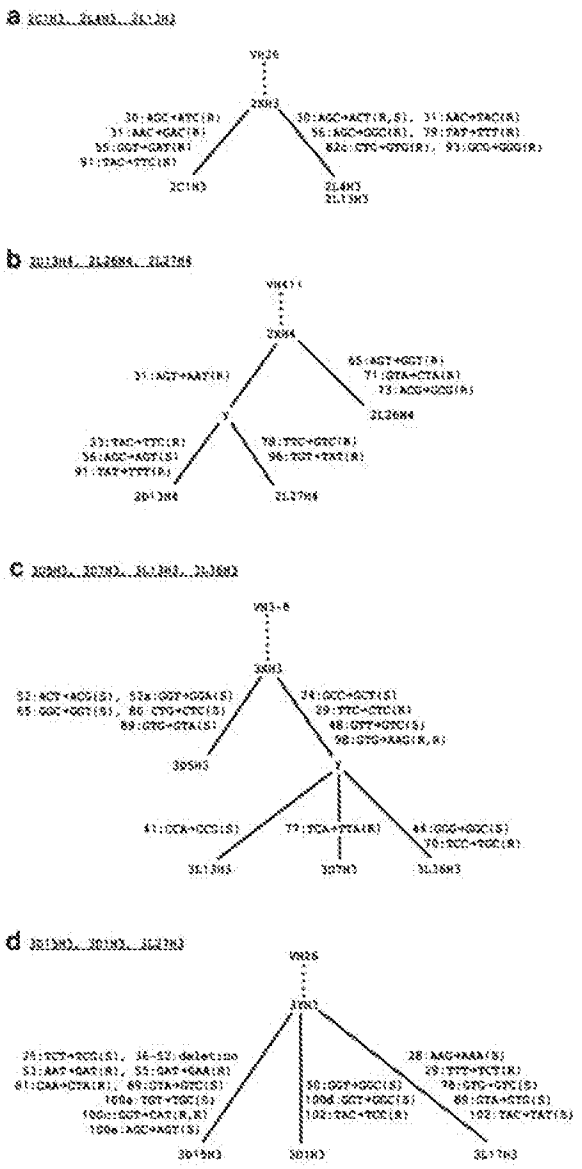

Fig. 4. Genealogical trees explaining the relationship between clonally related sequences. 2XH3, 2XH4, 3XH3, 3YH3 and Y represent hypothetical intermediates. Sequence comparisons between the intermediates 2XH3, 2XH4, 3XH3, 3YH3 and germline V_H, D_H and J_H genes are shown in Figure 3. Numbers along the branches indicate the amino acid number where the mutations are found. (a) Comparison of 2C1H3, 2L4H3 and 2L13H3; (b) comparison of 2D13H4, 2L26H4 and 2L27H4; (c) comparison of 3D5H3, 3D7H3, 3L13H3 and 3L36H3; (d) comparison of 3D15H3, 3D1H3 and 3L17H3.

2L28) and one of the cells from the dark zone (2D21) belong to one B cell clone (Table III, clone 1). This clone is characterized by an in-frame rearrangement of a V_H3 family gene and an out-of-frame V_H4 rearrangement. Two different V_κ rearrangements were obtained for this clone of cells, both of which are in-frame. Two of the three in-frame V_H3 rearrangements (2L4H3 and 2L13H3) are identical in sequence and differ from 2C1H3 by nine point mutations (Figures 3a and 4a). The sequences are most homologous to the germline gene VH26 with eight and 11 mutations for 2C1H3 and 2L4H3/2L13H3, respectively. On the assumption that VH26 is the V_H3 gene originally used in the V_H rearrangement of this clone a genealogical tree can be drawn up for the V_H3 rearrangements to explain the observed mutations in a stepwise manner (Figure 4a; in this and the other B cell clones identified the data on κ chain gene rearrangements were insufficient to design meaningful genealogical trees, however, there was no contradiction to the genealogical trees based on heavy chain V region sequences as depicted in Figure 4). The out-of-frame V_H4 rearrangements of cells 2C1 and 2C7 differ from each other by two nucleotides in the complementarity determining region (CDR) I (see Materials and methods for mutations of clonally related sequences not shown in Figures 3–5).

An in-frame $V_\kappa3$ rearrangement was obtained for three cells, 2C7, 2D21 and 2L28. Two of these, 2C7K3 and 2L28K3, are identical and differ by only one nucleotide from 2D21K3 and by two nucleotides from the $V_\kappa3$ germline gene L6 (not shown). A second V_κ rearrangement was amplified from 2C1, which harbours a $V_\kappa2$ gene rearranged in-frame to $J_\kappa2$ (not shown). The amplification of two in-frame V_κ rearrangements for this clone (2C1k2, 2C7k3; Table III), which seems to contradict the principle of allelic exclusion, could be explained by two consecutive rearrangements on one allele [first L6 to $J_\kappa1$ by deletion, then A3 to $J_\kappa2$ by inversion; see Lautner-Rieske *et al.* (1992) and Pargent *et al.* (1991)] as has been described earlier (Huber *et al.*, 1992). This would leave the first rearrangement in the opposite transcriptional orientation on the chromosome. Ongoing V_κ rearrangement has recently also been shown in murine B cells (Harada and Yamagishi, 1991). This process might allow autoreactive B cells to revise the specificity of their antigen receptor and thereby escape deletion (receptor editing; Tiegs *et al.*, 1993).

The two centroblasts 2D13 and 2D18 and the two centrocytes 2L26 and 2L27 represent a second clone of B cells in GC2 (Table III, clone 2). The sequences of an in-frame VH4 rearrangement—amplified from 2D13, 2L26 and 2L27—differ from each other by 5–7 bp and from the most homologous known V_H4 germline gene (VH411) by 16–18 bp (Figures 3b and 4b). A hypothetical genealogical tree of the three V_H4 sequences is shown in Figure 4b. In addition, the clone is characterized by an in-frame $V_\kappa1$ and an out-of-frame $V_\kappa4$ rearrangement. The $V_\kappa1$ rearrangements of 2L26 and 2L27 differ from each other by five mutations and harbour 18 and 15 bp differences, respectively, from the $V_\kappa1$ germline gene L9 (not shown). An unmutated $V_\kappa4$ gene, rearranged out-of-frame to $J_\kappa1$, was amplified from 2D18 and 2L27 (not shown).

Taken together, the sequence analysis of V_H and V_κ rearrangements amplified from cells of GC2 shows a distinction between centroblasts in the dark zone and centrocytes in the light zone (Figure 1b). Whereas V gene sequences of most centroblasts show little or no somatic mutation and most of the cells are clonally unrelated to other B cells in the germinal centre, seven of eight light zone cells for which sequences were obtained belong to either of two B cell clones whose members appear to be intermingled in the light zone area. Ongoing somatic mutation in the course of clonal growth is evident because of sequence differences between clonally related rearrangements.

Germinal centre 3, derived from a cervical lymph node of a 23 year old adult

In the case of GC3, sequence data were obtained for seven centroblasts from the dark zone and 10 centrocytes from the light zone (Figure 2 and Table IV). Thirteen of these 17 cells could be assigned to either of three different B cell clones.

Table IV. V region sequence analysis of germinal centre cells derived from GC3

Cell	V gene family	Germline gene[a]	Base pairs difference	Clone	In-frame?	Ref.
Dark zone						
3D1	VH3	VH26	18	1	+	1
3D5	VH3	VH3-8	20	2	+	2
3D7	VH3	VH3-8	19	2	+	2
3D15	VH3	VH26	19	1	–	1
3D15A	VK3	3L20K3	10	3	+	a
3D26	VK3	3L20K3	10	3	+	a
3D33	VK3	3L20K3	11	3	+	a
Light zone						
3L9	VH3	DP51	19		–	3
3L11	VH1	?		3	–	
3L13	VH3	VH3-8	19	2	+	2
3L17	VH3	VH26	20	1	+	1
3L19	VH3	VP	9		+	4
3L20	VK3	3D15AK3	10		+	a
3L31	VH1	DP14	18	3	+	3
	VH1	?			–	
	VK3	3L20K3	10		+	a
3L33	VK4	VK4	9		–	5
3L34	VK3	3L20K3	10	3	+	a
3L36	VH3	VH3-8	20	2	+	2

The sequences are compared with those of the most homologous germline genes. Most of the cells can be assigned to one of three B cell clones. An in-frame rearrangement is indicated by '+', a non-functional (out-of-frame) one by '–'. References for germline genes: 1: Chen (1990); 2: Winkler *et al.* (1992); 3: Tomlinson *et al.* (1992); 4: Denny *et al.* (1986); 5: Klobeck *et al.* (1985).
[a]The VK3 sequences of 3D15A, 3D26, 3D33, 3L31 and 3L34 are most homologous to the rearranged VK3 gene of 3L20.

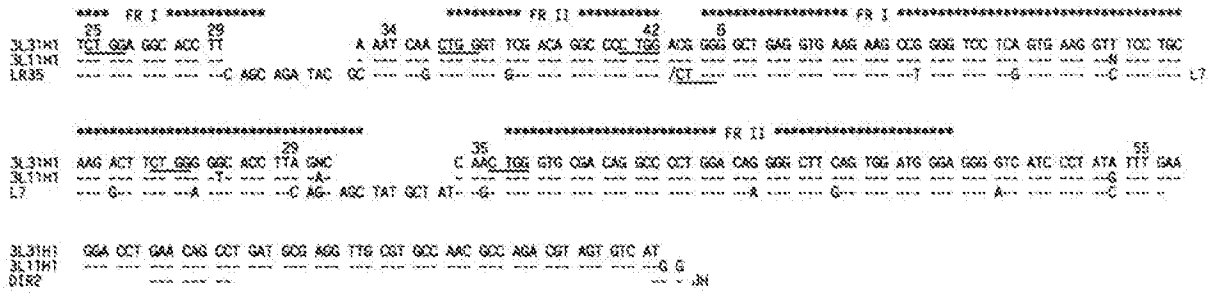

Fig. 5. Sequence analysis of the non-functional V_H1 rearrangement of cells 3L31 and 3L11. Dashes indicate sequence identity. Amino acid numbering is according to Kabat *et al.* (1987). Sequences are compared with those of the LR35 V_H1 gene (Ravetch *et al.*, 1981), the L7 V_H1 gene (Kipps and Duffy, 1991) and the DIR2 gene (Ichihara *et al.*, 1988). A common sequence motif found near non-homologous recombination breakpoints (Chou and Morrison, 1993) is underlined.

One clone, represented by cells 3D5, 3D7, 3L13 and 3L36 (Table IV, clone 2), is characterized by an in-frame rearrangement of a V_H3 family gene with 91% homology to the V_H3 germline gene VH3-8 (Figures 3c and 4c). The four rearrangements differ from each other by 2–11 point mutations. On the assumption that VH3-8 is the germline gene originally used for this rearrangement a genealogical tree can be drawn to explain the relationship between the four sequences (Figure 4c).

A second B cell clone in GC3, defined by cells 3D1, 3D15 and 3L17 (Table IV, clone 1), uses a V_H3 family gene with highest homology to V_H26 (Figures 3d and 4d). The two sequences of 3D1H3 and 3L17H3 represent in-frame rearrangements, whereas the sequence of 3D15H3 shows a 50 bp deletion of framework region (FR) II and part of CDRII. In addition to the deletion the three sequences differ from each other by 8–13 point mutations. The corresponding genealogical tree is shown in Figure 4d.

Sequences of six further cells could be assigned to a third B cell clone in GC3 (3D15A, 3D26, 3D33, 3L11, 3L31 and 3L34; Table IV, clone 3). The non-functional V_H1 rearrangements of 3L11 and 3L31 show several abnormalities: fragments of two V_H1 family gene segments are rearranged to each other, the first one being joined at amino acid position 42 to position 8 of the second one (Figure 5). Both V gene segments show a deletion of 11 nucleotides at the end of FRI and the beginning of CDRI. The homology to V_H1 sequences ends at position 55 in CDRII of the downstream V_H segment. At the 3′ end of the sequence only a short stretch of eight nucleotides shows homology to a D_H-like element (DIR2), and three nucleotides 5′ of the J_H primer sequence are homologous

to J_H germline sequences. Therefore, despite these abnormalities, the sequences show the characteristic features of $V_HD_HJ_H$ rearrangements. The fact that the two sequences harbour at least two mutations (Figure 5) is an additional indication that these unusual sequences represent true $V_HD_HJ_H$ rearrangements as somatic hypermutation is largely restricted to rearranged V genes (reviewed by Kocks and Rajewsky, 1989). Aberrant V_H-V_H genes have been described before (Deane and Norton, 1990; Brokaw *et al.*, 1992) and, interestingly, a recent report (Chou and Morrison, 1993) indicates that two specific tetranucleotide sequences, which are also found near the recombination sites in the sequences shown here, may be important in non-homologous recombinations involving immunoglobulin sequences (Figure 5). However, it is also possible that this unusual V_H sequence did not arise somatically but represents a rearrangement of a pseudogene encoded in the germline.

An in-frame V_x3 rearrangement was amplified for cells 3D15A, 3D26, 3D33, 3L31 and 3L34 (not shown). The sequence of 3D33K3 shows one point mutation relative to the other sequences which are identical to each other.

For the light zone of GC3 four additional sequences were obtained which could not be assigned to any B cell clone (Table IV), although three of them (the V_x rearrangements of 3L20K3 and 3L33K4 and the out-of-frame V_H rearrangement of 3L9H3) may well belong to one or the other of the two clones for which only an in-frame V_H rearrangement was identified.

In summary, GC3 is characterized by the proliferation of three B cell clones which as in the case of GC2 grow in an intermingled fashion and which are subject to ongoing somatic hypermutation (Figure 2b). However, in contrast to GC2, all cells analysed from the dark zone of GC3 appear to express mutated V region genes and belong to the three B cell clones which dominate both light and dark zones of this germinal centre.

Discussion

Reliability and efficiency of single cell gene amplification

The main technical risk of the present approach lies in its extreme sensitivity: since the amplification products from single cells should trace back to single genes, contamination by a single target molecule would be sufficient to give rise to a false positive result. Contamination by single molecules can never be formally excluded. In addition, the amplification reaction from a single cell cannot be repeated, so that at this level the method lacks an important element of scientific experimentation, namely reproducibility. It is the reproducibility of the *pattern* of gene rearrangements on which the present approach relies. Fortunately, in the analysis of rearranged V region genes contamination with cloned DNA from previous experiments can usually be easily identified in that mostly such genes are unique in sequence for a given B cell clone. On the other hand, if the same sequence was amplified from two or more germinal centre cells we cannot formally rule out the possibility that some PCR products result from cross-contamination. This could be true, for example, for the (identical) V_x3 sequences which we obtained from four cells of GC3 (3D15A, 3L31, 3D26 and 3L34).

Despite these problems, several lines of evidence indicate that the approach chosen in the present analysis yields a reliable picture of clonal diversification and expansion of B cells in human germinal centres. (i) In no case was the same sequence or pair of related sequences obtained for cells from two different germinal centres although the cells were amplified in parallel. (ii) A double blind control experiment in which isolated B and T cells were analysed in parallel yielded 25 amplification products from a total of 20 B cells, but only two such products from 19 T cells (see Materials and methods). (iii) The fact that a clear genealogy of somatic mutations in clonally related cells was seen within germinal centres can hardly be explained by cross-contamination of samples.

Ideally, we should be able to amplify from each B cell one (in-frame) or two $V_HD_HJ_H$ and V_xJ_x rearrangements, respectively. The latter would not necessarily be true for cells expressing λ chain (40% of the B cells in the human), but even those mostly retain one or two V_xJ_x joints in their genome (Graninger *et al.*, 1988).

It is apparent from our results that under the present experimental conditions successful amplifications are much less frequent than ideally expected. Even if the efficiency of amplification is expressed as the frequency of cells for which at least one V gene rearrangement could be amplified, the efficiencies range from 55% for mantle zone B cells to 25% for cells in the light zone. The former value was surprisingly constant in three experiments, namely the analysis of GC2 and 3 and the double blind control experiment. It may represent the upper limit of the sensitivity of the method whose limitations are manifold: the cells are micromanipulated from frozen sections with a thickness of about one cell diameter, so that in most cases part of the nucleus is missing. The oligonucleotide primers may not amplify all V_H and V_x rearrangements, because of either the usage of hitherto unknown V genes or somatic mutations. Incubation with proteinase K may not in every instance lead to amplifiable DNA in the absence of further purification steps. Finally, since the sections were not stained with a B cell-specific antibody, some of the isolated cells could be non-B, e.g. T cells [~7% in the mantle zone according to Lusheng *et al.* (1983)].

That the amplification efficiencies in germinal centre cells were lower than those in mantle zone cells (Table I) could again be merely due to technical reasons. Centroblasts are larger than both mantle zone B cells and centrocytes. Therefore, the likelihood of isolating the entire cell nucleus is lower for centroblasts than for the smaller cells. In the case of the centrocytes of the light zone the presence of a considerable fraction of non-B cells has to be taken into account (~14% T cells; Lusheng *et al.*, 1983). However, the low amplification efficiency observed for cells in the light zone of GC2 and 3, and the dark zone of GC3 (Table I), could also be explained by the oligoclonality and somatic hypermutation of the B cells within these structures: the amplification efficiency must drop dramatically if the cells of an entire clone are refractory to V gene amplification, be it because of the particular V genes involved or the occurrence of shared mutations at sites complementary to the primers. In addition, as discussed further below, the hypermutation process as such may negatively affect the amplification efficiencies. These possibilities have to be taken

into account when the clonal composition of GC2 and 3 is evaluated (see below). In the worst (and unlikely) case, the true number of major clones populating these germinal centres could be about double those observed experimentally.

Mantle zone B cells express a diverse repertoire of unmutated V region genes

The origin and function of the small, resting, IgM and IgD expressing B cells in the mantle zone have been a matter of much debate (Kroese *et al.*, 1990). The mantle zone surrounds the germinal centres and corresponds to what is called a primary B cell follicle when a germinal centre is absent. Nine of the 11 rearrangements isolated from mantle zone cells involve unmutated V region genes and this may be true for the remaining two also. In addition, there was no indication of any clonal relationships between mantle zone B cells or to cells in the adjacent germinal centre. These results support the hypothesis that the mantle zone (and therefore also primary B cell follicles) originates from and represents the large pool of small recirculating B cells in the body which are excluded from the rapidly proliferating cells in the germinal centre (Liu *et al.*, 1992) and, at least at young age, largely express germline encoded V region genes, in analogy to what is seen in the mouse (Weiss and Rajewsky, 1990; Gu *et al.*, 1991).

Pattern of ongoing somatic hypermutation in proliferating germinal centre cells

The present data formally demonstrate that somatic hypermutation is ongoing in germinal centre B cells in the course of proliferation, in that V region sequences differing by mutation were isolated from different members of proliferating B cell clones *in situ*. Although the PCR products were directly sequenced we never saw the incorporation of more than one base into a given position on the sequencing gels. This argues against models of somatic hypermutation which predict that a cell undergoing hypermutation should contain multiple copies of a given rearranged V gene which differ from each other by mutations (Manser, 1990; Steele, 1991). Even if mutations were introduced in the process of DNA replication, i.e. the S phase of the cell cycle, we would expect distinct copies of the mutating V gene in the cell in G_2 phase. Our sequence data are insufficient at this point to exclude this latter model, but the model could be directly tested by isolating G_2 phase germinal centre B cells by flow cytometry and subjecting them to single cell V gene amplification. Other models of somatic hypermutation, on the other hand, predict the results obtained in the present analysis, such as the classical model of Brenner and Milstein (1966), which is based on error-prone repair of lesions specifically introduced into rearrranged V genes. Indeed, if such a process occurs at high frequency in germinal centre B cells, the efficiency of V gene amplification by PCR might be negatively affected as we have experimentally observed (see above).

That somatic hypermutation can occur at a very high rate (on the order of one or more mutations per cell division) is again born out by the present results, in accordance with earlier work in the mouse (see Kocks and Rajewsky, 1989; Jacob *et al.*, 1991a). In most cases, homologous V gene sequences isolated from different members of a B cell clone expanding in the germinal centre differed from each other by mutation.

The analysis of genomic rearranged V genes from single cells appears ideally suited to investigate which types of somatic mutation are introduced into these genes. It was not surprising that we almost exclusively observed point mutations, in agreement with earlier data (Kocks and Rajewsky, 1989). An exception was a 50 bp deletion in the in-frame VH3 rearrangement of the centroblast 3D15. Deletions of a few base pairs have been observed in the past in the analysis of somatic hypermutation (see Kocks and Rajewsky, 1989). Although we cannot formally exclude a PCR artefact in the present case, we consider it more likely that larger deletions are occasionally introduced, but were previously missed for technical reasons (e.g. lost in the course of gel purification of cloned PCR products obtained from cell populations). Like in the case of the non-functional V_H1 rearrangement of clone 3 in GC3 (Figure 5) tetranucleotide motifs often found at non-homologous recombination breakpoints are seen near the left recombination site (Figure 3d; Chou and Morrison, 1993). The 50 bp deletion in centroblast 3D15 renders the in-frame VH gene rearrangement of this cell non-functional. It is known from earlier work that somatic mutants expressing non-functional antibodies or lacking antibody expression altogether are rapidly eliminated from the cell population (Weiss *et al.*, 1992). This is also evident from the distribution of replacement and silent mutations in clones 1 and 2 from GC3 where a striking counterselection against replacement mutations is observed (Table V). Because of such counterselection the hypermutation mechanism may introduce deletions and other 'lethal' mutations such as stop codons more frequently than the available data disclose.

In the two B cell clones expanding in GC2 the hypermutation mechanism appears to operate with different efficiency on different V gene rearrangements in the cells of either clone: in both cases significantly fewer mutations were seen in the rearranged V_κ than in the V_H genes (Table III and Figure 4a and b), a phenomenon which has also been observed in the analysis of clonally related hybridomas in the mouse (Clarke *et al.*, 1990; Rickert and Clarke, 1993). Perhaps V genes differ in hypothetical, *cis*-acting elements which target the hypermutation mechanism to the appropriate location. However, the absence of somatic hypermutation in a rearranged V_κ gene could also result from a secondary V_κ gene rearrangement which, if it involves inversion, could place the initial V_κ joint far away from the C_κ locus (Weichold *et al.*, 1990; Huber *et al.*, 1992). Work by Sharpe *et al.* (1991) has shown that an enhancer element downstream of C_κ is essential for full activation of the somatic hypermutation mechanism.

Clonal and intraclonal diversity, compartmentalization and dynamics of germinal centres

A large fraction of the cells in GC2 and 3 for which sequence data were obtained could be assigned to two or three major B cell clones (Figures 1b and 2b). As pointed out above, the true number of such major clones could be somewhat higher, up to a factor of 2. This is in very good agreement with earlier work in rodents, in which germinal centres were estimated to originate from one to six B cell precursors whose progeny was identified by genetic markers (Kroese *et al.*, 1987; Liu *et al.*, 1991a). As the present analysis shows, these clones grow in an intermingled fashion and presumably can reach very large sizes: we estimate that GC2 and 3 contain

Table V. Mutant selection in B cell clones expanding in GC2 and GC3: replacement versus silent mutations

GC	Clone	Length	No. of mutations		R/S ratio			
		FR/CDR	FR	CDR	Observed		Random	
					FR	CDR	FR	CDR
2	1	1.8:1	7	4	6:1	4:0	3.0:1	3.2:1
	2	1.6:1	4	5	4:0	4:1	2.7:1	3.5:1
				sum	10:1	8:1		
3	1	1.5:1	9	5	3:6	2:3	3.3:1	3.6:1
	2	1.3:1	8	9	3:5	4:5	3.3:1	3.2:1
				sum	6:11	6:8		

The designation of clones is as in Tables III and IV. R/S: ratio of replacement (R) to silent (S) mutations. Only nucleotide differences between clonally related sequences are considered. Two mutations in a codon are counted as two separate events. The random R/S ratio was calculated for the common precursors in the genealogical trees (2XH3, 2XH4, 3XH3 and 3YH3) taking the codon composition into account.

$\sim 1 \times 10^8$ B cells each (see Materials and methods). If the majority of these cells represent four to six clones of approximately equal size, then each clone has a size of $\sim$2000 cells and has thus gone through more than 10 generations. Within a clone, most cells apparently express distinct antibody V regions, so that each germinal centre generates many thousands of B cells with different antibody binding sites which are all derived from the few binding sites expressed by the clonal precursors and presumably mostly selected for binding to just a few epitopes of some immunizing antigen(s).

We were surprised to find in the dark zone of GC2 a large population of dividing cells most of which expressed germline-encoded V region genes unrelated to those expressed by the clones dominating the light zone of GC2 and which appeared clonally unrelated among each other. Since we identified five independent in-frame V_H rearrangements in these cells, they must originate from a minimum of five precursor cells. This, together with the presence of cells belonging to the two major clones of GC2, raises the number of B cell precursors for the dark zone of this germinal centre to a minimum of seven. However, the true number of precursor cells is likely to be substantially larger: considering the five independent V_H rearrangements, the probability of picking five clonally unrelated cells from a population of cells from five clones of equal size, is 0.04. This probability rises to 0.15 assuming seven and to 0.3 assuming 10 such clones.

Although other interpretations are possible, we take these data to suggest that initially, germinal centres are populated by a polyclonal set of antigen-activated B cells which proliferate in the dark zone and largely express unmutated V region genes. Through an unknown signal somatic hypermutation is turned on in these cells and rare somatic mutants expressing high affinity antibodies are selected for further expansion in the light zone of the germinal centre, as postulated by MacLennan and colleagues (MacLennan *et al.*, 1991). Unselected cells are rapidly eliminated, whereas high affinity mutants may go through multiple rounds of proliferation and mutation in the dark zone. At the end of this process, the germinal centre is exclusively populated by members of the few clones which have won in the competition. GC3 (Figure 2b) would be representative of this stage of germinal centre development. Significantly, in the clones which have survived in this germinal centre we see strong counterselection against replacement mutations as one might expect for a late stage of somatic evolution of antibody affinity (Table V). In contrast, at an earlier stage, when the system is still in search of an optimal selection, replacement mutations may predominate (GC2; Table V).

In this picture, the clonal complexity of germinal centres estimated in earlier work to be in the order 1 to 6 does not reflect the number of progenitor cells originally populating the germinal centre, but that of the surviving clones.

It is clear that the verification of this model will require a kinetic analysis which can be more easily performed in experimental animals. Such an analysis being under way, we would like to point out that the present approach of analysing cells picked from their histological microenvironment in molecular terms through gene amplification, should be useful in the context of many other physiological and pathological processes. The range of possible applications could be considerably broadened by modifying the technique such that it also allows the analysis of gene expression in single cells isolated from histological sections.

Materials and methods

Tissues

Two human lymph nodes which had been taken out for diagnostic reasons were analysed. GC2 is derived from an inguinal lymph node of a 6 year old child, and GC3 from a cervical lymph node of a 23 year old adult who presented with tonsillitis.

Staining of frozen sections

For immunostaining 10 μm thick frozen sections were put on glass slides, air dried and incubated either with the OKT3 antibody (Ortho Diagnostics) or with the antibody Ki-67 (gift of Dr H.Lemke, Kiel) for 30 min at room temperature. After short washes with Tris-buffered saline, the slides were incubated with biotinylated Fab fragments of a rabbit anti-mouse monoclonal antibody (E413, Dako Diagnostics, Hamburg, 1:400) for 30 min. Following another washing step the slides were incubated with streptavidin–biotin labelled with alkaline phosphatase (K391, Dako Diagnostics, Hamburg) for 30 min. After washing, bound alkaline phosphatase was visualized by staining with new fuchsin. The slides were counterstained with haematoxylin.

Micromanipulation of single cells

The stained sections were incubated with 5 mg/ml collagenase H (Boehringer, Mannheim) in PBS. Single cells were mobilized under the microscope (Olympus) with the help of a hydraulic micromanipulator (Narishige) using 600× magnification. The cells were then aspirated using a micropipette fixed to a second micromanipulator. After the isolation of a cell a photograph was taken to allow the exact localization of that cell in the histological microenvironment. Isolated cells were put into 20 μl PCR buffer (50 mM

Table VI. Sequences of oligonucleotides used as primers for the amplification of rearranged V_H and V_x genes

V_H1	5′-ACTAGTCGACCTCAGTGAAGGT<CT>TCCTGCAAGGC-3′
V_H2	5′-ACTAGTCGACGTCCTGCGCTGGTGAAA<GC>CCACAC-3′
V_H3	5′-ACTAGTCGACGGGTCCCTGAGACTCTCCTGTGCAG-3′
V_H4	5′-ACTAGTCGACCCTGTCCCTCACCTGC<AG>CTGTC-3′
V_H5	5′-ACTAGTCGACAAAAAGCCCGGGGAGTCTCTGA<AG>GA-3′
V_H6	5′-ACTAGTCGACCTGTGCCATCTCCGGGGACAGTG-3′
$3'J_H1,2,4,4$	5′-ACCTGAGGAGACGGTGACCAGGGT-3′
$3'J_H3$	5′-TACCTGAAGAGACGGTGACCATTGT-3′
$3'J_H6$	5′-ACCTGAGGAGACGGTGACCGTGGT-3′
$5'J_H1,3,4,5$	5′-ACTAGTCGACGGTGACCAGGGT<GCT>CC<CT>GGCC-3′
$5'J_H2$	5′-ACTAGTCGACAGTGACCAGGGTGCCACGGCC-3′
$5'J_H6$	5′-ACTAGTCGACGGTGACCGTGGTCCCTTGCC-3′
V_x1	5′-TGATGTCGACATCC<AG>G<TA>TGACCCAGTCTCC<AT>TC-3′
V_x2	5′-TGATGTCGACAG<TA>CTCCACTCTCCCTG<CT>CCGTCA-3′
V_x3	5′-TGATGTCGACTCCAG<GC>CACCCTGTCT<GT>TGTCTC-3′
V_x4	5′-TGATGTCGACTCCCTGGCTGTGTCTCTGGGC-3′
V_x5	5′-TGATGTCGACAGTCTCCAGCATTCATGTCAGCGA-3′
V_x6	5′-TGATGTCGACTT<CT>CTCTCTGTGACTCCA<GA><GA>GGAG-3′
$3'J_x1,2,4$	5′-ACTCACGTTTGAT<TC>TCCA<GC>CTTGGTCC-3′
$3'J_x3$	5′-GTACTTACGTTTGATATCCACTTTGGTCC-3′
$3'J_x5$	5′-GCTTACGTTTAATCTCCAGTCGTGTCC-3′
$5'J_x1,2$	5′-TGATGTCGACTTGAT<CT>TCCA<GC>CTTGGTCCC<CT>TGGC-3′
$5'J_x3$	5′-TGATGTCGACTGATATCCACTTTGGTCCCAGGGC-3′
$5'J_x4$	5′-TGATGTCGACTGATCTCCACCTTGGTCCCTCCGC-3′
$5'J_x5$	5′-TGATGTCGACTAATCTCCAGTCGTGTCCCTTGGC-3′

The V_H and V_x primers hybridize to the FR1 of the respective family. '< >' denotes a nucleotide mix at this position. The $5'J_H$ and $5'J_x$ primers were used at equimolar concentrations. The V_H1 primer also hybridizes to members of the newly defined V_H7 family (Mortari *et al.*, 1992).

KCl, 10 mM Tris–HCl pH 8.4, 0.01% gelatin, 1.5 mM $MgCl_2$) containing 1 ng/μl 5S rRNA and stored at −20°C.

PCR amplification

A set of oligonucleotides was chosen as primers for PCR amplification of rearranged V_H and V_x genes. Twelve V gene family specific primers were used for the six human V_H and six V_x families together with J_H and J_x specific oligonucleotides (see Table VI; the V_H1 primer also amplifies members of the newly defined V_H7 family (Mortari *et al.*, 1992)). A semi-nested PCR approach was chosen. In the first round of amplification the 12 V gene primers and the outer (3′) J_H and J_x primer mixes were used simultaneously in one tube. Taking into account the degeneracy of several of the primers a total of 45 different oligonucleotides is present in the first round. For the second round of amplification aliquots of the first round were reamplified using the same V_H and V_x primers but with nested J_H and J_x primer mixes in seperate reactions for each V gene family.

Single cells in 20 μl PCR buffer were incubated with 0.25 mg/ml proteinase K for 1 h at 50°C. The enzyme was inactivated by heating to 95°C for 10 min. The first round of amplification was carried out in the same reaction tube in a 50 μl volume containing 50 mM KCl, 10 mM Tris–HCl pH 8.4, 0.01% gelatin, 2.5 mM $MgCl_2$, 200 μM each dATP, dGTP, dCTP and dTTP, 2.8 nM each V_H, V_x, $3'J_H$ and $3'J_x$ primer (see Table VI) and 2.5 U of Taq DNA polymerase (Gibco BRL). Enzyme was added after the first denaturation step. The cycle program consisted of one cycle at 95°C for 2 min, 59°C for 4 min, 72°C for 80 s, followed by 34 cycles of 95°C for 90 s, 59°C for 30 s, 72°C for 80 s, followed by 5 min incubation at 72°C.

The second round of amplification was carried out in separate reactions for each of the six V_H and six V_x family specific primers using 1.5 μl of the first round reaction mixture in a 50 μl volume with 50 mM KCl, 10 mM Tris–HCl pH 8.4, 0.01% gelatin, 1.5 or 2.5 mM $MgCl_2$ (1.5 mM for the V_H primers, 2.5 mM for the V_x primers), 200 μM each dATP, dGTP, dCTP and dTTP, 0.125 μM of one of the V_H or V_x primers and 0.125 μM of the $5'J_H$ or $5'J_x$ primer mixes (see Table V). 2.5 U of Taq DNA polymerase were added after the first denaturation step. The cycle program consisted of one cycle at 95°C for 2 min, 61°C or 65°C for 4 min, 72°C for 80 s, followed by 44 cycles of 95°C for 90 s, 61°C or 65°C for 30 s (65°C for the V_H3 and V_H4 primers), 72°C for 80 s, followed by 5 min incubation at 72°C. All amplifications were carried out in a Trio-Thermoblock (Biometra). A 10 μl aliquot of the reaction was analysed on a 2% agarose gel.

Extreme care was taken throughout the procedure to avoid contamination by DNA: gloves were changed frequently, separate equipment and working space were used for pre- and post-PCR manipulations, and aerosol resistant pipette tips were used.

Sequence analysis

PCR products were purified by gel electrophoresis through 2.5% NuSieve GTG agarose (Biozym). An aliquot of the isolated DNA was sequenced using the ds cycle sequencing system (Gibco BRL) as recommended by the supplier. Both strands of the PCR product were sequenced with the primers used in the second round of amplification. The V gene sequences were analysed using DNASIS software (Pharmacia) and the GenBank data library (release 73).

Double blind control experiment

To test the method for efficiency and reliability of the PCR amplification from single micromanipulated cells a control experiment was carried out. For this experiment T cells were micromanipulated from the T cell zone of a lymph node section stained with an anti-CD3 antibody (OKT3) and mantle zone cells as a source of B cells were isolated from another section of the same lymph node stained with the Ki-67 antibody (Gerdes *et al.*, 1984), which allows the identification of the mantle zone of GC. Isolated cells were coded and the analysis was carried out as a double blind experiment. Twenty cells were B cells and 19 were T cells. Ten of the 20 B cells gave at least one PCR product—defined as a visible band of the expected length on an ethidium bromide stained gel—upon V_H and V_x amplification with 25 PCR products altogether. For the 19 T cells, four PCR bands were obtained (sequences not shown). Sequence analysis of these PCR products revealed that only two of them represent V gene rearrangements (an in-frame V_H and an out-of-frame V_x rearrangement), the others being due to non-specific priming in the PCR (e.g. part of the unrearranged J_H locus in one case). Such false positive sequences were never obtained for PCR products derived from B cells.

Estimation of the number of cells in the germinal centres

The number of cells seen on the sections was counted (750 and 760 cells for GC2 and GC3, respectively) and the ratio between length and width of the germinal centre sections was determined. Assuming that the volume of the germinal centres can be approximated as a spheroid and that the germinal centres are cut near the largest area, GC2 and GC3 harbour at least 12 000 and 14 000 cells, respectively. Taking into account that the dark zone of GCs mainly contains B cells whereas in the light zone >10% non-B cells are present (Lusheng *et al.*, 1993) the number of B cells in the two germinal centres may be $\sim 1 \times 10^8$.

Mutations of clonally related sequences not shown in figures

Sequences 2C1H4–2C7H4 had position 32 changed from ACT to CCT [replacement mutation (R)] and position 35a from GAC to AAC (R); 2L26K1–2L27K1 had position 32 changed from TAT to TCT (R), position 48 from ATG to ATC (R), position 52 from ACG to ACT [silent mutation (S)], position 63 from AGA to AGC (R) and position 83 from TCT to TTT (R); 2C7K3 and 2LK28K3–2D21K3 had position 29 changed from GTT to ATT (R); 3D15AK3, 3D26K3, 3D33K3 and 3L31K3–3L34K3 had position 33 changed from TTA to TTG (S).

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EXHIBIT 7

Peripheral Blood Mononuclear Cells of a Patient With Advanced Hodgkin's Lymphoma Give Rise to Permanently Growing Hodgkin-Reed Sternberg Cells

By Jürgen Wolf, Ursula Kapp, Heribert Bohlen, Martin Kornacker, Claudia Schoch, Bettina Stahl, Susanne Mücke, Christof von Kalle, Christa Fonatsch, Hans-Eckart Schaefer, Martin-Leo Hansmann, and Volker Diehl

A novel Hodgkin's disease (HD) derived cell line, L1236, was established from the peripheral blood of a patient with advanced Hodgkin's disease. Analysis of immunoglobulin (Ig) gene rearrangements revealed a biallelic Ig heavy chain and a monoallelic Ig kappa light chain gene rearrangement, pointing to a B-lymphoid origin of these cells. No DNA of Epstein-Barr virus was detected in L1236. The cells expressed the HD-associated surface antigens CD30 and CD15 as well as the transferrin receptor (CD71). Cytogenetic analysis of early passages of L1236 cells revealed a grossly disordered karyotype including cytogenetic aberrations described previously in other HD-derived cell lines. The Hodgkin/Reed-Sternberg (H-RS) cell origin of L1236 cells is further confirmed by Kanzler et al (*Blood* 87:3428, 1996), who found identical Ig gene rearrangement sequences in L1236 cells and H-RS cells of the same patient's bone marrow. L1236 cells expressed antigens necessary for efficient antigen presentation to T cells including HLA class I and II, B7.1 and B7.2, as well as adhesion molecules ICAM 1 and LFA 3. The cells secreted the interleukins (IL)-6, -8, -10, tumor necrosis factor (TNF) α, interferon (IFN) γ, transforming growth factor (TGF) β, and the granulocyte-macrophage colony stimulating factor (GM-CSF). After subcutaneous inoculation into SCID mice, a necrotic regression of initially growing tumors at the injection site was followed by disseminated intralymphatic growth. Our findings, together with the results of Kanzler et al, demonstrate that H-RS cells of B-lymphoid origin were present in the peripheral blood of a patient with advanced HD. These cells exerted a malignant phenotype with regard to their in vitro and in vivo characteristics.

HODGKIN'S LYMPHOMA is unique among malignant lymphomas in that the putative malignant cells, the mononucleated Hodgkin cells, and their bi- or polynucleated Reed-Sternberg cell derivatives, represent only a minority of 0.1% to 1% of the total cell population in affected lymphatic tissue. They are surrounded by reactive T lymphocytes, histiocytes, eosinophils, and stromal cells. Due to the scarcity of the Hodgkin/Reed-Sternberg (H-RS) cells and the resulting technical problems of their in situ characterization, the cellular origin and the clonality of these cells has been a matter of debate in the past decennia.[1] Immunophenotyping of H-RS cells yielded a heterogeneous pattern of lineage specific marker expression disallowing the determination of the normal cellular counterpart.[2] Cytogenetic analysis revealed the presence of numerous structural and numerical chromosomal aberrations, which are neither consistent nor specific.[3] A search for gene expression at the single cell level showed a heterogeneous pattern, which also did not elucidate the origin of these cells.[4]

In numerous human neoplasms the establishment of permanent cell lines, with limitations, has allowed biologic characterization of the tumor cell population. By comparison, the outgrowth of a permanent Hodgkin cell line from Hodgkin's lymphoma-derived tissue cultured in vitro is an extremely rare event.[5] Only 14 cell lines have been established to date, which may be considered to derive from H-RS cells.[6-16] These cell lines have been extensively studied with regard to karyotype, immunophenotype, immunoglobulin (Ig) and T cell receptor (TCR) gene rearrangements and expression of cytokine genes, cytokine receptor genes, and oncogenes. Similar to in situ analysis of biopsy material, results from the analysis of Hodgkin's disease (HD)-derived cell lines were heterogeneous. With the exception of the consistent expression of some surface antigens (CD30, CD15, CD71), no specific antigen expression pattern allowed the determination of the hematopoietic lineage derivation of H-RS cells. The cell lines either express T cell specific markers, B cell specific markers, both, or—in one case (HD-MyZ)—none of them. In analogy, Ig- and TCR-gene rearrangements were found. No specific cytogenetic aberration, consistent oncogene expression, or loss of tumor suppressor gene function could be identified in the cell lines.[17] In addition, validity of results obtained with these cell lines was discussed controversially, since the H-RS cell origin of these cells could not be proven on the molecular level.

Recently, isolation of single H-RS cells from frozen tissue sections by micromanipulation and subsequent polymerase chain reaction (PCR) amplification of Ig gene sequences was used as an experimental tool to determine lineage origin of H-RS cells in biopsy specimen.[18] In three of three cases clonal Ig gene rearrangements were found demonstrating unequivocally a B cell origin of these H-RS cells; however, no functional characterization of B cell-derived H-RS cells could be performed yet.

In this report we describe in vitro cultivation and characterization of H-RS cells with a B lymphoid origin from the peripheral blood of a patient with advanced HD. These cells expressed typical HD-associated surface markers. Cytogenetic analysis revealed a completely aberrant karyotype in-

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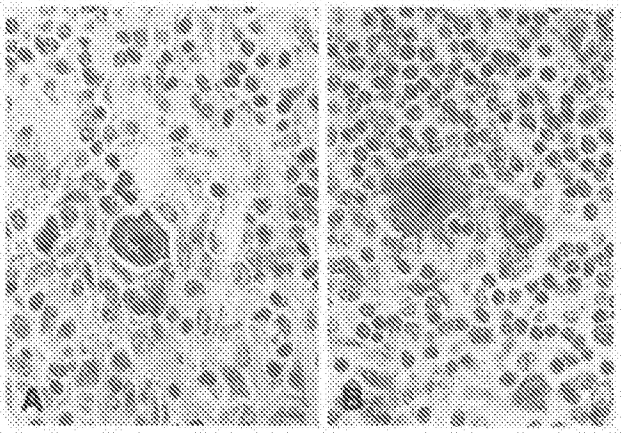


Fig 1. CD30 staining of H-RS cells in lymph node tissue excised for primary diagnosis of HD in 1991. Cervical lymph node, paraffin section, streptavidin-biotin immunostaining, original magnification × 600. (A) In the middle of the picture a Reed-Sternberg cell strongly positive for CD30 surrounded by small lymphocytes and histiocytes; (B) a large Reed-Sternberg cell and a Hodgkin cell positive for CD30.

cluding specific chromosomal rearrangements described previously in other H-RS cell lines. Analysis of Ig gene rearrangements showed a B lymphoid origin of this tumor cell population. The H-RS cell origin of L1236 cells was confirmed by Kanzler et al[9] who detected identical Ig gene rearrangement sequences in L1236 cells and the H-RS cells in the bone marrow of the same patient. Analysis of L1236 cells may thus provide valid information on biologic characteristics of H-RS cells of B lymphoid origin.

MATERIALS AND METHODS

Case report. In 1991, HD of the mixed cellularity subtype, clinical stage IA (cervical lymph node involvement) was diagnosed in a 31-year-old patient (Figs 1 and 2). After radiation therapy (40 Gy),

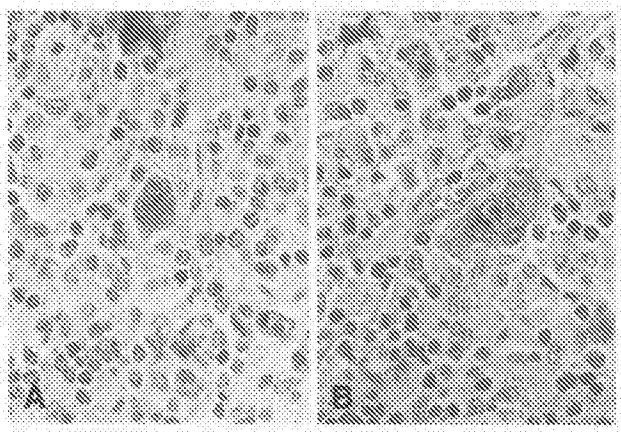


Fig 2. CD15 staining of H-RS cells in lymph node tissue excised for primary diagnosis in 1991. Cervical lymph node, paraffin section, streptavidin-biotin immunostaining, original magnification × 600. (A) A multinucleated Reed-Sternberg cell and a Hodgkin cell positively immunostained with CD15; (B) Hodgkin-infiltrate showing a Reed-Sternberg cell with intracytoplasmic and membrane bound positivity for CD15. In addition, histiocytes, epithelioid cells, and lymphocytes negative for CD15 are present.

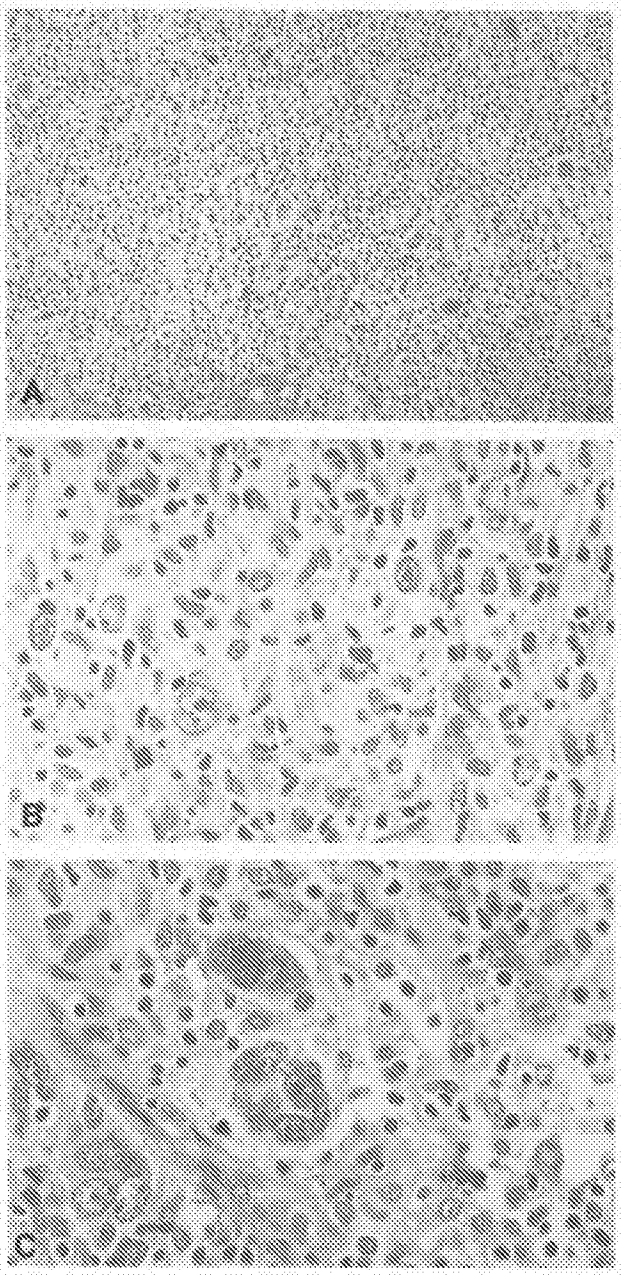


Fig 3. Hematoxylin-eosin staining of lymph node tissue excised for diagnosis of Hodgkin's lymphoma relapse in 1993. Abdomic lymph node, paraffin section. (A) Infiltration of lymph node tissue by HD, original magnification × 175; (B) numerous mononuclear Hodgkin cells, original magnification × 550; (C) polynucleated Reed-Sternberg cells, original magnification × 550.

a complete remission was obtained. A first relapse occurred in 1992 with involvement of the spleen and one splenic hilar lymph node. A splenectomy was performed without any adequate specific therapy. In 1993, a second relapse was diagnosed with involvement of abdominal lymph nodes and bone marrow (Fig 3). The treatment included three cycles of chemotherapy (COPP/ABVD) followed by high-dose chemotherapy with autologous bone marrow transplantation. Three months later the patient again relapsed with extended involvement of bone marrow and the liver. In April 1994, the patient

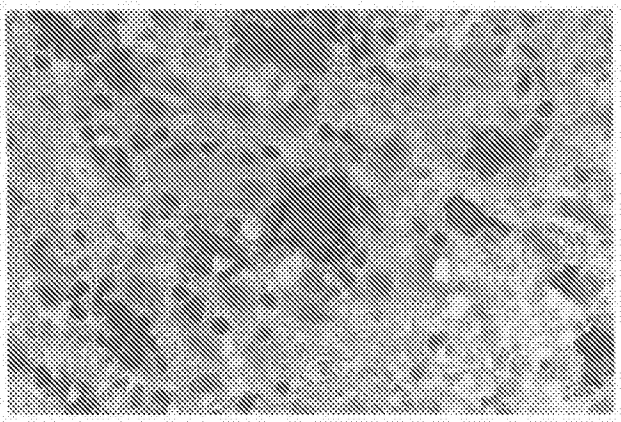


Fig 4. CD30 staining of H-RS cells in bone marrow obtained in 1994. Bone marrow sections, streptavidin-biotin immunostaining, original magnification × 600. In the middle, a Reed-Sternberg cell with intense CD30 staining.

was admitted to our hospital for experimental treatment with Ricin-A coupled anti-CD25 immunotoxins. Before therapy, a bone marrow biopsy was performed showing pronounced infiltration of the bone marrow with Hodgkin's lymphoma (Fig 4). After administration of the first course of immunotoxin therapy no response was observed. Subsequently, salvage chemotherapy (Dexa-BEAM, dose reduction 50%) was begun in April 1994, but had to be stopped due to severe liver toxicity. The patient's condition worsened progressively. He developed fever, pulmonary infiltrations, and died in May of 1994.

Immunohistology of biopsy specimen. Immunohistochemical investigations were performed using monoclonal antibodies against CD30, CD15, and CD80 (Becton Dickinson, Mountain View, CA). The streptavidin-biotin complex method (ABC) was applied. Briefly, the sections were digested with trypsin followed by incubation with the primary antibody (30 min). After a washing step (Tris-buffered saline) the slides were incubated with biotinylated rabbit antimouse

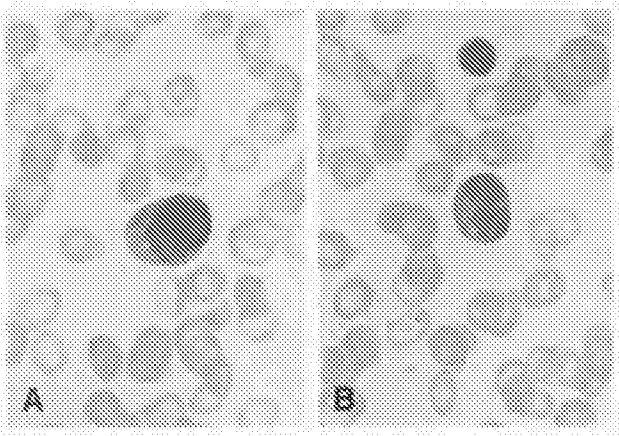
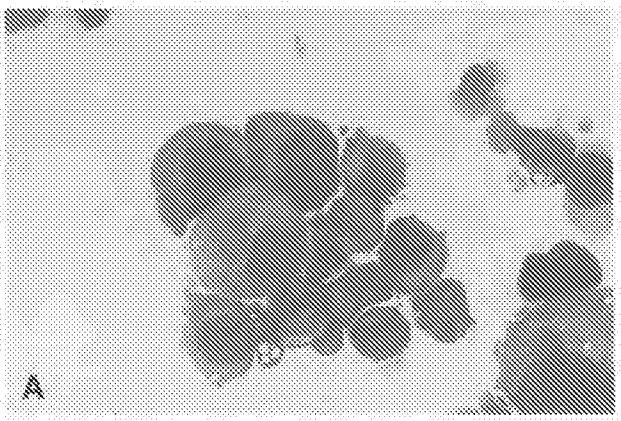


Fig 5. Presence of atypical lymphocytes in the peripheral blood. Blood smears obtained 6 weeks before establishment of the L1236 cell line in 1994, Pappenheim staining, original magnification × 875. (A) Atypical lymphoid cell with irregular nuclear profile and basophilic agranular cytoplasm (a monocyte origin of these cells was excluded by negative enzyme reaction for esterase); (B) in the middle an atypical lymphoid cell as described in (A), and at the top of the picture, a normal lymphocyte with about a half-sized diameter.



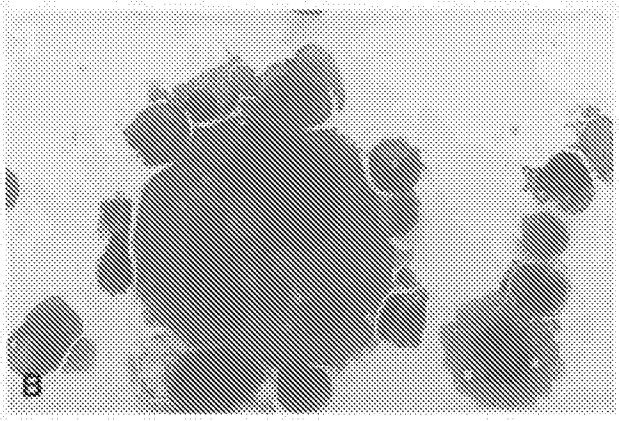


Fig 6. Morphology of in vitro cultivated L1236 cells. Cytospin preparations, original magnification × 880, hematoxylin-eosin staining. (A) Mononuclear cells partly with multiple nucleoli surrounding a binuclear cell; (B) giant cell with numerous nuclei and vacuolized cytoplasm.

F(ab)-fragments (30 min). After another washing step and incubation with streptavidin-biotin complex labeled with alkaline phosphatase (30 min), the enzyme reaction was developed with the Neufuchsin method and the slides were counterstained with hæmalaun and mounted.

Cell culture. Lymphocytes were separated from peripheral blood of the patient by density centrifugation (Ficoll-Hypaque). All cells (initial peripheral blood lymphocytes, established cell line L1236, control Burkitt lymphoma cell line BL 60-P7) were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 50 U/mL penicillin, 50 μg/mL streptomycin, and 4 mmol/L L-glutamine in a 5% CO_2 atmosphere at 37°C.

Analysis of immunophenotype. All monoclonal antibodies (MoAb) against surface antigens used in this study were obtained from Becton Dickinson. For staining, cells were incubated with the first antibody (5 μg/mL; 50 μL/1 × 10^6 cells) for 30 minutes at 4°C. The cells were then washed twice, stained with goat antimouse-FITC (15 min at 4°C) and, after another washing step, analyzed on a FACScan flow cytometer (Becton Dickinson). A minimum of 1 × 10^6 events was analyzed. Immunofluorescence data were displayed on a four-decade log scale. Data were evaluated by CellQuest software (Becton Dickinson).

Cytogenetics. After 3 months in tissue culture, L1236 cells were treated with colcemid (0.1 to 0.5 μg/mL medium) for 0.5 or 2 hours before harvesting. Then they were sedimented at 1,000 rpm, treated with hypotonic KCl solution (75 mmol/L) at room temperature for

20 minutes, fixed in methanol:acetic acid (3:1), dropped on ice-cold slides, and air dried. A modified Giemsa-Acid Saline-Giemsa (GAG) band staining of chromosomes was performed as previously described.[20]

Fluorescence in situ hybridization. Dual color chromosome painting was performed.[21] The air-dried slides were immersed in 70% formamide/2× SSC at 70°C for 2 minutes, transferred to ice-cold ethanol, 70%, 80%, 90%, and 100% sequentially, and air dried. Equal parts of one fluorescein isothiocyanate (FITC)- and one biotin-labeled whole chromosome probe readily prepared with hybridization buffer and competitor DNA were mixed. Whole chromosome probes of chromosomes 1, 2, 3, 4, 7, 8, 10, 11, 12, 14, 15, 16, 17, 20, and 21 were used (Angewandte Gentechnologie Systeme GmbH, Heidelberg, Germany and Oncor, Gaithersburg, MD). The probe mixture was heated in a water bath at 70°C for 5 minutes and then incubated at 37°C for 1 hour. Ten microliter of probe mixture was placed onto slides, covered with coverslips, sealed with rubber cement, and incubated overnight at 37°C. Posthybridization washes were performed at 42°C in 50% formamide/1× SSC and 2× SSC for 2 × 5 minutes each. Hybridization signals were detected and amplified using solution A containing mouse anti-FITC antibodies (0.4 μg/mL; Boehringer Mannheim, Germany) and Texas Red-conjugated streptavidin (5 μg/mL; Dianova, Hamburg, Germany), and solution B containing FITC-conjugated sheep antimouse (12 μg/mL) and biotinylated goat antistreptavidin antibodies (2.5 μg/mL; Vector Laboratories, Burlingame, CT). All reagents were made up in 4× SSC and 1% bovine serum albumin (BSA). Detection of hybridized chromosomes was achieved by covering the slides with a blocking solution (4× SSC, 3% BSA) followed by sequential incubations in the solutions A, B, and A for 20 minutes at 37°C each. Incubations were separated by washes in 4× SSC, 0.1% Tween 20 for 3 × 5 minutes at 42°C. After a final wash, the preparations were counterstained with 700 ng/mL 4,6-diamidino-2-phenylindol (DAPI) in McIlvaine's buffer (5.6 mmol/L citric acid, 87.2 mmol/L disodium phosphate, pH 7.0) for 2 minutes and mounted in 90% glycerol, 10% phosphate-buffered saline, and 1 mg/mL p-phenylenediamine. The hybridized metaphases were photographed under a *Zeiss* epifluorescence microscope equipped with Zeiss filter combination 02 (DAPI), 10 (FITC), and 15 (Texas Red) using Kodak Ektachrome 400 films.

Southern blotting. Extraction of cellular DNA and restriction endonuclease digestion were performed using standard protocols.[22] Briefly, 10 μg of cleaved cellular DNA were separated by agarose gel electrophoresis and transferred to a nylon filter (NEN, Gene Screen Plus, Boston, MA). Hybridization was performed in 50% formamide, 2× SSC at 42°C with ^{32}P-labeled DNA probes.[23] The following probes were used for detection of immunoglobulin gene rearrangements: a genomic 2.2 kb *Sau3a* fragment of the human immunoglobulin heavy chain joining region (IgH *J*),[24] a 2.1 kb *Sac* I-*Eco*RI fragment of the human immunoglobulin κ light chain constant region,[25] and a genomic 3.5 kb *Hind*III-*Eco*RI fragment of the human immunoglobulin λ light chain constant region.[26] T-cell receptor β gene rearrangement was analyzed with an 800 bp cDNA fragment.[27] To detect the presence of EBV DNA, the 3.2 kb *Bgl*II U fragment (nucleotides 13944-17016)[28] specific for the EBV internal repeat 1 (IR 1) was used.

Enzyme-linked immunosorbent assay (ELISA). Production of human cytokines interleukin (IL)-2, -4, -6, -7, -8, -10, interferon (IFN) γ, tumor necrosis factor (TNF) α, transforming growth factor (TGF) β, and granulocyte-macrophage colony stimulating factor (GM-CSF) by the cell line L1236 was determined by an ELISA (Quantikine™ for IL-2, -4, -6, -8, -10, IFNγ, TGFβ, GM-CSF, and TNF α; Biokine® for IL-7, Biermann Diagnostica GmbH, Bad Nauheim, FRG). ELISA was performed according to the manufacturer's instructions. The sensitivity thresholds were: 88 pg/mL (IL-2), 3 pg/mL (IL-4), 0.35 pg/mL (IL-6), 4.1 pg/mL (IL-7), 4.7 pg/mL (IL-8), 1.0 pg/mL (IL-10), 3.0 pg/mL (IFNγ), 0.17 pg/mL (TNFα), 1.5 pg/mL (GM-CSF).

Xenotransplantation. SCID mice were initially obtained from W. Schuler, Basel Institute of Immunology, Switzerland, under licensing of Melvin Bosma, Fox Chase Center, Philadelphia, PA. The animals were propagated under specific pathogen-free (SPF) conditions. Leakiness of the animals was excluded by measurement of their serum Ig levels as described.[29] Mice at the age of 4 to 8 weeks were used for transplantation experiments. 2×10^7 viable cells from exponentially growing cultures in a total volume of 0.2 mL RPMI 1640 without FCS and antibiotics were inoculated subcutaneously (SC) or intraperitoneally (IP) in each flank of an animal. Diameters of the grafts were measured twice weekly.

RESULTS

Peripheral blood mononuclear cells were obtained from the patient the day before the Dexa-BEAM salvage chemotherapy was started in April 1994. The patient's blood count showed 300 leukocytes/μL. In the differential count 60% atypical lymphocytes were described, but no H-RS cells were identified. These atypical lymphocytes with an almost double-size diameter compared with normal lymphocytes were characterized by irregular nuclear profiles and basophilic agranular cytoplasm (Fig 5). To discriminate them from monocytes, esterase-enzyme reaction was performed and showed negative results (data not shown). Lymphocytes were separated from peripheral blood by density centrifugation (Ficoll-Hypaque) and transferred into RPMI tissue culture medium as described earlier. Culture medium was exchanged twice weekly, and dead cells were removed by gentle centrifugation. The cells grew in suspension, forming small clumps up to a maximal density of 6×10^5 cells/mL, before growth arrest occurred. Heterogeneity with regard to size and form between the cells was observed. Most of the cells were mono- or binucleated with round to irregularly shaped large nuclei and a medium-sized basophilic cytoplasma, partly with vacuoles (Fig 6A). A minority of approximately 10% of the cultures consisted of multinucleated giant cells with vacuolated cytoplasm (Fig 6B).

Surface antigen expression of L1236 cells was analyzed on a FACSort. The cells showed surface expression of the HD-associated activation antigens CD30 (HD-associated antigen), CD15 (X-Hapten), and CD71 (transferrin receptor), while they did not express CD25 (IL2-receptor). L1236 cells also expressed CD23 (B-cell associated activation antigen), CD80 (B7-1 molecule), CD86 (B7-2 molecule), the adhesion molecules CD54 (ICAM-1), CD58 (LFA-3), HLA class I as well as class II (HLA-DP, -DR) antigens. With the exception of CD23 no expression of B-lineage antigens (CD19, CD20, CD38, s-Ig κ and λ light chain) was found. The cells were also negative for CD10 (CALLA), T-lineage antigens (CD3, CD4, CD5, CD8, CD45, CD45RO, CD45RA, TCR γ delta), the myeloid-lineage associated antigen CD33, the natural killer cell marker CD16, the monocyte antigen CD14, and the hematopoietic stem cell antigen CD34. Figure 7 shows representative FACS analysis of surface antigen expression on L1236 cells for CD30, CD15, CD71, CD58, CD54, CD23, CD80, HLA class I, and HLA class II.

Immunohistochemical analysis of the H-RS cells in the

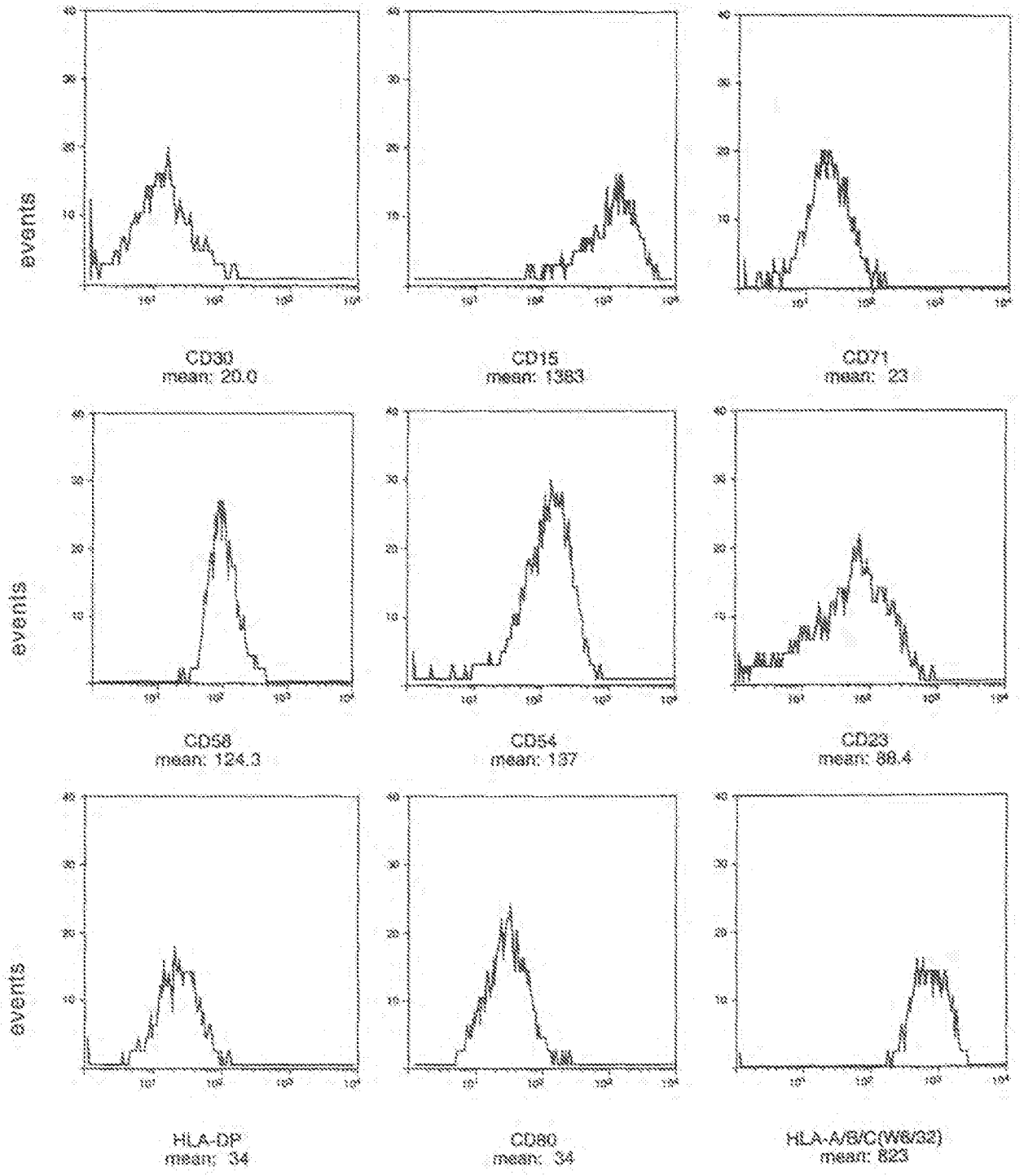


Fig 7. Surface antigen expression on the L1236 cell line. Cells were analyzed on a FACSort flow cytometer after staining with antibodies against the indicated antigens. The staining pattern is shown on a four decade log scale. The mean positivity is given below each histogram.

patient's bone marrow revealed, in concordance with FACS analysis of in vitro cultured L1236 cells, expression of the antigens CD30 (Fig 4), CD15, and CD80. Expression of further antigens was not tested due to the scarcity of the available bone marrow material.

Cytogenetic analysis of the L1236 cells revealed a near-triploid grossly disordered karyotype with numerous structural and numerical aberrations. Nearly all chromosomes were affected by duplications, deletions, inversions, and mainly translocations (Fig 8). To identify the origin of chro-

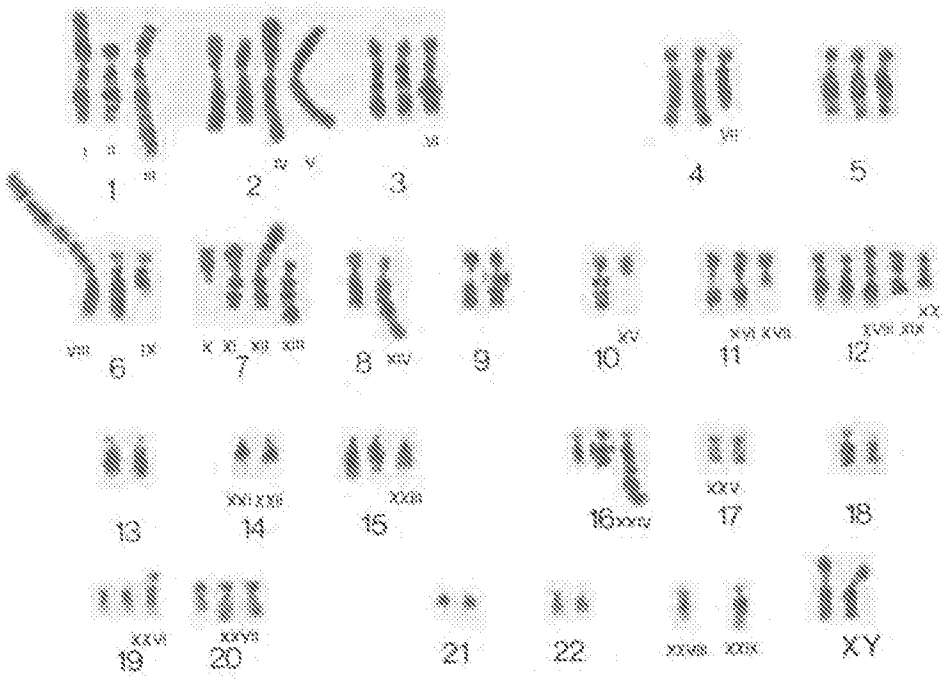


Fig 8. Giemsa banded karyotype of L1236 cells.

mosome segments in marker chromosomes. FISH analysis with painting probes was used. Two examples for the characterization of marker chromosomes using FISH are given in Fig 9A and B. Table 1 summarizes the cytogenetic aberrations identified in L1236 cells.

Absence of EBV DNA in cell line L1236 was demonstrated by Southern blot analysis. L1236 DNA was probed with the *Bgl*II-U 1 fragment of the EBV genome, which hybridizes to the internal repeat 1 (IR 1) of EBV. In contrast, DNA of the EBV positive BL60-P7 cell line harboring about 10 genome copies of integrated EBV,[30] yielded a strong positive hybridization signal even after dilution with EBV-negative placenta DNA in a 1:50 ratio confirming the sensitivity of Southern blot analysis to be below 1 copy of the EBV genome per cell (data not shown).

DNA of L1236 cells was analyzed by Southern blot hybridization for rearrangements of Ig and TCR genes. After restriction enzyme digestion with either *Eco*RI or *Hind*III and subsequent hybridization with an Ig heavy chain joining (IgH J) region fragment, two rearranged fragments were detected, indicating a biallelic Ig heavy chain gene rearrangement. Hybridization with an Ig κ light chain probe after digestion with *Eco*RI, *Hind*III, and *Bam*HI showed each one rearranged and one germline Ig κ light gene. Only germline fragments were detected after hybridizing *Eco*RI or *Hind*III digested L1236 DNA with an Ig λ light chain probe. Figure 10 shows Southern blot analysis for Ig heavy and light chain gene rearrangements with each one representative restriction enzyme. No TCR rearrangements were detected using Southern blot analysis (data not shown).

Cytokine concentrations in the supernatant of exponentially growing L1236 cells were measured using ELISA. L1236 cells produced detectable amounts of the IL-6, -8, -10, INF γ, TGF β, GM-CSF and, with strikingly high amounts, TNF α. No secretion of IL-2 and IL-4 was detected (Table 2).

Native SCID mice ($n = 3$) were inoculated SC with 2×10^7 L1236 cells each. Another group of 3 native SCID mice were inoculated IP with 2×10^7 L1236 cells each. At SC inoculation sites after a latency period of 6 to 8 weeks, initial tumor growth could be observed. When tumors reached a size of 0.5 to 1 cm in diameter, all SC tumors underwent extended necrosis and regressed completely within about 2 weeks. After 4 months the overall condition of all animals (SC and IP inoculations) worsened and they were killed. Autopsy revealed disseminated intralymphatic tumor growth in three of three animals of the SC group and in two of three animals of the IP group. Axillary, inguinal, mesenterical, and portal lymph nodes were enlarged to about 5 mm. Infiltration of extralymphatic tissue was not observed. Figure 11 shows the histology of an enlarged inguinal lymph node. Massive infiltration with L1236 cells exerting a basophilic cytoplasm and irregularly shaped nuclei with one or more nucleoli has taken place resembling the histologic picture of anaplastic large cell lymphoma (ALCL).

DISCUSSION

In the present study we have shown that malignant H-RS cells of B-lymphoid origin are present in the peripheral blood of a patient with advanced HD. These cells gave rise to the permanent cell line L1236 after in vitro cultivation. L1236 cells exerted an H-RS cell morphology and expressed HD-associated activation antigens CD30 (Ki 1), CD71 (transferrin receptor), as well as CD15. They had a grossly disordered karyotype including clonal chromosomal aberrations previously described in HD-derived cell lines L428 and L540.[31,32] In addition to these historically accepted criteria for defining an H-RS cell line, the H-RS cell origin of this

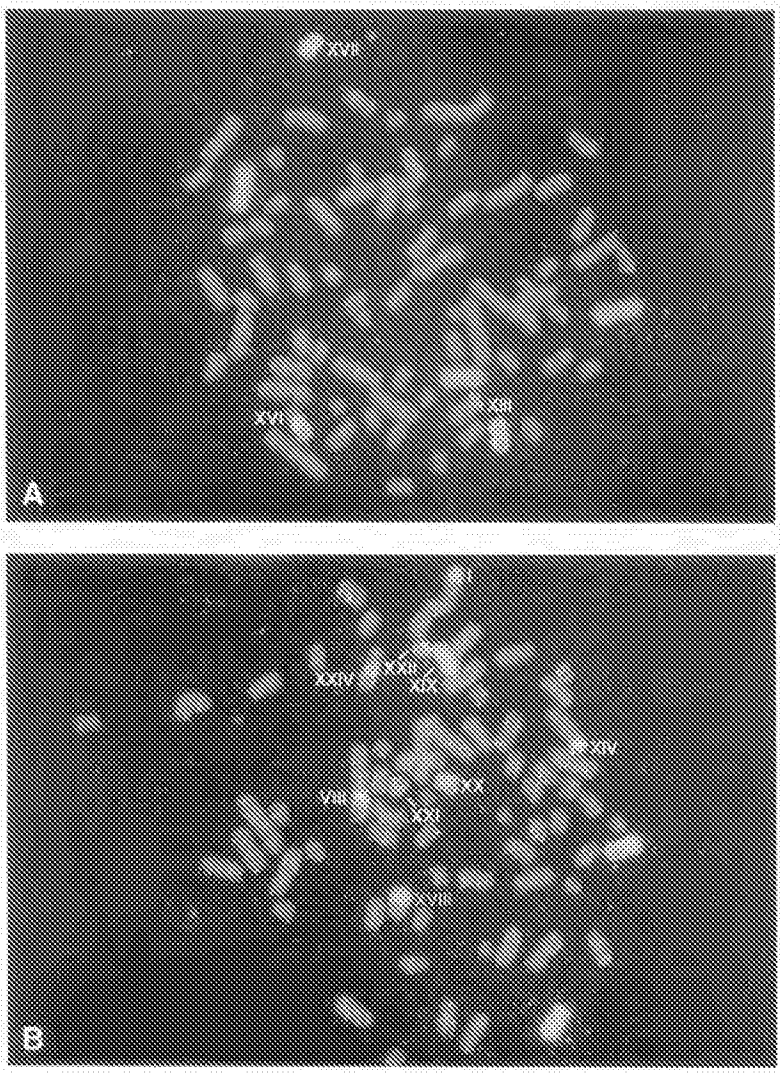


Fig 9. Metaphases of L1236 cells after FISH with painting probes to identify marker chromosomes derived from chromosomes 3, 11, 12, and 14. The painted marker chromosomes are indicated corresponding to Fig 8 and Table 1. (A) Two color fluorescence in situ hybridization with probes for chromosome 3 (TRITC, red) and chromosome 11 (FITC, green). (B) Two color fluorescence in situ hybridization with probes for chromosome 12 (FITC, green) and chromosome 14 (TRITC, red).

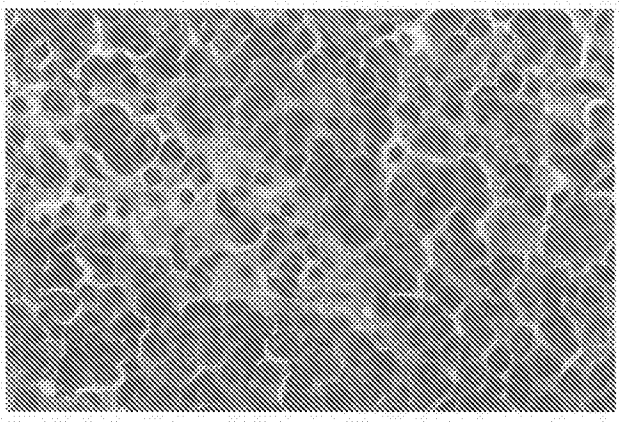


Fig 11. Dissemination of L1236 cells into an inguinal SCID mouse lymph node. Paraffin section, hematoxylin-eosin staining, original magnification × 600. The lymph node infiltration shows features of anaplastic large cell lymphoma (ALCL) with numerous large tumor cells with round to irregularly shaped nuclei and a broad basophilic cytoplasm and some multinucleated cells.

cell line was confirmed by Kanzler et al,[14] who demonstrated identical immunoglobulin rearrangements in L1236 cells and H-RS cells of the patient's bone marrow.

Lineage origin and clonality of H-RS cells is controversely discussed. Analysis of lineage specific antigen expression in HD biopsy specimens or HD-derived cell lines yielded heterogeneous results. Expression of B- or T-cell specific antigens as well as their absence on H-RS cells has been described. Similarly, Ig- and TCR-gene rearrangements or absence of both have been found in HD biopsies and HD-derived cell lines using Southern blot analysis.[39-45] Recently, single cell PCR has been used as an experimental tool to address lineage origin and clonality in H-RS cells. Küppers et al,[18] who picked single H-RS cells from frozen lymph node sections detected clonal Ig gene rearrangements in three of three HD cases analyzed (one nodular sclerosis, one mixed cellularity, one lymphocyte predominant subtype). Thus, in these three cases, as well as in the L1236 cells and one further case (unpublished observation; Kanzler, Küppers, Hansmann, Rajewsky) a clonal B-cell origin of the H-RS cells has definitely been proven. By comparison, Roth et al[46]

Table 1. Marker Chromosomes in L1236 Identified by Giemsa Banding and FISH Analysis

I:	der(1)t(1;14)(p34;?)
II:	der(1)t(1;6)(p32;?)
III:	der(1)dup(1)(?q21;q44)add(1)(p31-32)
IV + V:	dup(2)(p15p23)
VI:	der(3)t(3;18)(p25;?)
VII:	der(4)t(4;6)(q31;?)
VIII:	der(6)dup(6)(p11p25)*
IX:	der(6)t(1;6)(q34;q16)
X:	del(7)(q11)
XI:	der(7)t(7;17)(p22;?)
XII:	der(7)t(7;?)(p22;q22)
XIII:	der(7)†
XIV:	der(8)‡
XV:	del(10)(q11)
XVI:	add(11)(p13)§
XVII:	del(11)(q13)§
XVIII:	der(12)¶
XIX:	del(12)(q22)
XX:	del(12)(q15)
XXI:	der(14)t(1;14)(p34-35;q22)
XXII:	del(14)(q22-24)
XXIII:	der(15)t(15;20)(q22;q11)
XXIV:	der(16)#
XXV:	del(17)(q23q24)
XXVI:	der(18)t(7;18)(?;p13)
XXVII:	add(20)t(15;20)(q22;q11)§
XXVIII:	mar1
XXIX:	mar2

See also Figs 4 and 6.

* Material of chromosomes 14 and 10 is added to the short arm of der(6).

† Pericentric inversion of X1 with additional material of chromosome 11 at the short arm.

‡ Material of chromosomes 1 and 14 is added to 8q24.

§ Material of chromosomes 17 and Y is added to 18p11.

¶ Material of chromosome 14q and 10q is added to 18q24.

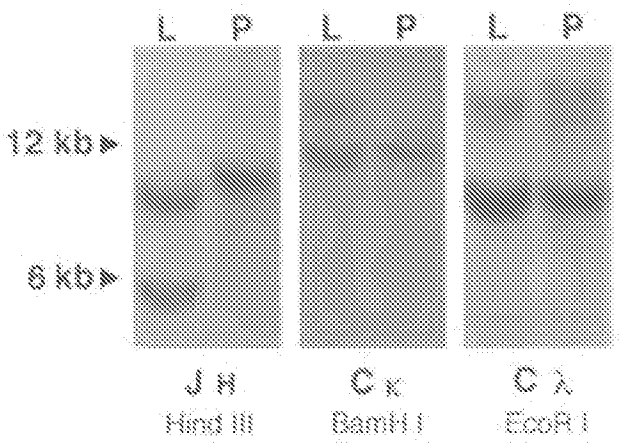


Fig 10. Southern blot analysis for detection of Ig gene rearrangements in L1236 cells. DNA of L1236 cells (L) and of human placenta (P) as germline control was digested with either *Hin*dIII, *Bam*HI, or *Eco*RI. Hybridization with an Ig heavy chain joining region fragment (JH) revealed a biallelic Ig heavy chain gene rearrangement. Hybridization with an Ig κ light chain probe (C κ) resulted in each one rearranged and one germline Ig κ light gene fragment in L1236 DNA. Only germline fragments were detected in L1236 after hybridization with an Ig λ light chain probe (C λ).

isolated H-RS cells from fresh lymph node suspensions of 13 patients with various subtypes of HD and reported the absence of Ig rearrangements in all of them. Delabie et al[37] analyzed resuspended single cells from formalin-fixed paraffin-embedded tissue of lymphocyte predominant HD (LPHD). In four cases analyzed, the CDR3 region of the Ig heavy chain gene rearrangement was amplified from the L&H cells, the putative malignant H-RS cell equivalents of LPHD. Since the amplified regions differed in length and sequence within each case, these experiments suggested a polyclonal B-cell nature of the tumor cells in LPHD. Tamaru et al[38] analyzed HD cases with a B-cell phenotype by PCR amplification of IgH gene rearrangements in whole tissue sections and also found evidence for a B-cell origin of H-RS cells. However, in this analysis it remained unclear whether the clonal Ig gene rearrangements detected originated from the H-RS cells. The question of clonality of H-RS cells was further addressed using interphase cytogenetic and FISH analysis.[39] These authors found evidence for the clonality of H-RS cells in seven of seven cases analyzed. At present it remains unclear whether methodological aspects or sample diversity account for these discrepancies. Possibly in HD different subentities with different lineage origin of H-RS cells exist. In addition, HD may start as a polyclonal disorder and progress to a monoclonal neoplasm in the course of the disease.[40] More cases will have to be analyzed to answer these questions. Nevertheless, the results of Küppers et al[18] clearly demonstrated that at least in a portion of HD-cases the H-RS cells derive from B lymphocytes at various stages of differentiation. The in vitro cultivation of L1236 cells carrying a biallelic heavy chain and a monoallelic κ light chain gene rearrangement provides evidence that in HD of B-cell origin in advanced stages, the H-RS cells can be present in the peripheral blood even if they are not identified as H-RS cells. In addition, the H-RS cell origin of the L1236 cell line is proven not only by analysis of

Table 2. Cytokine Production of L1236 Cells

Cytokine	ELISA
IL-3	neg
IL-4	neg
IL-5	503
IL-7	neg
IL-8	152
IL-10	72
INFγ	23
TNFα	8,348
TGFβ	516
GM-CSF	805

Cytokine production was measured in the supernatant of exponentially growing L1236 cells (4×10^5 cells/mL). The values given represent cytokine concentrations (pg/mL). Each value is a mean of two independently measured values. The sensitivity thresholds of ELISA for each cytokine is given in Materials and Methods. Negative (neg) means beyond the indicated sensitivity threshold.

morphology, surface antigen expression, and cytogenetics, but also on the molecular level by detection of identical Ig gene rearrangement sequences in the H-RS cells of the patient's bone marrow.[19] Further genetic probes as well as specific monoclonal antibodies against L1236 cells may be developed. This cell line, thus, represents a valid biological model for the study of biology and homing pattern of HD and its possible relation to B-cell differentiation.

HD shares many clinical and biological characteristics with an inflammatory process, such as, eg, fluctuating fever, nightsweats, and elevated levels of IL-2 receptor in the patients serum. In affected lymphatic tissue H-RS cells are surrounded mostly by T lymphocytes. Expression of CD4, CD45RO, and CD45RB characterizes these lymphocytes as T helper cells, expression of CD38 may indicate their previous activation.[41] These observations led to the hypothesis, that in HD an atypic, ie, non–self-limited immune response takes place.[40] L1236 cells express HLA class I and class II molecules, the B7.1 and B7.2 molecules (CD80, CD86) and the adhesion molecules ICAM-1 (CD 54) and LFA-3 (CD 58). All these molecules have been found to be crucial for physiological T-cell recruitment and activation; the B7 molecule via ligation to the CD28 molecule on the T cell and the adhesion molecules ICAM-1/LFA-3 by binding to their T-cell counterparts CD2/LFA3. It is tempting to speculate that expression of these antigens on L1236 cells and other HD-derived cell lines (authors' own unpublished data) indicates an original function as antigen-presenting cells in an (unsuccessful) T-cell response against a still unknown viral or cellular target antigen.

Although no specific chromosome aberration has been delineated in HD up to now, cytogenetic peculiarities can be observed that differ from other lymphomas. In most cases near triploid to tetraploid chromosome numbers and an excess of structural aberrations were observed. The chromosome bands 1p13-21, 2p16-p21, 4q25-q28, 6q15-q21, 7q11.2-q36, 11q13-q23, 12p11-p13, 12q22-q23, and 19p13 are nonrandomly involved in rearrangements in HD.[42-45] Moreover, the short arms of acrocentric chromosomes, harboring genes for the ribosomal RNA, the so-called nucleolus organizer regions, seem to be affected by chromosome aberrations.[42] The new established cell line L1236 is characterized by a near-triploid karyotype with multiple structural rearrangements involving chromosome bands that have been reported to be consistently rearranged in HD. As observed in the HD-derived cell lines L428 and L540, chromosomes 1, 2, 6, 7, 11, and 12 are involved in structural aberrations in L1236, too. A deletion in the long arm of chromosome 11—del(11)(q13-q14)—was observed in all three cell lines, a tetrasomy of chromosome 2 occurred in L540 and L1236, a rearrangement of the short arm of chromosome 2 could be identified in L428 as well as in L1236, the marker chromosome XX[del(12) (q15)] of L1236 was also found in L428.[31,42] By comparison, the very complex composition of marker chromosomes VIII and XXIV in L1236 cells suggests that these markers developed during in vitro cultivation and do not represent HD specific anomalies. In other malignant diseases with less complex aberrant karyotypes specific so-called primary chromosome aberrations were identified that are thought to cause malignant transformation, such as, eg, Ig-gene/c-myc translocations in Burkitt's lymphoma or the bcr/c-abl translocation in chronic myeloid leukemia. In contrast, in HD cytogenetic analysis of primary tumor material as well as of HD-derived cell lines show complex chromosome anomalies, so that no primary chromosomal aberration could be delineated up to now. It might be conceivable that in HD several karyotype changes have to be acquired before the disease becomes clinically apparent[46] and before cultivation for karyotype analysis is possible. In late stages of the disease, a complex aberrant karyotype as present in L1236 cells might then correspond to an aggressive growth of H-RS cells no more restricted to lymphatic tissue and resistant to radiation and polychemotherapy.

The malignant growth potential of L1236 cells is reflected by their intralymphatic dissemination in SCID mice after SC and IP inoculation. While HD-like lesions were only exceptionally observed after transplantation of HD biopsy material into SCID mice,[47] disseminated intralymphatic growth of HD-derived cell lines has been described.[13,48] The dissemination pattern of L1236 cells resembles that of the HD-derived cell lines L540 and its subline L540Cy with involvement of axillary, mediastinal, mesenteric, and inguinal lymph nodes. Because of the similarity with the spread of HD in humans, L1236 represents a suitable tool for studying in vivo growth characteristics of H-RS cells as well as for preclinical testing of new treatment modalities. After SC inoculation into SCID-mice, HD-derived cell lines formed progressively growing tumors at the injection site.[49] In contrast, L1236 cells inoculated SC only initially formed small tumors that underwent necrosis and regression. This resembles the in vivo growth pattern of EBV-immortalized lymphocytes in SCID mice. Despite intralymphatic dissemination of LCL cells after SC inoculation, tumors at the injection site regressed with necrosis.[50] In nude mice there is evidence that regression of LCL tumors after SC inoculation is caused by a cytokine-induced host response.[51,52] It remains to be established whether one of the numerous cytokines secreted by L1236 cells is responsible for a locally restricted anti-tumor host response in nonlymphatic SCID mouse tissue.

The cultivation of L1236 H-RS cells from the peripheral blood of a patient with advanced stage disease might also have clinical implications. After failure of first line chemotherapy, an increasing number of HD patients are treated by high-dose chemotherapy followed by autologous bone marrow transplantation or blood stem cell transplantation. This therapeutic procedure has been reported to improve rates of complete remissions and disease free survival.[53,54] Up to 50% of the patients, however, suffer from lymphoma relapse. Recently, a case of NHL relapse with extended pulmonary infiltrations early after autologous bone marrow transplantation was determined to be due to contamination of the infused bone marrow with tumor cells.[55] Similarly, a fulminant course of HD relapse after autologous peripheral stem cell transplantation was observed in our clinic (unpublished observation). At present, it remains an open question whether early relapse in these cases reflects survival of H-RS cells during high-dose chemotherapy, or, alternatively, tumor cell contamination of the grafted cells and fulminant

spread due to the missing T-cell control after intensive cytotoxic therapy. The results presented here, together with the data of Kanzler et al,[40] for the first time formally demonstrate the presence of H-RS cells in the peripheral blood of a patient with advanced HD. Thus, autologous blood stem cell transplantation after high-dose chemotherapy in these patients includes the risk of reinfusing malignant cells. Absence of CD34 expression on L1236 cells, however, suggests that CD34 enrichment before autologous stem cell transplantation possibly represents an efficient purging procedure for H-RS cells.

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EXHIBIT PA-1

In a future paper, we will examine strategies to search for the stringiness of an anisotropy pattern in more detail by running Monte Carlo experiments on our anisotropy map. We will also use Monte Carlo experiments to determine the proper limits on $G\mu/c^2$ from small-angular-scale experiments. Finally, we will also attempt a more quantitative estimate of other anisotropies predicted by cosmic string models, which might interfere with the stringy nature of the anisotropies discussed here.

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Amplification and analysis of DNA sequences in single human sperm and diploid cells

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The use of the polymerase chain reaction for analysing DNA sequences in individual diploid cells and human sperm shows that two genetic loci can be co-amplified from a single sperm, which may allow the analysis of previously inaccessible genetic phenomena.

THE construction of genetic maps in higher organisms requires analysis of the progeny of selected matings or calculation of linkage relationships by pedigree analysis. In humans only the latter is possible. Using restriction fragment length polymorphisms (RFLPs), there has been significant progress towards the construction of a human linkage map (for review see ref. 1). To locate genes with known phenotypic effects relative to RFLP markers there has been a concerted effort to establish a panel of genetic markers at about 10 cM (1 cM = 1% recombination) intervals so that no gene will be further than 5 cM away from an RFLP (ref. 2). Pedigree analysis is thought to be able to measure genetic distances of about 1 cM encompassing about 1,000 kilobases (kb) of DNA with statistical reliability. The analysis of smaller distances requires such a large number of individuals from informative familes that it is impractical. If the genotype of large numbers of individual sperm could be determined, the measurement of genetic recombination over shorter physical distances could be accomplished at a resolution far greater than that currently possible and without family studies. We have therefore attempted to define the conditions required to analyse DNA sequences in single cells using the polymerase chain reaction (PCR, refs 3 and 4). We studied single diploid cells and then single sperm from an individual heterozygous at two genetic loci found on non-homologous chromosomes. For each locus we determined which of the two alleles were present in any one sperm and analysed the independent assortment of the alleles at a single locus and the independent segregation of genes on non-homologous chromosomes.

Analysis in single diploid cells

We studied the human β-globin gene locus in individual diploid cells from two tissue culture cell lines. One was derived from a homozygous individual for the sickle-cell mutation at codon six (β^S); the other was homozygous for the normal β^A allele. PCR primers that amplify a β-globin fragment containing codon six and allele-specific oligonucleotide probes (ASO) which can distinguish between these two alleles have already been described[3,5]. We co-cultivated the cells homozygous for β^A and cells homozygous for β^S in the same tissue culture flask for several days. Individual cells from this mixture were drawn into a thin plastic pipette during observation under a phase-contrast microscope. Each individual cell was delivered into a PCR tube containing a lysis solution and, after incubation, PCR buffer containing deoxyribonucleotides, *Taq* DNA polymerase[6] and a set of PCR primers that amplify the informative region of the globin gene was added. After DNA denaturation 50 cycles of amplification were performed[6]. Aliquots from each sample of amplified product were hybridized separately with the β^A and β^S probes after fixation to nylon membranes[5,7]. The results of this co-cultivation experiment are shown in Fig. 1. Out of the 37 cells analysed, 84% hybridized with only one of the two allele-specific probes; 19 with the β^A probe and 12 with the β^S probe. None of the 12 control tubes, which received water instead of a cell, was positive, indicating that DNA contamination was insignificant. No sample hybridized with both probes, indicating that a single cell only was introduced into each tube and that DNA from lysed cells present in the co-cultivation mixture did not adhere to individual cells.

The amount of amplified β-globin gene product produced by PCR of a single cell was determined by comparing the intensity of the hybridization signal obtained with that from known amounts of plasmid DNA carrying the globin gene spotted on the same filter (Fig. 1). We estimate that, starting with a diploid amount of globin DNA (3.3×10^{-9} fmol), between 5 and 500 fmol of PCR product was produced in 50 cycles. This is equivalent to an average amplification ratio of 7.6×10^{10} with

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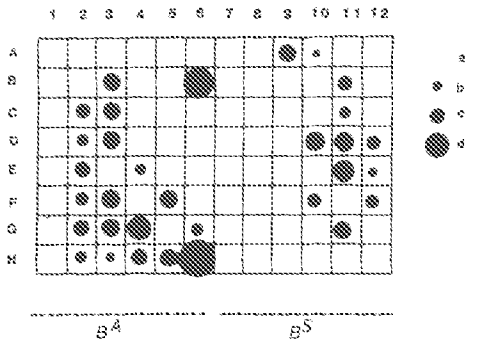

Fig. 1 PCR analysis of the β-globin gene in individual tissue culture cells. Each of two aliquots from the PCR products of a single cell were placed in the same row of the dot blot apparatus separated by six columns. Half of the filter was hybridized to the β^A ASO and the other to the β^S ASO. 1A–1H (7A–7H) and 2A, 2B (8A, 8B) were water blanks. 6H and 12H are aliquots of PCR product of purified β^A DNA. One, 3, 10 and 30 fmol of β^S gene containing plasmid were dotted as hybridization standards at positions a, b, c and d respectively. The remaining samples were individual tissue culture cells. After washing three times single cells were selected from a cell suspension ($1–3 \times 10^6$ ml^{-1}) with a Zeiss phase-contrast microscope at ×100 using a plastic needle with a 0.1-mm-diameter opening made by pulling a flamed disposable 1-ml-graduated plastic pipet. Each single cell sample was delivered into a 0.5 ml plastic microfuge tube containing 10 μl autoclaved distilled water. The cells were lysed using a slight modification of a published sperm lysis procedure[16]. The sample was adjusted to a final volume of 20 μl containing 1 × PCR reaction buffer (ref. 6; 50 mM KCl, 10 mM Tris-HCl *pH* 8.3, 2.5 mM $MgCl_2$, 0.1 mg ml^{-1} gelatin), 0.05 mg ml^{-1} proteinase K, 20 mM DTT, and 1.7 μM SDS. After one hour at 37 °C, samples were heated to 85 °C, and suspended in 100 μl PCR reaction mix containing 1 × PCR buffer, 1 μM each oligonucleotide PCR primer, 187.5 μM each of dATP, dCTP, dGTP and dTTP, 100 ng *E. coli* DNA, 2 units of *Thermus auquaticus* thermostable DNA polymerase (Perkin Elmer-Cetus Instruments), and 60 μl mineral oil to prevent evaporation. The PCR reactions were carried out on a DNA Thermal Cycler (Perkin Elmer-Cetus Instruments). After heating at 95 °C for 10 min to ensure DNA denaturation, each cycle of PCR consisted of incubation at 95 °C for 15 s, 15 s incubation at 54 °C and a 1 min incubation at 72 °C. After fifty cycles of PCR, dot blot analysis of 20 μl samples of the PCR reaction were carried out using β^S- and β^A-allele specific probes[5].

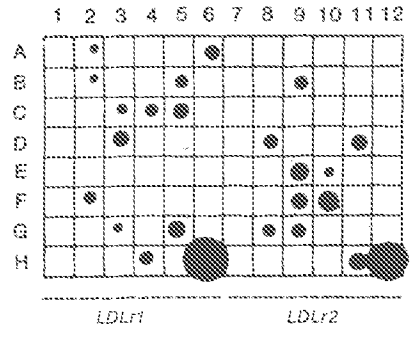

Fig. 2 PCR analysis of the LDL receptor locus of individual human sperm. 1A–1H (7A–7H) are water blanks. 6H and 12H are aliquots of amplified DNA from an *LDLr*1/*LDLr*2 heterozygote. The remaining samples are from individual sperm. The organization of the samples on the dot blot is as in Fig. 1. Sperm were purified from semen by a modification of a published procedure[10]: 0.5 ml semen was mixed with 3 ml of 40% sucrose. The mixture was applied to the top of a sucrose step gradient made by adding 3.5 ml 90%, 70% and 50% (w/v) sucrose successively to a 15-ml graduated plastic tube (Falcon 2095). The sample was spun at 14,500g for two hours at room temperature. 0.5 ml of the interface between 70% and 90% sucrose was collected and applied to an identical sucrose gradient. This was repeated twice more. Single sperm were isolated in the same way as individual diploid cells using a sperm suspension at a concentration of 1×10^6 sperm ml^{-1}. The sequence of the two primers used for PCR of the *LDLr* locus were 5'AGTGCCAACCGCCTCACAGG3' and 5'CCTCTCACACCAGTTCACTC3'. The ASO for the *LDLr*1 allele had the sequence 5'AGGATATGGTCCTCTTCCA3' whereas the *LDLr*2 ASO had the sequence 5'TGGAAGAGAACCATATCCT3'. The PCR and dot blot analysis were performed as in Fig. 1 except that the final washes of the filters hybridized with the *LDLr* probes (and *HLA DQA* probes, see Fig. 3) were at 56 °C.

an average efficiency per cycle of 65%. The precise extent of amplification may have been slightly lower if these cell lines contained more than two copies of chromosome 11. Considering the extent of amplification, elimination of all sources of possible contamination is critical to the success of these experiments.

Analysis in single human sperm

The genotype of single sperm derived from an individual heterozygous at the gene coding for the low density lipoprotein receptor (the *LDLr* gene), which has been localized to chromosome 19 (ref. 8), was analysed next. We adapted the detection of an *LDLr* polymorphism[9] to PCR and ASO analysis using DNA sequence information (D. Russell, unpublished data), with the PCR primers and probes shown in the Fig. 2 legend. The size of the expected PCR product was 254 base pairs (bp).

Sperm were purified from a semen sample by centrifugation through a sucrose step gradient[10] and stored for 8 months at −20 °C. Individual sperm were drawn into a fine plastic needle under microscopic observation and delivered to a tube for lysis and amplification. In a series of experiments we analysed the *LDLr* genotypes in 80 individual sperm: typical results from one such experiment are shown in Fig. 2. Altogether 55% of the sperm gave a hybridization signal. Twenty-two carried one allele; 21 the other. Only one sample was positive with both probes. Sixteen additional control tubes which received all of the reagents but no sperm did not give any hybridization signal. The distribution of the two amplified alleles obeyed Mendel's law of independent segregation, indicating that the PCR reactions were initiated with a single meiotic product and that no contaminating diploid DNA sequences were present.

Independent assortment of chromosomes

We attempted to amplify simultaneously DNA sequences at two different loci on non-homologous chromosomes in a single sperm. Our sperm donor was heterozygous at the *HLA DQ-α* locus (*DQA*) on chromosome six, as well as at the *LDLr* gene. Primers for amplification of the first gene and probes to distinguish between allelic variants have been described previously[5,11]. The predicted size of the PCR product was 242 bp. Initial experiments in which primers for both loci were present throughout the entire amplification experiment were unsuccessful, so we performed only the first 20 amplification cycles in the presence of both primer pairs. After this primary amplification, 1/50 of the reaction mixture was placed in a tube and diluted with a PCR solution containing the *HLA* primers only and another aliquot was diluted with a PCR mix containing the *LDLr* primers only. After an additional 45 cycles of amplification, part of each secondary reaction was hybridized to either of the two ASOs for that locus. A total of 150 individual sperm were analysed, in a series of such experiments (Table 1; representative data are shown in Fig. 3). Twenty-seven samples did not exhibit amplification of either locus, but we did detect hybridization signals in 123 samples (82%). In nine of these samples, two alleles from at least one of the two loci were

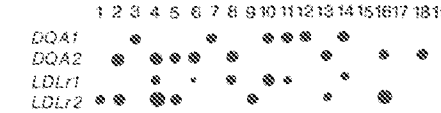

Fig. 3 PCR analysis of individual sperm simultaneously amplified with *HLA DQA* and *LDLr* primers and analysed with allele-specific probes for the *DQA* alleles 1 and 2 (*DQA1*, *DQA2*) and *LDLr* alleles *1* and *2* (*LDLr1*, *LDLr2*). Water blanks are in positions 19–21. Examples of sperm where one allele at each locus is amplified are shown in positions 2, 5, 6, 8, 10, 11, 13, 14 and 16. The remaining samples can be divided into those that amplified more than one allele at a locus (position 4), only one allele at one locus (positions 1, 3, 7, 9, 12, 17, 18) or neither allele (position 15). During the first 20 cycles both loci were amplified simultaneously with both sets of primers present at 1 μM. A 2 μl aliquot from each reaction was then added to each of two tubes with 100 μl fresh PCR reaction buffer containing only one of the primer pairs. Forty-five additional PCR cycles were then performed.

detected and these samples were excluded from further analysis. These samples may have contained two sperm of different genotypes. On the other hand these nine samples might have resulted from non-disjunction events. The analysis of only two loci cannot distinguish between these possibilities.

Among the remaining 114 sperm, 96 could be typed at the *LDLr* locus with 45 having *LDLr*1 and 51 having *LDLr*2. The two alleles segregate in the expected 1:1 ratio (Chi square = 0.375, $0.75 > P > 0.5$, 1 degree of freedom). Eighty-eight could be typed at the *DQA* locus: 53 had the *DQA*1 allele, 35 the *DQA*2. The segregation of the *DQA* alleles with the expected 1:1 ratio is at the borderline of statistical significance (Chi square = 3.68, $0.1 > P > 0.05$, 1 degree of freedom). This could represent a statistical fluctuation, unequal amplification of the two *DQA* alleles resulting from base mismatches between the PCR primers and the *DQA* DNA sequence of our particular donor due to the extensive polymorphism of this locus, or some unusual genetic phenomenon such as segregation distortion.

Seventy of the 114 sperm (61%) that gave hybridization signals could be typed at both loci. The independent segregation of chromosome six and chromosome 19 should result in the equally frequent occurrence of the four possible gametes: *DQA*1, *LDLr*1; *DQA*1, *LDLr*2; *DQA*2, *LDLr*1; *DQA*2, *LDLr*2. We actually observed 21, 18, 14 and 17 of each type respectively; the difference is not statistically significant (Chi square = 1.43, $0.75 > P > 0.5$, 3 degrees of freedom). These results show that we can reliably and with reasonable efficiency simultaneously determine the genotype of individual sperm at two distinct genetic loci.

Among the 114 samples that gave a hybridization signal, 18 showed one of the two *DQA* alleles but an *LDLr* product could not be detected. Twenty-six sperm showed one of the two *LDLr* alleles but no amplification of the *HLA* locus. These sperm could be nullosomic for one of the two chromosomes we studied due to a non-disjunction event. There may also have been a failure to amplify one of the two loci. Because we only looked at a single locus on each chromosome we cannot distinguish between these possibilities.

The relative frequency of successful amplification of the *LDLr* and *DQA* loci in single sperm is approximately the same. Among 141 samples, 88 amplified *DQA* and 96 amplified *LDLr*. We are unable to calculate accurately the absolute frequency of success because we cannot be sure that every one of the 141 samples did in fact contain a sperm. As a result, we cannot use our data to calculate accurately the expected frequency of samples with a single allele amplified at both loci, at only one locus or at neither locus assuming that amplification at each locus is an independent event. Amplification of both loci may not be independent events if sperm lysis is not uniform from sample to sample. Thus the assessibility of one of the target DNA molecules to the PCR reagents might be positively correlated with the accessibility of the other. Improving the absolute rate of successful amplification of single sperm may depend upon improving lysis procedures, thereby enhancing target DNA accessibility.

Discussion

The analysis of the genotype of single sperm at the DNA level is a unique tool for the study of problems in human genetics considered intractable using current approaches. To date there have been no practical methods for accurate measurement of genetic distances of less than 1 cM. A significant advantage of the approach described here is that a large number of meiotic products can be examined from a single individual allowing determination of the recombination frequency between genetic markers which are physically very close. In conjunction with gel electrophoresis procedures for very large DNA fragments and chromosome-walking data it should be possible to measure the frequency of recombination between genetic markers whose physical distance apart is known precisely. This would allow the analysis of recombination frequency as a function of physical distance and a test of the conventionally accepted value of one per cent recombination per million base pairs[12] for specific chromosomal regions. Because it should be possible to obtain statistically significant data on recombination frequencies from a single individual, it should also be possible to determine for the first time whether different individuals have the same or different rates of recombination for the same interval.

The ability to measure recombination over short physical distances will be especially useful in the study of recombination hot spots. The effect that a hot spot can have on recombination between flanking markers depends upon how far away the flanking marker loci are from the ends of the hot spot. Thus a 1-kb DNA segment with a recombination potential which is 10-fold greater than normal DNA will have very little effect on recombination measured between markers that are 500 kb to either side of the hot spot. Pedigree analysis cannot measure recombination over the short intervals typical of many of the hot spot regions that have been deduced from population genetics data[13–15], given the number of informative families required and the effort involved in obtaining the data. With PCR, we can envisage typing as many as 500 meiotic products in a week. Decreasing the number of samples containing two sperm and increasing the efficiency of amplification of both loci simultaneously will be required for the highest resolution

Table 1 Amplification of sequences at two different loci

Total number of sperm examined	150
No signal	27
*DQA*1, *LDLr*1	21
*DQA*1, *LDLr*2	18
*DQA*2, *LDLr*1	14
*DQA*2, *LDLr*2	17
*DQA*1	14
*DQA*2	4
*LDLr*1	10
*LDLr*2	16
*DQA*1, *LDLr*1, *LDLr*2	2
*DQA*2, *LDLr*1, *LDLr*2	1
*DQA*1, *DQA*2	1
*LDLr*1, *LDLr*2	2
*DQA*1, *DQA*2, *LDLr*1, *LDLr*2	3
Controls	32
No signal	29
*LDLr*1	2
*LDLr*2	1

studies. In linkage experiments a fraction of the samples that contain two sperm and which have amplified only one of the two alleles at each locus will generate 'false recombinants'. We expect that the frequency of false recombinants can be minimized by careful attention to sperm isolation and the enhancement of sperm lysis and amplification efficiency. If very large numbers of sperm could be analysed with great reliability, some mutational events which cannot be analysed by conventional methods might eventually be studied.

Our ability to haplotype sperm at the DNA level will provide a fundamentally new approach to studying human recombination and may also be useful in determining the physical order of DNA polymorphisms which are so tightly linked that they cannot be resolved by additional family analysis. This may be especially significant in the case of random RFLPs tightly linked to disease-causing loci. The genetic distances between the random RFLPs could be accurately determined and the RFLPs ordered with respect to one another by three point crosses, provided that simultaneous amplification of 3 marker loci could be made efficient enough for single sperm experiments. Such fine structure maps might be of great value in attempts to locate the disease-causing locus itself. Of course because sperm do not exhibit disease phenotypes, an unknown disease locus cannot be directly mapped relative to polymorphisms in this way.

The analysis of single sperm in species that cannot be extensively bred or have exceptionally long generation times may be the only practical way of making genetic maps for these species.

One immediate practical application of the analysis of individual sperm is in the area of forensic medicine. *HLA* typing for paternity determinations or identification of criminals is often hampered by the inability to determine the haplotype of the suspected individuals because this would require the analysis of close relatives. *HLA* analysis of individual sperm from a suspect would allow the linkage phase of the *HLA* markers to be unambiguously determined and thus increase the probability of inclusion or exclusion.

Finally, the ability to study DNA sequences in individual diploid cells will make it possible to study cell-to-cell variation in developmental processes involving DNA rearrangements or other genetic alterations. It is also likely that analysis of messenger RNAs in single cells would be possible if efficient reverse transcription could be carried out before PCR was initiated. Prenatal diagnosis on a single cell derived from a preimplantation embryo resulting from *in vitro* fertilization is also conceivable.

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LETTERS TO NATURE

No cometesimals in the inner Solar System

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Ultraviolet measurements made by Voyager 2, apparently showing a rapid decrease in hydrogen Lyman-α emission with distance from the Sun, were taken by Donahue *et al.*[1] as evidence for a source of atomic hydrogen in the very local interstellar medium (VLISM). The suggested source[1] is a class of small comets, at solar distances of ~1 AU, that produce atomic hydrogen as their icy mantle is evaporated. This claim has been adduced as evidence for a theory[2] that the Earth is subject to a large influx of cometary material, significantly affecting atmospheric evolution. Here we analyse again the Voyager 2 data, and show that no source of hydrogen in the VLSIM is required other than the inflow of neutral atoms; the original analysis was apparently flawed by an erroneous transcription of tabulated data (T. M. Donahue, personal communication).

Although Donahue *et al.*[1] claimed to find a cometary source of hydrogen, the estimated flux was ~seven orders of magnitude smaller than required by the theory of Frank *et al.*[2]. But by postulating a different and more numerous kind of comet, Frank *et al.* have claimed[3] that the Voyager data, the most important direct evidence for small comets and the crucial data in obtaining limits on sources of volatile compounds such as H_2O, can be made to agree with their theory.

The observations in question are obtained with Voyager 2 shortly after launch[1]. Figure 1 shows the observing geometry looking down on the north pole of the Solar System. A number of observations, in a direction approximately downstream with respect to the inflowing VLISM neutral gas, were obtained with the spacecraft located near 1 AU. Further measurements were obtained with the spacecraft located at $r_0 \sim 1.3$ AU and beyond, with all observations approximately downstream as shown in Fig. 1. The analysis of these data by Donahue *et al.*[1] indicated that the measurements near 1 AU in a direction almost tangential to the Earth's orbit demonstrated intensities in excess of a normal VLISM model. The excess near 1 AU was attributed to the influx of cometesimals. We argue that the stronger signal obtained near 1 AU is simply a consequence of observing geometry.

The search for a local source of hydrogen in the Voyager data was limited by Donahue *et al.*[1] to observations in the VLISM downstream direction (Fig. 1). The observations obtained with the spacecraft positioned near 1 AU were necessarily at a high angle ($\beta \approx 90°$; see Fig. 1) to the antisolar direction because the spacecraft was positioned in the vicinity of 0° right ascension (α) (Fig. 1). Later observations with the spacecraft positioned near 2 AU were obtained with a viewing direction more closely aligned with the antisolar direction. A non-negligible fraction

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Whole genome amplification from a single cell: Implications for genetic analysis

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ABSTRACT **We have developed an *in vitro* method for amplifying a large fraction of the DNA sequences present in a single haploid cell by repeated primer extensions using a mixture of 15-base random oligonucleotides. We studied 12 genetic loci and estimate that the probability of amplifying any sequence in the genome to a minimum of 30 copies is not less than 0.78 (95% confidence). Whole genome amplification beginning with a single cell, or other samples with very small amounts of DNA, has significant implications for multipoint mapping by sperm or oocyte typing and possibly for genetic disease diagnosis, forensics, and the analysis of ancient DNA samples.**

The sensitivity of the polymerase chain reaction (PCR; refs. 1–3) is great enough to allow the analysis of DNA in a single cell (4, 5). This led to the development of preimplantation genetic disease diagnosis using single cells from early embryos or polar bodies (6–11) and genetic recombination analysis using a single sperm (12–15) or oocyte (16). In all these cases the single cell can be analyzed only once and independent confirmation of the genotype of any one cell is impossible. We have developed a method to circumvent this limitation. Multiple copies of the DNA sequences present in a single cell are made by an *in vitro* method that we call *primer-extension preamplification* (PEP). Multiple rounds of extension with the *Taq* DNA polymerase and a random mixture of 15-base oligonucleotides as primers produce multiple copies of the DNA sequences originally present in the sample. It is estimated that at least 78% of the genomic sequences in a single human haploid cell can be copied no less than 30 times. As a result, only a small aliquot of the amplified sample has to be used to analyze any one gene and material remains for additional analyses. Our method not only extends the possible applications of single cell studies but also has implications for the analysis of any small DNA sample.

MATERIALS AND METHODS

PEP of Single-Sperm DNA. The DNA sequences in individual sperm cells were copied by multiple rounds of primer extension using a collection of 15-base oligonucleotides in which any one of the four possible bases could be present at each position. Theoretically, the primer was composed of a mixture of 4^{15} (1×10^9) sequences.

Single human sperm were sorted by flow cytometry into 96-well Falcon microtiter dishes containing 5 μl of an alkaline lysis solution (200 mM KOH/50 mM dithiothreitol) as described (17, 18). After a 10-min incubation at 65°C, 5 μl of neutralization solution (900 mM Tris·HCl, pH 8.3/300 mM KCl/200 mM HCl) was added. To the lysed and neutralized sample was added 5 μl of a 400 μM solution of random primers (Operon Technologies, Alameda, CA), 6 μl of 10× K^+ free PCR buffer [25 mM $MgCl_2$/gelatin (1 mg/ml)/100 mM Tris·HCl, pH 8.3], 3 μl of a mixture of the 4 dNTPs (each at 2 mM), and 1 μl of *Taq* polymerase (Perkin–Elmer/Cetus, 5 units), and brought to 60 μl with water. Fifty primer-extension cycles were carried out in a MJ Research thermocycler (Cambridge, MA). Each cycle consisted of a 1-min denaturation step at 92°C, a 2-min annealing step at 37°C, a programmed ramping step of 10 sec/degree to 55°C, and a 4-min incubation at 55°C for polymerase extension. Each sample was then divided into aliquots and analyzed for specific DNA sequences.

Specific Gene Analysis. We tested the aliquots from a single sperm subjected to PEP for the presence of specific DNA sequences by using conditions that are capable of detecting single DNA molecules. A total of 12 loci were studied. We used a hemi-nesting strategy that enhances yield and specificity when starting with one target DNA molecule and allows the product to be detected by ethidium bromide staining (17, 18). The first round of PCR utilizes a pair of primers that flank the target sequence. The second round uses one of the two original primers and a second internal primer. In every case we took 2 μl of first-round product for the second round of PCR.

The PCR conditions and primer sequences for seven loci are described in ref. 18 (*PTH*, *LDLR*, and *HBG2*), ref. 13 (*D3S2*, *D3S11*, and *D3S12*), and ref. 14 (*D3S3*) with the modification that all four dNTPs (each at 100 μM) were used in the second round. Three microsatellite repeat polymorphisms (*D9S52*, *APOC2*, and *D19S49*) were analyzed (R.H., J. L. Weber, and N.A., unpublished data). The X chromosome-linked *STS* locus and the Y chromosome-linked *STS* pseudogene locus are amplified in the first round with the same set of primers (5′-GAGTGAAACTCACTCAGCAC-3′ at 0.1 μM and 5′-CCTTAGGAACCAGGAGATAC-3′ at 0.1 μM) at 92°C for 30 sec and 60°C for 4 min (11 cycles) or 3 min (next 31 cycles). Standard PCR buffer was used with all four dNTPs (each at 100 μM). The second round of amplification [92°C for 30 sec, 65°C for 1 min, and 72°C for 30 sec for 26 cycles; all four dNTPs (each at 8 μM)] included the first primer above and a mixture of two primers. One (5′-ACCGTACTTGCATGAGAAGCTGTCCCAAAGGA-3′ at 0.5 μM) is specific for the Y chromosome-linked pseudogene and the other (5′-TGGGAGACTGTCCCGAAGGT-3′ at 2 μM) is specific for the X chromosome-linked gene. Because the gene-specific primers differ in length, the size of the PCR products for each locus is also different. The primers for the DNA segment 25 kilobases distal to the pseudoautosomal boundary (pa) are 5′-GGAGTAAGACGCCATCTCAA-3′ and 5′-GATGTCGGCAAACTAGAACC-3′, and each is used at 0.2 μM. Only one round of a PCR using the conditions for the first round of *STS* amplification is required.

RESULTS

Our first experiment was designed to estimate the efficiency of the PEP procedure. Twelve single sperm were sorted by flow cytometry, lysed, and subjected to PEP for 50 primer-

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Abbreviation: PEP, primer-extension preamplification.

extension cycles using the mixture of random primers. After PEP, each sperm sample was divided into 30 aliquots that contained 2 μl of the original 60-μl PEP reaction mixture. For each sperm all 30 aliquots were tested for the presence of 1 of 12 specific DNA sequences by using PCR methods shown to be capable of detecting a single DNA molecule (17). Representative data for one of the sperm amplified by PEP and tested for the *PTH* locus are shown in Fig. 1. The controls in lanes 1–4 show that no specific gene product is produced if no DNA is added to the PEP reaction mixture. The 4 samples in lanes 5–8 come from a single sperm that was not subjected to PEP but was divided into 5 aliquots after lysis. Only 1 sample gave the specific *PTH* product. All but 1 of the 30 samples that received PEP aliquots were positive for *PTH*. The fraction of the 30 aliquots that were positive for the gene tested in each of the 12 sperm is shown in Table 1.

We can estimate a lower bound for the number of copies of each specific DNA segment present after PEP by using these data. In the worst case shown in Table 1, only 24 of the 30 aliquots were positive (*APOC2*). If the PEP product of this sperm had less than 30 copies of the *APOC2* DNA segment, then the chance that 24 or more of the 30 aliquots would be positive is less than 0.005. Therefore, we can assert with 99.5% confidence that at least 30 copies were present in this PEP reaction mixture. We are virtually certain that in the remaining 11 samples there also must have been at least 30 and probably more copies of the amplified sequence. The fact that the likelihood of detecting a single molecule without PEP can range from 72 to 96% (12–14) could contribute to underestimating the actual copy number.

The above experiment fails to show explicitly that the products of all 12 loci could have been detected in a single sperm. To test this possibility, 18 additional single sperm from the same donor were subjected to PEP. Twelve aliquots, each comprising 1/24th (2.5 μl) of the total PEP product, were taken for each sperm, and each aliquot was examined for a different one of the 12 loci. The success with which each of the 12 loci were detected in each of the 18 sperm tested is given in Table 2. Fig. 2 shows the results from one of the sperm.

We can also use these data to estimate the average number of copies per DNA segment produced by PEP. Of a total of

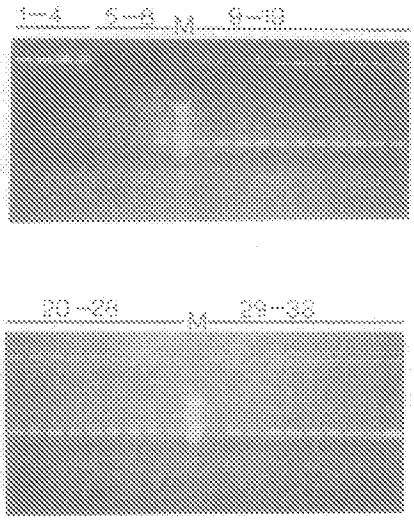

FIG. 1. Testing for the presence of *PTH* sequences in DNA from a single sperm subjected to PEP. Lanes: 1–4, *PTH* amplification of aliquots from a PEP sample to which no DNA was added; 5–8, amplification of *PTH* sequences in 4 aliquots of a lysed single sperm not subjected to PEP; 9–38, *PTH* amplification of 30 aliquots from a single sperm subjected to PEP; M, *Msp* I digest of pBR322 as molecular size markers.

Table 1. Analysis of PEP products from 12 sperm

Sperm	DNA segment	No. positive signals in 30 aliquots
1	*PTH*	29
2	*D9S52*	30
3	*D19S49*	30
4	*APOC2*	24
5	*D3S2*	30
6	*D3S3*	28
7	*D3S11*	30
8	*D3S12*	30
9	*HBG2*	30
10	*LDLR*	27
11	*STS*	30
12	pa	29

216 aliquots (12 × 18), 16 or 7.4% failed to give a positive signal. If the number of copies in a 2.5-μl aliquot is a Poisson random variable with an expected value of λ, then the maximum likelihood estimate of λ is 2.60. Since each PEP sample contained 60 μl, the average total number of copies is estimated to be 62.46 with a 95% confidence interval of 53.0–78.2. The results from the first experiment are not suitable for such a calculation since the 30 trials for any one locus are not independent.

The sperm donor used in this experiment was heterozygous at the microsatellite containing locus *APOC2* on chromosome 19 and, as a male, had the *STS* gene on the X chromosome and the *STS* pseudogene on the Y chromosome. The PCR systems for these loci were designed so that the allelic state at *APOC2* (the number of CA repeats) and the presence of the X or Y chromosome could be determined from the size of the PCR product alone. Among the 18 sperm, 9 contained one *APOC2* allele and 9 contained the other. Similarly, among the 17 samples that were positive for *STS*, 9 carried the X chromosome and 8 carried the Y chromosome. Fig. 3 shows the genotype of 9 of the 18 sperm. The segregation pattern of the *APOC2* alleles and the X and Y chromosomes can be clearly seen. The independent assortment of the sex chromosomes from chromosome 19 is also observed.

Our data can be used to estimate what fraction of the genome in a single cell can be expected to be amplified by PEP. It is likely that not all of the DNA sequences in a cell are capable of being amplified by a PCR just as not all sequences are capable of being cloned by conventional methods. Our estimate is made by using PCR systems designed from 12 cloned and sequenced genomic DNA segments. Therefore, it is more accurate to say that we are estimating what fraction of all the genomic sequences in a single cell capable of being cloned and amplified by a PCR can be amplifed by PEP. To make this estimate, we assume that the fraction of the genome that is amplified is approximately the same for each individual sperm. The data from the second experiment support this assumption. Of 216 amplification attempts (12 loci × 18 sperm), 200 amplifications were positive. Under the assumption above and further by assuming that each DNA segment that can be cloned and amplified by a PCR is equally likely to be amplified by PEP, the number of positive loci for any given sperm is a binomially distributed random variable with a probability of success equal to approximately 0.9259 (200 of 216). The actual frequency distribution of positive amplifications among the 18 sperm is consistent with that expected from 18 independent random variables with the binomial distribution described above (the Kolmogorov–Smirnov statistic d is 0.057, $P > 0.2$; see ref. 19). Since the PEP procedure appears to amplify approximately the same fraction of the whole genome in each sperm, we can treat the 12 loci amplified in the first experiment as if

Table 2. Analysis of 12 loci in 18 sperm

Sperm	Locus												Total positive loci, no.
	PTH	*D9S52*	*D19S49*	*APOC2*	*D3S2*	*D3S3*	*D3S11*	*D3S12*	*HBG2*	*LDLR*	*STS*	pa	
1	+	+	–	+	+	+	+	+	+	+	+	+	11
2	+	+	+	+	+	+	+	+	+	+	+	+	12
3	+	+	+	+	–	+	+	–	+	+	+	+	10
4	+	+	+	+	+	+	–	–	+	+	+	+	10
5	–	+	+	+	+	+	–	+	+	+	–	+	9
6	+	+	+	+	+	+	+	+	+	+	+	+	12
7	+	+	+	+	+	+	+	+	+	+	+	+	12
8	+	+	+	+	–	+	+	+	+	+	+	+	11
9	+	+	+	+	–	+	–	+	+	+	+	+	10
10	+	–	+	+	+	+	+	+	+	+	+	+	11
11	+	+	+	+	+	–	+	+	–	+	+	+	10
12	+	+	+	+	+	+	+	+	+	+	+	+	12
13	+	+	+	+	+	+	+	–	+	+	+	+	11
14	–	+	+	+	+	+	+	+	+	+	+	+	11
15	+	+	+	+	+	+	+	+	+	+	+	+	12
16	+	+	+	+	+	+	+	+	+	+	+	+	12
17	+	+	+	+	+	+	+	+	+	+	+	+	12
18	+	+	+	+	+	+	+	+	+	+	+	+	12
Total no. positive sperm	16	17	17	18	15	17	15	15	17	18	17	18	

they were chosen at random from among all the DNA sequences present in a single sperm. Since we are virtually certain that all 12 loci were amplified to at least a level of 30 copies (from the first experiment), we calculate with 95% confidence that the probability P of amplifying any sequence in the genome to a minimum level of 30 copies is no less than 0.78 ($P^{12} \geq 0.05$). Given the nature of our assumptions, this is a conservative estimate.

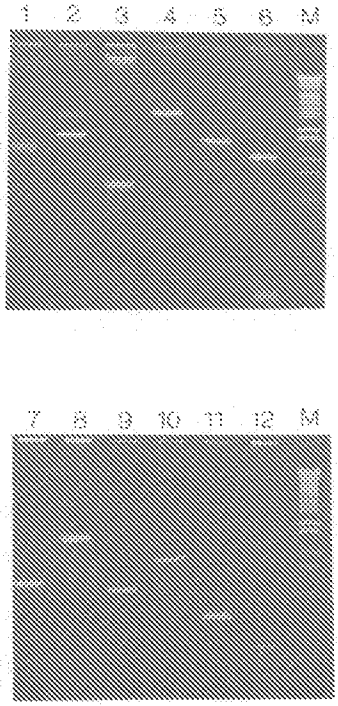

FIG. 2. Analysis of aliquots from a single-sperm (Table 2, sperm 12) PEP preparation for the presence of DNA sequences from 12 genetic loci. Each aliquot contained 1/24th of the PEP product. Lanes 1–12 show the PCR products obtained using primers specific for *D19S49* [132 base pairs (bp)], *D9S52* (152 bp), *APOC2* (101 bp), *PTH* (177 bp), X chromosome-linked *STS* (144 bp), pa (122 bp), *LDLR* (96 bp), *HBG2* (139 bp), *D3S2* (95 bp), *D3S3* (128 bp), *D3S11* (75 bp), and *D3S12* (62 bp), respectively. Lanes M show a *Msp* I digest of pBR322.

DISCUSSION

Methods for amplifying a random collection of cellular DNA sequences rather than one specific sequence have been used for cloning portions of microdissected chromosomes (20, 21) and in a scheme to select for genomic DNA segments that are capable of binding specific proteins (22). Amplification is achieved by ligating specific sequences to both ends of the fragments and by using primers complementary to these specific sequences for a PCR. In these approaches considerable manipulation of the sample is required before amplification including isolation of double-stranded DNA, restriction enzyme digestion, and ligation. From the data presented

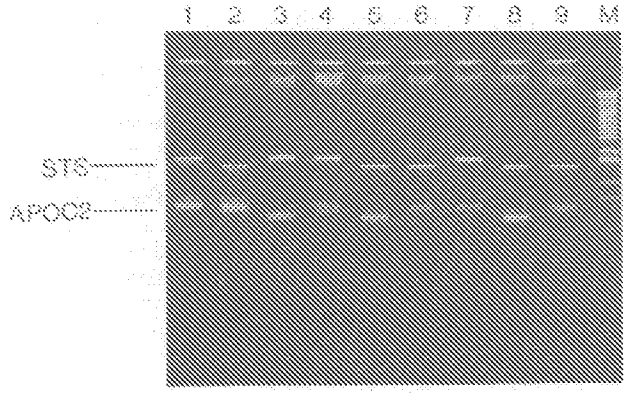

FIG. 3. *APOC2* and *STS* (or *STS* pseudogene) amplification products from nine sperm (lanes 1–9, respectively). Each lane contains a mixture of the products from the two amplifications, each using 1/24th of the PEP product. Lane M contains a *Msp* I digest of pBR322. Two bands can be seen for each sperm. The upper and lower *STS* bands represent the Y chromosome-linked (153 bp) and X chromosome-linked (144 bp) genes, respectively, and the two *APOC2* bands are the two alternative alleles in this individual.

in these studies, it is not possible to estimate what fraction of the total starting DNA was represented in the PCR product. We did not think it was likely that these approaches would be useful when starting with a single cell since it was expected that portions of the single genome would be lost during the many manipulations required.

On the contrary, PEP does not involve extensive manipulations of the sample. Only cell lysis, the addition of reagents, and repeated rounds of primer extension are required. Because of the random nature of the primers, it is also unlikely that any one primer-extension product will undergo primer extension at a subsequent cycle more than any other primer-extension product. Our data are consistent with these expectations. With our current protocol, we are 95% confident that PEP produces a minimum of 30 copies of not less than 78% of the DNA sequences present in a single cell.

Our first PEP experiments (data not shown) were carried out with random hexanucleotides and were not successful using different combinations of annealing (25–37°C) and extension (50–72°C) temperatures. Switching to random 15-mers dramatically improved our results. The PEP protocol we report uses 5 units of *Taq* polymerase and primer at 33 μM. Two units of *Taq* polymerase and primer at 20 μM also works (data not shown) but the results of these experiments have not been subjected to the extensive quantitative evaluation reported above. It is possible that further changes in primer length, primer-extension conditions, dNTP concentration, or other aspects of the PEP reaction could lead to increases in copy number beyond what has been achieved.

We do not know the size distribution of the PEP products since the amount of DNA present after 50 cycles is too low to be detected by conventional means. Some molecules must be greater than 335 bp in length, which is the size of the longest first-round PCR product (*HBG2*, ref. 12) in the data reported here. Recent experiments have shown that some PEP fragments must be at least 800 bp in length (data not shown) but their copy number has not been quantitated.

Could the use of a random-primer mixture introduce mutations into the PEP products and will this affect the subsequent analysis of specific sequences by PCR? This is not likely for the following reason. For a specific locus to be amplified from a PEP product in a subsequent experiment, the PEP product must contain at least one molecule with the specific upstream and downstream primer sequences that flank the target region for the particular locus under investigation. If random 15-mers anneal to the target region at the polymorphic site during PEP, the extension products will not contain the upstream-specific PCR primer sequences and exponential amplification by PCR cannot be efficiently achieved.

Implications of PEP for Single Cell Analysis in Single Gamete Typing. Single gamete typing using a sperm or oocyte has been used to study genetic recombination in humans (4, 12–15), mice (16), and cattle (23). However, the genotype of any one sperm could not be confirmed by a second PCR analysis. Not only can the typing be repeated using the PEP method but the possible exchange of additional flanking DNA polymorphisms detected in other aliquots from the same sperm could confirm rare recombination events.

The original sperm typing method for ordering DNA polymorphisms was laborious and required a series of independent three-point crosses (13, 24, 25). The development of the PEP procedure, however, will allow a more conventional multipoint mapping strategy where the same set of meiotic products can be studied for a number of polymorphisms. A very conservative estimate is that we can study at least 24 2.5-μl aliquots out of 60 μl of PEP product from a single sperm. The ability to type this many polymorphisms, coupled with the fact that informative males are easily found for virtually any combination of polymorphisms due to the very large number of sperm donors that are available, will allow map construction using standard multipoint mapping statistical procedures. In addition to determining order, the large number of sperm that can be examined would also allow the recombination fraction to be estimated with great accuracy. Sperm typing is also amenable to automation, which could enhance the speed of mapping. Aliquots of the semen samples would be used to determine which donors were informative for the polymorphisms to be mapped. Once multiply informative donors were identified, single sperm from those donors would be sorted, their genome would be amplified using PEP, and aliquots would be typed for the appropriate polymorphisms as described above.

The PEP approach to multipoint mapping by sperm typing would be possible in any species where single sperm can be isolated and would be especially valuable in those cases where selected breeding is difficult or extensive pedigrees are unavailable. In species where multiple oocytes can be obtained the same approach could be used (16, 26).

Preimplantation Genetic Diagnosis and Small DNA Samples. Another potential use of the PEP procedure is in preimplantation genetic disease diagnosis. A number of studies in mice and humans have shown that, after *in vitro* fertilization, analysis of DNA in individual cells from early embryos (6–9) or the polar body accompanying the oocyte before fertilization (10, 11) could be a useful tool for prenatal diagnosis before implantation. Since a PCR is not error free, clinical use of single-cell PCR data is accompanied by some risk since it has not been possible to confirm the genotype of any one embryonic cell or polar body. Prospective patients need to be counseled as to the risk of errors because, after implantation and a confirmatory chorion villus sampling or amniocentesis, a therapeutic abortion might still be warranted. Risk assessment based on a probabilistic method that takes PCR errors into consideration has been developed for both autosomal and sex-linked dominant and recessive diseases diagnosed using single-cell analysis (27, 28). However, these risks could be appreciably lowered if the PEP procedure was first carried out on the embryonic cell or polar body so that the genotype could be confirmed. In addition, the number of loci studied could be substantially increased.

Often, only very small DNA samples are available for forensic analysis or studies of ancient DNA. The PEP procedure could prove useful in these cases. When more molecules are examined in any one PCR experiment, the likelihood that the sample will be typed accurately increases. Also, if the isolated DNA is first subjected to PEP, significantly more genetic loci could be examined from an initially limited sample.

Considerations in Analyzing Single Diploid Cells and DNA. When a single diploid cell or very small cell-free DNA samples are to be typed, careful consideration must be given to the size of each aliquot and the number of aliquots taken from the PEP reaction. This is due not only to the small amounts of starting material but also to the relatively few copies made of each DNA segment during PEP (average, 60). In single diploid cell analysis, the sample subjected to PEP contains two copies of each autosomal gene. PEP should produce approximately equal numbers of DNA fragments representing both alleles at each locus. However, if the size of the aliquot taken from the sample after PEP is too small, the two different allelic DNA fragments may not be present in equal amounts due to sampling error. One allele might be missing altogether, although typing another aliquot could reveal the missing allele. Thus, the genotype determined by typing only one small aliquot may be different from that of the original sample. This is also true for very small cell-free forensics or ancient DNA samples that, in addition, could have had a biased representation of the two alleles even before PEP. Thus both aliquot size and number are important

for correct typing. Statistical considerations and suggested experimental approaches relevant to this sampling problem are discussed in Navidi *et al.* (29).

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EXHIBIT 8

Int. J. Cancer: 67, 632–635 (1996)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

DUPLICATION OF CHROMOSOME SEGMENT 12q15-24 IS ASSOCIATED WITH ATYPICAL LIPOMATOUS TUMORS: A REPORT OF THE CHAMP COLLABORATIVE STUDY GROUP

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Ordinary lipomas are cytogenetically characterized by a variety of balanced rearrangements involving chromosome segment 12q13-15, and atypical lipomatous tumors (ALT) by supernumerary ring chromosomes or giant markers known to contain amplified 12q sequences. In a series of 228 cytogenetically analyzed and histopathologically reexamined ordinary lipomas and ALT, 10 tumors showed unbalanced chromosome-12 aberrations. All 4 tumors with loss of segments from 12q were classified as ordinary lipomas, whereas 5 of the 6 tumors showing gain of 12q material were diagnosed as ALT. One or three extra copies of 12q15-q24 were present in all 5 ALT. We conclude that duplication of 12q sequences may be a sufficient level of amplification for development of the microscopic appearance that characterizes ALT.

The group of adipose-tissue tumors is the best characterized cytogenetically of all soft-tissue tumors; more than 300 cases with chromosome aberrations have been reported (Mitelman, 1994). Several characteristic, and even specific, chromosome rearrangements that correlate with histopathologic diagnosis have been identified (Sandberg and Bridge, 1994; Heim and Mitelman, 1995; Fletcher *et al.*, 1996). The majority of ordinary lipomas with abnormal karyotypes can be sub-divided into 3 cytogenetic sub-groups characterized by rearrangements of 12q13-15, 6p or 13q. These aberrations are not mutually exclusive, and have been found in different combinations in a few tumors. About two thirds of ordinary lipomas with chromosome changes show 12q13-15 aberrations. The vast majority of these are seemingly balanced translocations, inversions or insertions, which have been seen in 114 of 120 cases so far reported (Mitelman, 1994). The 12q13-15 segment has been found to recombine with a variety of other segments, the most frequent being 3q27-28. Recent data indicate that the molecular genetic consequence of these aberrations is the rearrangement of the gene *HMGIC* localized to 12q15, most likely leading to the formation of a variety of chimeric proteins, depending on the segment with which 12q15 recombines (Ashar *et al.*, 1995; Schoenmakers *et al.*, 1995).

Atypical lipomas and well-differentiated liposarcomas, here collectively referred to as atypical lipomatous tumors (ALT), are cytogenetically indistinguishable (Sreekantaiah *et al.*, 1992; Mandahl *et al.*, 1994). These tumors are typically hyperdiploid, with one or more supernumerary ring chromosomes and/or giant marker chromosomes, the origin of which cannot be determined by G-banding analysis. Fluorescence *in situ* hybridization (FISH), comparative genomic hybridization (CGH) and molecular genetic analyses have shown that the rings and markers are composed entirely or partly of segments from chromosome 12 (Dal Cin *et al.*, 1993; Forus *et al.*, 1993; Leach *et al.*, 1993; Nilbert *et al.*, 1994; Pedeutour *et al.*, 1994; Suijkerbuijk *et al.*, 1994; Szymanska *et al.*, 1996). The aberrations give rise to amplification of chromosome-12 sequences that frequently involve the *MDM2* gene.

In order to investigate whether other types of chromosomal imbalances (such as numerical changes, deletions and duplications) of chromosome 12 might also be associated with a particular histologic appearance in adipose-tissue tumors, we assessed the karyotypic profile in a series of 228 cytogenetically analyzed ordinary lipomas and ALT.

MATERIAL AND METHODS

The 228 tumors, investigated cytogenetically at the Department of Clinical Genetics in Lund and the Center for Human Genetics in Louvain between 1987 and 1994 were, as part of this study, re-examined cytogenetically by the cytogeneticists of the CHAMP (CHromosomes And MorPhology) collaborative study group, and histopathologically by the CHAMP pathologists, who were unaware of the cytogenetic findings and clinical data. For cytogenetic analysis, fresh tumor samples were disaggregated, cultured, harvested and stained as described (Limon *et al.*, 1986; Mandahl *et al.*, 1988). Chromosome preparations were made after 3 to 10 days of culture. Chromosome aberrations and karyotypes were described according to ISCN (1995). Sections were cut from all paraffin blocks available from each tumor, *i.e.*, 1 to 10 blocks, and analyzed histopathologically. Histological criteria for classification have been reported elsewhere (Fletcher *et al.*, 1996; Rosai *et al.*, 1996). The consensus diagnoses were ordinary lipoma in 169 cases and ALT in 59 cases. Ten tumors showed cytogenetically detectable imbalances involving chromosome 12, and these are the subject of this report. The clinical data are shown in Table I.

RESULTS

Of the 10 selected tumors with unbalanced aberrations involving chromosome 12, 5 were classified as ordinary lipomas and 5 as ALT with varying degrees of atypia (Table II). Spindle-cell areas were found in 3 tumors (cases 6, 9 and 10), demonstrating focal morphological overlap with spindle cell liposarcoma. In 4 tumors, all ordinary lipomas, there was net loss of 12q material, with deletion of segments 12q14-qter in 3 and 12q13-q15 in 1. Two tumors, one ordinary lipoma and one ALT, were trisomic for chromosome 12 (Fig. 1). The remaining 4 tumors, all ALT, showed duplication or triplication of

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varying 12q segments, with q15-q24 as the minimal segment in common. All cases had various additional aberrations; in 2 ordinary lipomas and 3 ALT, chromosome 13 was involved, and one tumor of each type had trisomy 8.

DISCUSSION

Two cytogenetic types of aberrations affecting chromosome segment 12q13-15 are known to be associated with ordinary lipoma and with ALT. These are recombinations through translocations, inversions and insertions in the former, and ring or marker chromosome formation in the latter. In ordinary lipomas, the essential event seems to be fusion of the *HMGIC* gene in 12q15 with an as yet unknown number of genes localized to a variety of chromosome segments, leading to the creation of chimeric genes (Ashar *et al.*, 1995; Schoenmakers *et al.*, 1995). In ALT, amplification of genes in chromosome arm 12q seems to be the important consequence of the aberrations (Leach *et al.*, 1993; Nilbert *et al.*, 1994). Although it has not been possible to verify the involvement of chromosome 12 by chromosome banding techniques, it is known from FISH studies that the rings and giant markers of ALT are composed, exclusively or partly, of 12q sequences (Dal Cin *et al.*, 1993; Pedeutour *et al.*, 1994). Analysis with CGH has identified 2 amplification units in these tumors, one in 12q14-15 and the other in 12q21.3-22 (Suijkerbuijk *et al.*, 1994). Although several cancer-associated genes, *e.g.*, *MDM2*, *SAS*, *CDK4*, *CHOP* and *GLI*, have been found to be amplified in at least some adipose-tissue tumors, the essential gene(s) in the amplicon has (have) not been identified.

In the present study, including 169 ordinary lipomas and 59 ALT, all 4 tumors with loss of segments from 12q were classified as ordinary lipomas, just like the vast majority of tumors with balanced chromosome-12 aberrations. Six tumors with duplication or triplication of chromosome-12 material were identified. Only one (case 5) of these was diagnosed as an ordinary lipoma, whereas all of the remaining 5 showed varying degrees of atypia and were accordingly classified as ALT; spindle-cell areas were found in 3 tumors (cases 6, 9 and 10). The latter tumors demonstrated focal morphological overlap with spindle-cell liposarcoma, a recently described spindle-cell variant of well-differentiated liposarcoma (Dei Tos *et al.*, 1994). The minimal gained segment in common was 12q15-q24, which overlaps with the amplification units identified by FISH and CGH in ALT with the characteristic rings or giant markers. Molecular genetic studies have estimated that the copy number of genes located in 12q13-15 is 3 to 5 times greater in ALT with ring chromosomes than in normal diploid cells (Nilbert *et al.*, 1994). This is to be compared with the low-level amplification, resulting in 1.5 to 2.5 times the normal copy number, seen in the ALT of the present series.

Apart from the cases included in the present series, 8 ordinary lipomas with unbalanced chromosome-12 aberrations have been reported (Mandahl *et al.*, 1988, 1994; Sreekantaiah *et al.*, 1991). Of these, 3 showed net gain of material from 12q in the form of duplication of 12q13-22 in 2 cases and duplication of the entire long arm except the 12q13-15 segment in one. All 3 cases were classified as ordinary lipomas, which is at odds

TABLE I – CLINICAL DATA

Case number	Gender	Age (in decades)	Site[1]	Depth[2]	Size category[3]
1	F	8	E	S	2
2	F	5	E	D	1
3	F	4	E	S	1
4	M	6	E	S	1
5	F	5	E	D	1
6	F	4	E	D	3
7	F	7	E	D	2
8	M	6	T	D	3
9	F	4	E	D	3
10	F	4	T	S	1

[1]E, extremity; T, trunk wall.–[2]S, superficial; D, deep-seated.–[3]1, 0–5 cm; 2, 6–10 cm; 3, ≥ 11 cm.

TABLE II – ORDINARY LIPOMAS AND ALT WITH UNBALANCED CHROMOSOME-12 REARRANGEMENTS AS DETECTED BY G-BANDING ANALYSIS

Case number[1]	Karyotype	Lost segment	Gained segment	Diagnosis
1	46,XX,der(11)t(11;12)(q11;p12),der(12) del(12)(q13q15)inv(12)(p12q13)t(12;15) (q13;q12),der(15)t(11;15)(q11;q12)	q13-q15		L
2	46,XX,der(3)t(3;6)(q23;q21),t(6;12) (q27; q14),del(6)(q21),der(12)t(3;12)(q23;q14)	q14-qter		L
3	45,XX,–2,der(12)t(2;12)(q14–21;q14),t(13; 22)(q14;q13)	q14-qter		L
4	45,XY,der(1)del(1)(p11)ins(1;?)(q32; ?),add(11)(p11),add(12)(q14),–15	q14-qter		L
5	48,XX,+8,+12,del(13)(q11q14)		pter-qter	L
6	46,XX,der(12)dup(12)(q24q13)add(12)(q13), der(19)t(12;19)(q13;p13)dup(12) (q24q13) add(12)(q13)		q13-q24	ALT
7	45,X,dic(X;?13)(q13;q11),dup(12) (q24q15)/ 46,X,–X,dup(12),–13		q15-q24	ALT
8	48,XY,t(1;12)(p36;q13),+8,+12,del(13) (q12q22),del(15)(q22q25)		pter-qter	ALT
9	47,XX,add(8)(q11),+der(?)t(?;8)(?;q11) ins(?; 12)(?;q24q11)/46,idem,–8/47,XX,t(8;10; 14)(q12;q11;p13),+ins(?;12) (?;q24q11)/ 46,XX,der(10)t(8;10) (q12;q11),der(14)t(10; 14)(q11;p13)		q11-q24	ALT
10	46,XX,der(5)t(5;?15)(q13;?q15),der(8) t(8; 12)(q24;q13),t(12;16)(p11; p11),–13,–22,der(?)t(?;5)(?;q15)		q13-qter	ALT

[1]Cases 1–9 were primary tumors; case 10 was a local recurrence. Cases 1 and 6 have been reported earlier (Mandahl *et al.*, 1994). Case 6 was reclassified from ordinary lipoma (L) to ALT.

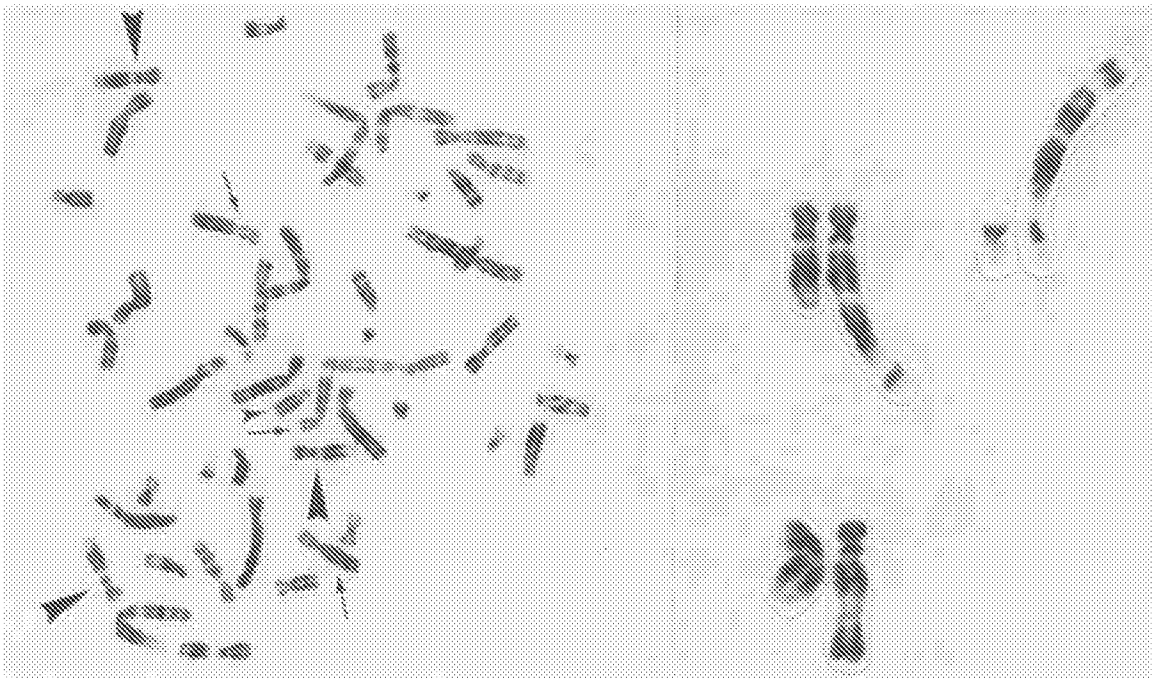

FIGURE 1 – *Left:* Metaphase plate from case 5 showing trisomy 12 (large arrowheads), trisomy 8 (arrows), and a deleted chromosome 13 (small arrowhead). *Upper right:* Chromosomes 12 and 19 from case 6 showing 2 copies of 12q13-24 in each of the derivative chromosomes. *Lower right:* Chromosomes 12 from case 7 showing an inverted duplication.

with our findings. One possible explanation for this discrepancy is that cytologic atypia—required for the diagnosis of ALT—can be focal and therefore overlooked histologically, unless multiple sections are scrutinized. Furthermore, the minimal gained segment in common in our series, 12q15-q24, was duplicated in only 1 of the 3 previously reported cases.

We conclude that duplication of 12q seems to be common in the rare sub-set of ALT having focal spindle-cell features. Furthermore, our findings indicate that even a modest increase in the dosage of genes localized in the distal half of 12q may be sufficient for development of the histopathologic features associated with ALT. Whether the degree of atypia is related to the size of the duplicated chromosomal segment and/or to the level of amplification in ALT remains to be determined.

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EXHIBIT 2

PTO/SB/08a (01-10)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)					
				Application Number	Unknown
				Filing Date	June 17, 2013
				First Named Inventor	Bert Vogelstein
				Art Unit	Unknown
				Examiner Name	Unknown
Sheet	1	of	2	**Docket Number**	LT00831 REX

U.S.PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

U.S.PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2]	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]

NON-PATENT LITERATURE DOCUMENTS

Examiner Initial*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	C1	BURG, J. L. et al., "Direct and Sensitive Detection of a Pathogenic Protozoan, Toxoplasma gondii, by Polymerase Chain Reaction", Journal of Clinical Microbiology, Vol. 27, No. 8, 1989, 1787-1792	
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	C7	KANZLER, H et al., "Molecular single cell analysis demonstrates the derivation of a peripheral blood-derived cell line (L1236) from the Hodgkin/Reed-Sternberg cells of a Hodgkin's lymphoma patient", Blood, Vol. 87, No. 8, 1996, 3429-3436	
	C8	MARCUCCI, GUIDO et al., "Detection of Unique ALL1 (MLL) Fusion Transcripts in Normal Human Bone Marrow and Blood: Distinct Origin of Normal versus Leukemic ALL1 Fusion Transcripts", Cancer Research, Vol. 58, 1998, 700-793	
	C9	PONTEN, FREDRIK et al., "Genomic analysis of single cells from human basal cell cancer using laser-assisted capture microscopy", Mutation Research Genomics, Vol. 382, No. 1-2, 1997, 45-55	

PTO/SB/08a (01-10)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)				Application Number	Unknown
				Filing Date	June 17, 2013
				First Named Inventor	Bert Vogelstein
				Art Unit	Unknown
				Examiner Name	Unknown
Sheet	2	of	2	Docket Number	LT00831 REX

	C10	TRÜMPER, LORENZ H. et al., "Single-Cell Analysis of Hodgkin and Reed-Stemberg Cells: Molecular Heterogeneity of Gene Expression and p53 Mutations", Blood, Vol. 81, No. 11, 1993, 3097-3115	

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter the office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document.
[4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re *Ex Parte* Reexamination of
Bert VOGELSTEIN *et al.*

U.S. Patent No. 6,440,706

Issue Date: August 27, 2002

For: DIGITAL AMPLIFICATION

Reexam Filing Date: To Be Assigned

Control No.: To Be Assigned

Examiner: To Be Assigned

Art Unit: To Be Assigned

Confirmation No.: To Be Assigned

REQUEST FOR *EX PARTE* REEXAMINATION UNDER 37 C.F.R. § 1.510

Mail Stop *Ex Parte* Reexam
ATTN: Central Reexamination Unit
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Dear Sir:

On behalf of Life Technologies Corp. (hereinafter "Requester"), under provisions of 37 C.F.R. § 1.510 *et seq.*, the undersigned hereby submits a Request for Reexamination of claims 1-12, 14-16, 19-32, 38-44, 46-48 and 51-64 of U.S. Patent No. 6,440,706 entitled "DIGITAL AMPLIFICATION" ("the '706 patent"). The '706 patent indicates on its face that it is assigned to Johns Hopkins University.

Entry and consideration are respectfully requested.

Pursuant to 37 C.F.R § 1.510, included with this Request are:

- the fee for requesting *ex parte* reexamination (37 C.F.R. § 1.20(c)(1));

- an identification of the reexamined patent by patent number and every claim for which reexamination is requested;
- a citation of the patents and printed publications that are presented to provide a substantial new question of patentability, listed on form PTO/SB/08A;
- a statement identifying each substantial new question of patentability based on the cited patents and printed publications, and a detailed explanation of the pertinence and manner of applying the patents and printed publications to every claim for which reexamination is requested;
- a copy of every patent or printed publication relied upon or referred to in the Request;
- a copy of the entire patent including the front face, drawings, and specification/claims (in double-column format) for which reexamination is requested, and a copy of any disclaimer, certificate of correction, or reexamination certificate issued in the patent as Exhibit 1;
- a certification that the Request has been served in its entirety on the patent owner (through the attorney of record during prosecution) at the address shown in the accompanying Certificate of Service;
- a showing that the attorney filing this request has the authority to act on behalf of the real party in interest pursuant to 37 C.F.R. § 1.34(a) under either a power of attorney from that party or in a representative capacity pursuant to § 1.34.

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TABLE OF EXHIBITS

Patent for which *Ex Parte* Reexamination is Requested

Exhibit 1:	U.S. Patent No. 6,440,706 titled "DIGITIAL AMPLIFICATION," filed July 11, 2000 and issued August 27, 2002 to Bert Vogelstein *et al.*

Prior Art References Relied Upon for SNQ

Exhibit PA-1:	Li *et al.*, "Amplification and analysis of DNA sequences in single human sperm and diploid cells." *Nature*. 29;335(6189):414-7 (1988)
Exhibit PA-2:	Zhang *et al.*, "Whole genome amplification from a single cell: implications for genetic analysis." *PNAS USA*, 89(13):5847-51 (1992)
Exhibit PA-3:	Jeffreys *et al.*, "Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells." *Nucl. Acids. Res.*, vol 16, no. 23, pages 10953-10971 (1988)
Exhibit PA-4:	Kalinina *et al.*, "Nanoliter scale PCR with TaqMan detection," *Nucl. Acids. Res.* vol 25, 1999-2004 (1997)
Exhibit PA-5:	Chou *et al.*, "Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications," *Nucleic Acids Res.*, 20(7): 1717–1723 (April 11, 1992)
Exhibit PA-6:	Burg, *et al.*, "Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction." *J. Clin. Microbiol.* 27, 1787-1792 (1989)
Exhibit PA-7:	Trumper *et al.*, "Single-Cell Analysis of Hodgkin and Reed-Sternberg Cells: Molecular Heterogeneity of Gene Expression and p53 Mutations," *Blood*, 81: 3097-3115 (1993)
Exhibit PA-8:	Kanzler *et al.*, "Molecular Single Cell Analysis Demonstrates the Derivation of Peripheral Blood-Derived Cell Line (L1236) From the Hodgkin/Reed-Sternberg Cells of a Hodgkin's Lymphoma Patient," *Blood*, 87: 3429-3436 (1996)
Exhibit PA-9:	Gravel *et al.*, "Single-cell analysis of the t(14;18)(q32;q21) chromosomal translocation in Hodgkin's disease demonstrates the absence of this translocation in neoplastic Hodgkin and Reed-Sternberg cells," *Blood* 91(8):2866-74 (Apr 15, 1998)
Exhibit PA-10:	Marcucci *et al.*, "Detection of Unique ALLÃ•(MLL) Fusion Transcripts in Normal Human Bone Marrow and Blood: Distinct Origin of Normal versus Leukemic ALL1 Fusion Transcripts," *Cancer Res*, 58:790-793. (February 15, 1998)
Exhibit PA-11:	Flint *et al.*, "NR2A Subunit Expression Shortens NMDA Receptor Synaptic Currents in Developing Neocortex," *J. Neurosci.*, 17(7):2469–2476 (April 1, 1997)
Exhibit PA-12:	Ponten *et al.*, "Genomic analysis of single cells from human basal cell cancer using laser-assisted capture microscopy," *Mutation Research Genomics* 382, 45–55 (1997).

Additional Documents

Exhibit 2:	PTO Form SB/08A
Exhibit 3:	Prosecution History File Wrapper for U.S. Patent No. 6,440,706
Exhibit 4:	U.S. Provisional Application No. 60/146,792, filed August 2, 1999.
Exhibit 5:	Relevant Portions of Prosecution History File Wrapper for App. No. 13/071,105.
Exhibit 6:	Kuppers *et al.*, The EMBO Journal vol.12 no.13 pp.4955-4967, 1993.
Exhibit 7:	Wolf *et al.*, Blood. 87: 3418-3428 (1996).
Exhibit 8:	Mandahl *et al.*, Int. J. Cancer: 67,632-635 (1996)

I. SUMMARY OF SUBSTANTIAL NEW QUESTIONS OF PATENTABILITY AND PROPOSED REJECTIONS

Ex parte reexamination is respectfully requested under 35 U.S.C. §§302-307 and 37 C.F.R. § 1.510 of claims 1-12, 14-16, 19-32, 38-44, 46-48 and 51-64 of U.S. Patent No. 6,440,706 to Vogelstein *et al.* ("the '706 patent"), and currently assigned to The Johns Hopkins University. The '706 patent issued on August 27, 2002 and claims a priority date of August 2, 1999.

This request presents 24 substantial new questions of patentability (SNQs) as to the '706 patent. The SNQs are summarized in **Table I** below.

The SNQs listed in Table I are based on the references cited herein and summarized in **Table II** below. The proposed rejections for each SNQ are summarized in **Table III** below.

Table I: Summary of SNQs	
SNQ No. 1:	Claims 1-3, 7-9, 14-16, 19, 21, 22, 27 and 32 are anticipated by Li under 35 U.S.C. § 102(b)
SNQ No. 2:	Claims 4 and 5 of the '706 patent are obvious under 35 U.S.C. § 103(a) over Li in view of Zhang
SNQ No. 3:	Claim 6 is obvious under 35 U.S.C. § 103(a) over Li in view of Jeffreys
SNQ No. 4:	Claims 10 and 11 are obvious under 35 U.S.C. § 103(a) over Li
SNQ No. 5:	Claim 12 is obvious under 35 U.S.C. § 103(a) over Li in view of Kalinina
SNQ No. 6:	Claim 20 is obvious under 35 U.S.C. § 103(a) over Li in view of Chou
SNQ No. 7:	Claim 23 is obvious under 35 U.S.C. § 103(a) over Li in view of Burg
SNQ No. 8:	Claim 24 is obvious under 35 U.S.C. § 103(a) over Li in view of Trumper
SNQ No. 9:	Claim 25 is obvious under 35 U.S.C. § 103(a) over Li in view of Kanzler
SNQ No. 10:	Claim 26 is obvious under 35 U.S.C. § 103(a) over Li in view of Gravel
SNQ No. 11:	Claim 28 and 29 are obvious under 35 U.S.C. § 103(a) over Li in view of Marcucci
SNQ No. 12:	Claim 30 is obvious under 35 U.S.C. § 103(a) over Li in view of Flint
SNQ No. 13:	Claim 31 is obvious under 35 U.S.C. § 103(a) over Li in view of Ponten

SNQ No. 14:	Claims 38, 39, 46 & 51 are anticipated by Zhang under 35 U.S.C. § 102(b)
SNQ No. 15:	Claims 40-43, 47, 48, 59 and 64 are obvious under 35 U.S.C. § 103(a) over Zhang in view of Li
SNQ No. 16:	Claim 44 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Kalinina
SNQ No. 17:	Claim 52 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Chou
SNQ No. 18:	Claims 53-55 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Burg
SNQ No. 19:	Claim 56 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Trumper
SNQ No. 20:	Claim 57 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Kanzler
SNQ No. 21:	Claim 58 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Gravel
SNQ No. 22:	Claims 60 and 61 are obvious under 35 U.S.C. § 103(a) over Zhang in view of Marcucci
SNQ No. 23:	Claim 62 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Flint
SNQ No. 24:	Claim 63 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Ponten

Table II: Summary of References Applied[1]

Exh. No	Reference	Art Under:	Originally Cited?	Originally Relied On Or Discussed?
PA-1	**"LI"** Li *et al.*, "Amplification and analysis of DNA sequences in single human sperm and diploid cells." *Nature*. 29;335(6189):414-7 (1988)	**102(b)/ 103**	**YES**	**NO**
PA-2	**"ZHANG"** Zhang *et al.*, "Whole genome amplification from a single cell: implications for genetic analysis." *PNAS USA*, 89(13):5847-51 (1992)	**102(b)/ 103**	**YES**	**NO**
PA-3	**"JEFFREYS"** Jeffreys *et al.*, "Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells," *Nucl. Acids. Res*., vol 16, no. 23, pages 10953-10971 (1988)	**102(b)/ 103**	**NO**	**NO**
PA-4	**"KALININA"** Kalinina *et al.*, "Nanoliter scale PCR with TaqMan detection," *Nucl. Acids. Res*. vol 25, 1999-2004	**102(b)/ 103**	**NO**	**NO**

[1] Applied references that are newly cited in this request are listed on the attached form SB/08A (Exhibit 2).

Table II: Summary of References Applied[1]				
	(1997)			
PA-5	**"CHOU"** Chou *et al.*, "Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications," *Nucleic Acids Res.*, 20(7): 1717–1723 (April 11, 1992)	**102(b)/ 103**	**NO**	**NO**
PA-6	**"BURG"** Burg, *et al.*, "Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction." *J. Clin. Microbiol.* 27, 1787-1792 (1989)	**102(b)/ 103**	**NO**	**NO**
PA-7	**"TRUMPER"** Trumper *et al.*, "Single-Cell Analysis of Hodgkin and Reed-Sternberg Cells: Molecular Heterogeneity of Gene Expression and p53 Mutations," *Blood*, 81: 3097-3115 (1993)	**102(b)/ 103**	**NO**	**NO**
PA-8	**"KANZLER"** Kanzler *et al.*, "Molecular Single Cell Analysis Demonstrates the Derivation of Peripheral Blood-Derived Cell Line (L1236) From the Hodgkin/Reed-Sternberg Cells of a Hodgkin's Lymphoma Patient," *Blood*, 87: 3429-3436 (1996)	**102(b)/ 103**	**NO**	**NO**
PA-9	**"GRAVEL"** Gravel *et al.*, "Single-cell analysis of the t(14;18)(q32;q21) chromosomal translocation in Hodgkin's disease demonstrates the absence of this translocation in neoplastic Hodgkin and Reed-Sternberg cells," *Blood* 91(8):2866-74 (Apr 15, 1998)	**102(b)/ 103**	**NO**	**NO**
PA-10	**"MARCUCCI"** Marcucci *et al.*, "Detection of Unique ALLÃ•(MLL) Fusion Transcripts in Normal Human Bone Marrow and Blood: Distinct Origin of Normal versus Leukemic ALL1 Fusion Transcripts," *Cancer Res*, 58:790-793. (February 15, 1998)	**102(b)/ 103**	**NO**	**NO**
PA-11	**"FLINT"** Flint *et al.*, "NR2A Subunit Expression Shortens NMDA Receptor Synaptic Currents in Developing Neocortex," *J. Neurosci.*, 17(7):2469–2476 (April 1, 1997)	**102(b)/ 103**	**NO**	**NO**
PA-12	**"PONTEN"** Ponten *et al.*, "Genomic analysis of single cells from	**102(b)/ 103**	**NO**	**NO**

Table II: Summary of References Applied[1]				
	human basal cell cancer using laser-assisted capture microscopy," *Mutation Research Genomics* 382, 45–55 (1997).			

These applied references are listed on the attached form SB/08A (**Exhibit 2**).

Table III: Summary of Proposed Rejections
Proposed Rejection No. 1: Claims 1-3, 7-9, 14-16, 19, 21, 22, 27 and 32 are anticipated by Li under 35 U.S.C. § 102(b)
Proposed Rejection No. 2: Claims 4 and 5 of the '706 patent are obvious under 35 U.S.C. § 103(a) over Li in view of Zhang
Proposed Rejection No. 3: Claim 6 is obvious under 35 U.S.C. § 103(a) over Li in view of Jeffreys
Proposed Rejection No. 4: Claims 10 and 11 are obvious under 35 U.S.C. § 103(a) over Li
Proposed Rejection No. 5: Claim 12 is obvious under 35 U.S.C. § 103(a) over Li in view of Kalinina
Proposed Rejection No. 6: Claim 20 is obvious under 35 U.S.C. § 103(a) over Li in view of Chou
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Proposed Rejection No. 8: Claim 24 is obvious under 35 U.S.C. § 103(a) over Li in view of Trumper
Proposed Rejection No. 9: Claim 25 is obvious under 35 U.S.C. § 103(a) over Li in view of Kanzler
Proposed Rejection No. 10: Claim 26 is obvious under 35 U.S.C. § 103(a) over Li in view of Gravel
Proposed Rejection No. 11: Claim 28 and 29 are obvious under 35 U.S.C. § 103(a) over Li in view of Marcucci
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Proposed Rejection No. 16: Claim 44 is obvious under 35 U.S.C. § 103(a) over Zhang in view of

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Proposed Rejection No. 17: Claim 52 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Chou
Proposed Rejection No. 18: Claims 53-55 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Burg
Proposed Rejection No. 19: Claim 56 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Trumper
Proposed Rejection No. 20: Claim 57 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Kanzler
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Proposed Rejection No. 22: Claims 60 and 61 are obvious under 35 U.S.C. § 103(a) over Zhang in view of Marcucci
Proposed Rejection No. 23: Claim 62 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Flint
Proposed Rejection No. 24: Claim 63 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Ponten

II. THE CLAIMS OF THE '706 PATENT ARE GIVEN THEIR BROADEST REASONABLE INTERPRETATION IN REEXAMINATION

As set forth in detail later in this Request, the claims of the '706 Patent do not need to be "interpreted" in any particular manner to be found unpatentable over the prior art (*e.g.,* by their plain terms each of the limitations is found in the prior art). Nevertheless, claim interpretation in the reexamination process differs from that in other contexts, such as litigation in the District Court. Therefore, Requester here summarizes the standards applicable in reexamination and emphasizes that this Request addresses the claims using that claim interpretation standard, rather than the standards that are applicable outside the reexamination context.

In the context of reexamining patent claims, "the PTO must apply the broadest reasonable meaning to the claim language, taking into account any definitions presented

in the specification." *In re Bass*, 314 F.3d 575, 577 (Fed. Cir. 2002) (citing *In re Yamamoto*, 740 F.2d 1569, 1571 (Fed. Cir. 1984)); *see also* 37 C.F.R. § 1.555(b). Giving claims their broadest reasonable construction "serves the public interest by reducing the possibility that claims, finally allowed, will be given broader scope than is justified." *In re Yamamoto*, 740 F.2d at 1571. "Construing claims broadly during prosecution is not unfair to the applicant (or, in this case, the patentee), because the applicant has the opportunity to amend the claims to obtain more precise claim coverage." *In re Am. Acad. of Sci. Tech Ctr.*, 367 F.3d 1359, 1363 (Fed. Cir. 2004) (citing *Yamamoto*, 740 F.2d at 1571-72).

While district courts interpret claim language in issued patents in light of the specification, prosecution history, prior art and other claims, this is not the mode of claim interpretation to be applied during examination (including reexamination). During examination, the claims must be interpreted as broadly as their terms reasonably allow. "The USPTO uses a different standard for construing claims than that used by district courts; during examination the USPTO must give claims their broadest reasonable interpretations." MPEP § 2111.01 (citing *Am. Acad. of Sci. Tech Ctr.,* 367 F.3d at 1363). The words of the claim must be given their plain meaning unless the applicant has provided a clear definition in the specification. *In re Zletz*, 893 F.2d 319, 321, 13 U.S.P.Q.2d 1320, 1322 (Fed. Cir. 1989). "[I]n proceedings before the PTO, claims in an application are to be given their broadest reasonable interpretation consistent with the specification . . . as it would be interpreted by one of ordinary skill in the art." *In re Cortright*, 165 F.3d 1353, 1359 (Fed. Cir. 1999) (citing *In re Bond*, 910 F.2d 831, 833

(Fed. Cir. 1990)). Thus, in the analysis and discussion presented below, the identified claims are given their broadest reasonable interpretation.

Because the standards of claim interpretation used in the courts in patent litigation are different from the claim interpretation standards used in the Office in claim examination proceedings (including reexamination), any and all claim interpretations discussed or submitted herein, and all applications of the prior art to the claims, are under the broadest reasonable interpretation specifically for the purpose of demonstrating a SNQ for reexamination within the PTO and are neither binding upon Requester in any litigation related to the '706 patent, nor necessarily the construction of the claims that would result under legal standards that are mandated to be used by the Courts in litigation. *See* 35 U.S.C. § 314; *see also* MPEP § 2686.04 II (determination of a SNQ is made independently of a Court's decision on validity because of different standards of proof and claim interpretation employed by the District Courts and the Office); *see also Trans Texas,* 498 F.3d at 1297-98; *In re Zletz*, 893 F.2d 319, 322 (Fed. Cir. 1989).

The interpretation and/or construction of the claims in the '706 patent presented either implicitly or explicitly herein should not be viewed as constituting, in whole or in part, Requester's own interpretation and/or construction of such claims, but instead should be viewed as constituting an interpretation and/or construction required by the standards applicable in the reexamination context and by Patent Owner's use of broad (and often expansive and undefined) terminology in the claims. Furthermore, Requester expressly reserves the right to present its own interpretation of such claims at a later time, which interpretation may differ, in whole or in part, from that presented herein.

III. SUMMARY OF THE CLAIMS

U.S. Patent No. 6,440,706 (the '706 patent) is generally drawn to methods of dividing templates into a plurality of assay samples and comparing the numbers of assay samples containing a selected and reference sequence. The claims for which reexamination is requested read as follows:

1. A method for determining the ratio of a selected genetic sequence in a population of genetic sequences, comprising the steps of:

diluting nucleic acid template molecules in a biological sample to form a set comprising a plurality of assay samples;

amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence;

comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample.

2. The method of claim 1 wherein the step of diluting is performed until at least one-tenth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required for the step of analyzing to determine the presence of the selected genetic sequence.

3. The method of claim 1 wherein the step of diluting is performed until between 0.1 and 0.9 of the assay samples yield an amplification product when subjected to a polymerase chain reaction.

4. The method of claim 1 wherein the step of diluting is performed until all of the assay samples yield an amplification product when subjected to a polymerase chain reaction and each assay sample contains less than 10 nucleic acid template molecules containing the reference genetic sequence.

5. The method of claim 1 wherein the step of diluting is performed until all of the assay samples yield an amplification product when subjected to a polymerase chain reaction and each assay sample contains less than 100 nucleic acid template molecules containing the reference genetic sequence.

6. The method of claim 1 wherein the biological sample is cell-free.

7. The method of claim 1 wherein the number of assay samples within the set is greater than 10.

8. The method of claim 1 wherein the number of assay samples within the set is greater than 50.

9. The method of claim 1 wherein the number of assay samples within the set is greater than 100.

10. The method of claim 1 wherein the number of assay samples within the set is greater than 500.

11. The method of claim 1 wherein the number of assay samples within the set is greater than 1000.

12. The method of claim 1 wherein the step of amplifying and the step of analyzing are performed on assay samples in the same receptacle.

14. The method of claim 1 wherein the step of analyzing employs gel electrophoresis.

15. The method of claim 1 wherein the step of analyzing employs hybridization to at least one nucleic acid probe.

16. The method of claim 1 wherein the step of analyzing employs hybridization to at least two nucleic acid probe.

19. The method of claim 1 wherein the step of amplifying employs a single pair of primers.

20. The method of claim 1 wherein the step of amplifying employs a polymerase which is activated only after heating.

21. The method of claim 1 wherein the step of amplifying employs at least 40 cycles of heating and cooling.

22. The method of claim 1 wherein the step of amplifying employs at least 50 cycles of heating and cooling.

23. The method of claim 1 wherein the step of amplifying employs at least 60 cycles of heating and cooling.

24. The method of claim 1 wherein the biological sample is selected from the group consisting of stool, blood, and lymph nodes.

25. The method of claim 1 wherein the biological sample is blood or bone marrow of a leukemia or lymphoma patient who has received anti-cancer therapy.

26. The method of claim 1 wherein the selected genetic sequence is a translocated allele.

27. The method of claim 1 wherein the selected genetic sequence is a wild-type allele.

28. The method of claim 1 wherein the selected genetic sequence is within an amplicon which is amplified during neoplastic development.

29. The method of claim 1 wherein the selected genetic sequence is a rare exon sequence.

30. The method of claim 1 wherein the nucleic acid template molecules comprise cDNA of RNA transcripts and the selected genetic sequence is present on a cDNA of a first transcript and the reference genetic sequence is present on a cDNA of a second transcript.

31. The method of claim 1 wherein the selected genetic sequence comprises a first mutation and the reference genetic sequence comprises a second mutation.

32. The method of claim 1 wherein the selected genetic sequence and the reference genetic sequence are on distinct chromosomes.

38. A method for determining the ratio of a selected genetic sequence in a population of genetic sequences, comprising the steps of:

amplifying template molecules within a set comprising a plurality of assay samples to form a population of amplified molecules in each of the assay samples of the set;

analyzing the amplified molecules in the assay samples of the set to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence, wherein at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence; comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample.

39. The method of claim 38 wherein the number of assay samples within the set is greater than 10.

40. The method of claim 38 wherein the number of assay samples within the set is greater than 50.

41. The method of claim 38 wherein the number of assay samples within the set is greater than 100.

42. The method of claim 38 wherein the number of assay samples within the set is greater than 500.

43. The method of claim 38 wherein the number of assay samples within the set is greater than 1000.

44. The method of claim 38 wherein the step of amplifying and the step of analyzing are performed on assay samples in the same receptacle.

46. The method of claim 38 wherein the step of analyzing employs gel electrophoresis.

47. The method of claim 38 wherein the step of analyzing employs hybridization to at least one nucleic acid probe.

48. The method of claim 38 wherein the step of analyzing employs hybridization to at least two nucleic acid probe.

51. The method of claim 38 wherein the step of amplifying employs a single pair of primers.

52. The method of claim 38 wherein the step of amplifying employs a polymerase which is activated only after heating.

53. The method of claim 38 wherein the step of amplifying employs at least 40 cycles of heating and cooling.

54. The method of claim 38 wherein the step of amplifying employs at least 50 cycles of heating and cooling.

55. The method of claim 38 wherein the step of amplifying employs at least 60 cycles of heating and cooling.

56. The method of claim 38 wherein the template molecules are obtained from a body sample selected from the group consisting of stool, blood, and lymph nodes.

57. The method of claim 38 wherein the template molecules are obtained from a body sample of a leukemia or lymphoma patient who has received anti-cancer therapy, said body sample being selected from the group consisting of blood and bone marrow.

58. The method of claim 38 wherein the selected genetic sequence is a translocated allele.

59. The method of claim 38 wherein the selected genetic sequence is a wild-type allele.

60. The method of claim 38 wherein the selected genetic sequence is within an amplicon which is amplified during neoplastic development.

61. The method of claim 38 wherein the selected genetic sequence is a rare exon sequence.

62. The method of claim 38 wherein the nucleic acid template molecules comprise cDNA of RNA transcripts and the selected genetic sequence is present on a cDNA of a first transcript and the reference genetic sequence is present on a cDNA of a second transcript.

63. The method of claim 38 wherein the selected genetic sequence comprises a first mutation and the reference genetic sequence comprises a second mutation.

64. The method of claim 38 wherein the selected genetic sequence and the reference genetic sequence are on distinct chromosomes.

IV. RELEVANCE OF THE PROSECUTION HISTORY TO THIS REEXAMINATION

A copy of the prosecution history file wrapper for the '706 patent is provided as Exhibit 3. The relevant portions of the prosecution of the '706 patent are outlined below.

The application that matured into the '706 patent, U.S. Patent Appl. No. 09/613,826 (the "'826 Application") was filed July 11, 2000. The '826 application claimed priority to a provisional application no. 60/146,792, filed August 2, 1999, included here as Exhibit 4. The '826 application was filed with sixty-four (64) claims, of which claims 1, 33, 37 and 38 are independent.[2]

On April 12, 2001, the Examiner mailed a non-final Office Action in which all 64 claims were rejected. In particular, claims 1-32 and 38-64 were rejected under 35 U.S.C. § 112, second paragraph, as being "incomplete for omitting essential steps," such as omitting the step of "serially diluting to form a set of assay samples and testing

[2] The numbering of the originally-filed claims correspond to the numbering of the issued claims. In this summary of the prosecution history, issues relating to claims that are not the subject of the request for reexamination will not be addressed here unless relevant.

by PCR."[3] Moreover, the Examiner stated that "[i]t appears that the initial concentration of sample at the start of the assay is essential to the invention. Such a step would be critical because it is unclear as to how otherwise the initial concentration would be achieved with testing by PCR."[4] The Examiner also rejected claims 1-32 and 38-64 under 35 U.S.C. § 112, second paragraph, for omitting essential steps, in particular the step of "linear amplification by PCR" which the Examiner deemed to be "essential to the invention."[5]

The Examiner also rejected claims 1, 3, 4-11, 14-16 and 19-32 under 35 U.S.C. § 103(a) as being unpatentable over U.S. Patent No. 5,927,870 ("Lapidus") and PNAS vol. 87, pp. 6296-6300 ("Ruano"). The Examiner stated that Lapidus taught a "method of determining a subpopulation of genomically transformed cells . . . by enumerating [the] number of molecules of a target sequence and comparing with a number of molecules of reference genetic sequence."[6] The Examiner stated that Lapidus "[did] not teach dilution to one half genomic equivalent in samples," but that Ruano taught "single molecule dilution (SMD) in which genomic DNA concentration is one haploid equivalent per aliquot."[7] The Examiner concluded that "[o]ne of ordinary skill would have been motivated to apply Ruano et al SMD method to Lapidus et al's comparison method in order to determine actual allele concentration ratios" and that "[i]t would have been prima facie obvious to apply Ruano et al's dilution method to Lapidus et al's method in order to accurately determine allele ratios."

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3 April 12, 2001 Office Action, page 4 (Exhibit 3).
4 April 12, 2001 Office Action, page 4 (Exhibit 3).
5 April 12, 2001 Office Action, page 4 (Exhibit 3).
6 April 12, 2001 Office Action, pages 5-6 (Exhibit 3).
7 April 12, 2001 Office Action, page 6 (Exhibit 3).

Moreover, the Examiner also stated that "it would have been prima facie obvious to further optimize the assay conditions as in increasing the number of PCR cycles or increasing the dilution schema."[8]

In response to the Examiner's rejection under 35 U.S.C. § 112, second paragraph, relating to a lack of an essential diluting step, the Applicants stated that with respect to claim 38, "this [diluting] step is neither essential nor required. If samples are sufficiently dilute, no dilution is required. Thus dilution is not a necessary step."[9]

In response to the Examiner's second grounds for rejection under 35 U.S.C. § 112, second paragraph, the Applicants stated that in independent claims 1 and 38, "the amplified molecules in the assay samples of the set are analyzed, but a particular analysis method is not required"[10] and "linear amplification is not essential to the method of the invention."[11]

In response to the rejections under 35 U.S.C. § 103(a) based on Lapidus and Ruano, the Applicants argued that the combination of references did not teach "the step of analyzing or the step of comparing as specified in claim."[12] In particular, the Applicants stated that "Lapidus instead teaches determining concentration" which they contend "is different from determining the number of assay samples containing a genetic sequence."[13] Furthermore, the Applicants argued that since "the numbers of assay samples are not determined according to Lapidus, neither are the numbers compared, as

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[8] April 12, 2001 Office Action, page 6 (Exhibit 3).
[9] July 12, 2001 Amendment, page 8 (Exhibit 3).
[10] July 12, 2001 Amendment, page 9 (Exhibit 3).
[11] July 12, 2001 Amendment, page 10 (Exhibit 3).
[12] July 12, 2001 Amendment, page 12 (Exhibit 3).
[13] July 12, 2001 Amendment, page 12, emphasis in original (Exhibit 3).

required in step 4 [of claim 1]."[14] The Applicants then argued that "[t]his difference leads to an advantage of the present invention over Lapidus" and "the present invention eliminates the quantitative bias which exponential amplification introduces into a nucleic acid sample."[15]

In a final Office Action mailed September 20, 2001, the Examiner stated that claims 1-32 and 38-64 were allowable, stating that "[t]here is no prior art that teach or suggest diluting a nucleic acid template in a sample to a plurality of sample and amplifying the template molecule in the samples and analyzing amplified molecules to determine the first number of samples containing the selected genetic sequence and second number assay samples which contain a reference genetic sequence and comparing the two numbers."[16] The Examiner also stated that "there is no prior art that teach or suggest that one tenth or one fiftieth of samples in a set comprise N molecules such that 1/N is larger than the ratio of selected genetic sequence to total genetic sequences required for the step of analyzing to determine presence of selected genetic sequence."[17] The Examiner finally stated that "[t]he closest prior art is Lapidus et al who teach a reference and target nucleic acid amplification and concentration determination. However, his determination of concentration is within a sample and they do not teach or suggest a dilution."[18]

A Notice of Allowability was mailed on March 19, 2002. In the Notice, the Examiner again stated that Lapidus was the closest prior art which taught "a reference

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[14] July 12, 2001 Amendment, page 12 (Exhibit 3).
[15] July 12, 2001 Amendment, page 12 (Exhibit 3).
[16] September 20, 2001 Office Action, page 5 (Exhibit 3).
[17] September 20, 2001 Office Action, page 5 (Exhibit 3).
[18] September 20, 2001 Office Action, page 5 (Exhibit 3).

and target nucleic acid amplification and concentration determination," but distinguished it from the claims because "his determination of concentration is within a sample and they do not teach or suggest a dilution." The '706 patent subsequently issued on August 27, 2002 with 64 claims.

V. SUBSTANTIAL NEW QUESTIONS OF PATENTABILITY

Ex parte reexamination of claims 1-12, 14-16, 19-32, 38-44, 46-48 and 51-64 of the '706 patent is respectfully requested. During prosecution, Applicants distinguished the claims over the references that were relied upon by the Examiner (which did not include any of the references cited in this Request) by arguing that they did not teach the claimed methods. The references cited in Table II, either alone or in combination, provide precisely the teachings that anticipate or render obvious claims 1-12, 14-16, 19-32, 38-44, 46-48 and 51-64 of the '706 patent. Most of these references are not of record, the few that are were not discussed or relied on by the Examiner. Accordingly, the references cited in the Table II, either alone or in combination in the manner described in this Request, raise substantial new questions ("SNQs") of patentability. A brief statement of the SNQs of patentability is set forth immediately below. A detailed explanation of the pertinence and manner of applying the cited prior art to each claim for which reexamination is sought is presented in **Section VI** below.

A. SNQ No. 1: Claims 1-3, 7-9, 14-16, 19, 21, 22, 27 and 32 are anticipated by Li under 35 U.S.C. § 102(b)

Li was published on September 29, 1988, and is thus prior art to the '706 patent under 35 U.S.C. § 102(b).[19] Under the broadest reasonable interpretation of the claims, Li discloses methods that meet all of the limitations of the methods of claims 1-3, 7-9, 14-16, 19, 21, 22, 27 and 32.

SNQ No. 1 based on Li is new for at least two reasons: (i) Although Li was cited in an IDS by the applicants of the '706 patent, Li was not relied upon or discussed by the PTO during original prosecution; and (ii) the explanation presented herein of how Li anticipates claims 1-3, 7-9, 14-16, 19, 21, 22, 27 and 32 was not before the original Examiner.

SNQ No. 1 based on Li is substantial at least because Li teaches all aspects of claims 1-3, 7-9, 14-16, 19, 21, 22, 27 and 32 and squarely anticipates these claims under their broadest reasonable interpretation. Requester notes that the PTO recently found that Li anticipated claims having substantially identical limitations in App. No. 13/071,105, which is a pending continuation of the '706 patent. In contrast, none of the art that was discussed or relied on during the original prosecution of the '706 patent was found to anticipate the '706 claims.

Thus, a substantial new question of patentability is raised by Li with respect to claims 1-3, 7-9, 14-16, 19, 21, 22, 27 and 32.

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[19] Li *et al.*, "Amplification and analysis of DNA sequences in single human sperm and diploid cells." *Nature*. 29;335(6189):414-7 (1988); **Exhibit PA-1.**

B. SNQ No. 2: Claims 4 and 5 of the '706 patent are obvious under 35 U.S.C. § 103(a) over Li in view of Zhang

Li has been discussed above. Zhang was published on July 1, 1992 and is prior art to the '706 patent under 35 U.S.C. § 102(b).[20]

Li and Zhang raise a new question of patentability as to claims 4 and 5 for at least two reasons: (i) Although Li and Zhang were submitted by the applicants during original prosecution of the '706 patent, and neither reference was discussed or relied on by the PTO; (ii) the explanation presented herein of how Li in view of Zhang renders claims 4 and 5 obvious was not before the original Examiner, and (iii) the Zhang and Li references present non-cumulative technological teachings that were not previously discussed or relied on and discussed on the record during the prosecution of the '706 patent.

Li and Zhang raise a substantial question of patentability at least because (i) Li teaches all of the steps of base claim 1, (ii) Zhang teaches the additional recitations of claims 4 & 5, and (iii) Li and Zhang are readily combinable to yield the subject matter of claims 4 & 5. A detailed explanation of why Li's and Zhang's combined teachings would have rendered the claims obvious is presented in more detail in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Li and Zhang with respect to claims 4 and 5.

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[20] Zhang *et al.*, "Whole genome amplification from a single cell: implications for genetic analysis." *PNAS USA*, 89(13):5847-51 (1992); **Exhibit PA-2**

C. SNQ No. 3: Claim 6 is obvious under 35 U.S.C. § 103(a) over Li in view of Jeffreys

Li has been discussed above. Jeffreys was published on December 9, 1988 and is prior art to the '706 patent under 35 U.S.C. § 102(b).[21] Jeffreys is newly cited in the present request.

Li and Jeffreys raise a new question of patentability as to claim 6 for at least two reasons: (i) Jeffreys was not considered during original prosecution of the '706 patent and Li, although cited, was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Li in view of Jeffreys renders claim 6 obvious was not before the original Examiner, and (iii) Li and Jeffreys present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Li and Jeffreys raise a substantial question of patentability at least because (i) Li teaches all of the steps of base claim 1, (ii) Jeffreys teaches the additional recitations of claim 6, and (iii) Li and Jeffreys are readily combinable to yield the subject matter of claim 6. A detailed explanation of why Li's and Jeffreys's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Li and Jeffreys with respect to claim 6.

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[21] Jeffreys *et al.*, "Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells", *Nucl. Acids. Res.*, vol 16, no. 23, pages 10953-10971 (1988); **Exhibit PA-3.**

D. SNQ No. 4: Claims 10 and 11 are obvious under 35 U.S.C. § 103(a) over Li

Li has been discussed above. Li raises a substantial question of patentability because it would have been obvious to those of ordinary skill in the art to practice the methods of claims 10 and 11 in light of the Li. Exemplary rationales as to why Li's teachings would have rendered the claims obvious are presented in more detail in the next section applying the art to the claims.

Li raises a new question of patentability as to claim 10 and 11 for at least two reasons: (i) Li teaches all of the steps of base claim 1, (ii) Li presents non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent, which new teachings render the additional recitations of claims 10 & 11 obvious. A detailed explanation of why Li's teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Li raises a substantial question of patentability at least because (i) Li teaches all of the steps of base claim 1, (ii) Li contains new non-cumulative teachings that render obvious the additional recitations of claims 10 & 11.

Thus, a substantial new question of patentability is raised by Li with respect to claims 10 and 11.

E. SNQ No. 5: Claim 12 is obvious under 35 U.S.C. § 103(a) over Li in view of Kalinina

Li has been discussed above. Kalinina was published on May 1997 and is prior art to the '706 patent under 35 U.S.C. § 102(b).[22] Kalinina is newly cited in the present request.

Li and Kalinina raise a new question of patentability as to claim 12 for at least two reasons: (i) Kalinina was not considered during original prosecution of the '706 patent and Li, although cited, was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Li in view of Kalinina renders claim 12 obvious was not before the original Examiner, and (iii) Li and Kalinina present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Li and Kalinina raise a substantial question of patentability at least because (i) Li teaches all of the steps of base claim 1, (ii) Kalinina teaches the additional recitations of claim 12, and (iii) Li and Kalinina are readily combinable to yield the subject matter of claim 12. A detailed explanation of why Li's and Kalinina's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Li and Kalinina with respect to claim 12.

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[22] Kalinina *et al.*, "Nanoliter scale PCR with TaqMan detection", *Nucl. Acids. Res.* vol 25, 1999-2004 (1997); **Exhibit PA-4.**

F. SNQ No. 6: Claim 20 is obvious under 35 U.S.C. § 103(a) over Li in view of Chou

Li has been discussed above. Chou was published on April 11, 1992 and is prior art to the '706 patent under 35 U.S.C. § 102(b).[23] Although Chou is cited in the specification of the '706 patent, Chou was not cited in an information disclosure statement to the original examiner. Chou is therefore newly cited in the present request.

Li and Chou raise a new question of patentability as to claim 20 for at least two reasons: (i) Chou was not considered during original prosecution of the '706 patent and Li, although cited, was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Li in view of Chou renders claim 20 obvious was not before the original Examiner, and (iii) Li and Chou present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Li and Chou raise a substantial question of patentability at least because (i) Li teaches all of the steps of base claim 1, (ii) Chou teaches the additional recitations of claim 20, and (iii) Li and Chou are readily combinable to yield the subject matter of claim 20. A detailed explanation of why Li's and Chou's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Li and Chou with respect to claim 20.

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[23] Chou *et al.*, "Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications", *Nucleic Acids Res.*, 20(7): 1717–1723 (April 11, 1992); **Exhibit PA-5.**

G. SNQ No. 7: Claim 23 is obvious under 35 U.S.C. § 103(a) over Li in view of Burg

Li has been discussed above. Burg was published on August 1989 and is prior art to the '706 patent under 35 U.S.C. § 102(b).[24] Burg is newly cited in the present request.

Li and Burg raise a new question of patentability as to claim 23 for at least two reasons: (i) Burg was not considered during original prosecution of the '706 patent and Li, although cited, was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Li in view of Burg renders claim 23 obvious was not before the original Examiner, and (iii) Li and Burg present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Li and Burg raise a substantial question of patentability at least because (i) Li teaches all of the steps of base claim 1, (ii) Burg teaches the additional recitations of claim 23, and (iii) Li and Burg are readily combinable to yield the subject matter of claim 23. A detailed explanation of why Li's and Burg's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Li and Burg with respect to claim 23.

H. SNQ No. 8: Claim 24 is obvious under 35 U.S.C. § 103(a) over Li in view of Trumper

Li has been discussed above. Trumper was published on June 1, 1993 and is prior art to the '706 patent under 35 U.S.C. § 102(b).[25] Trumper is newly cited in the present request.

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[24] Burg, *et al.*, "Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction." *J. Clin. Microbiol.* 27, 1787-1792 (1989); **Exhibit PA-6.**

Li and Trumper raise a new question of patentability as to claim 24 for at least two reasons: (i) Trumper was not considered during original prosecution of the '706 patent and Li, although cited, was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Li in view of Trumper renders claim 24 obvious was not before the original Examiner, and (iii) Li and Trumper present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Li and Trumper raise a substantial question of patentability at least because (i) Li teaches all of the steps of base claim 1, (ii) Trumper teaches the additional recitations of claim 24, and (iii) Li and Trumper are readily combinable to yield the subject matter of claim 24. A detailed explanation of why Li's and Trumper's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Li and Trumper with respect to claim 24.

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[25] Trumper *et al.*, "Single-Cell Analysis of Hodgkin and Reed-Sternberg Cells: Molecular Heterogeneity of Gene Expression and p53 Mutations," *Blood*, 81: 3097-3115 (1993); **Exhibit PA-7.**

I. SNQ No. 9: Claim 25 is obvious under 35 U.S.C. § 103(a) over Li in view of Kanzler

Li has been discussed above. Kanzler was published on April 15, 1996 and is prior art to the '706 patent under 35 U.S.C. § 102(b).[26] Kanzler is newly cited in the present request.

Li and Kanzler raise a new question of patentability as to claim 25 for at least two reasons: (i) Kanzler was not considered during original prosecution of the '706 patent and Li, although cited, was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Li in view of Kanzler renders claim 25 obvious was not before the original Examiner, and (iii) Li and Kanzler present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Li and Kanzler raise a substantial question of patentability at least because (i) Li teaches all of the steps of base claim 1, (ii) Kanzler teaches the additional recitations of claim 25, and (iii) Li and Kanzler are readily combinable to yield the subject matter of claim 25. A detailed explanation of why Li's and Kanzler's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Li and Kanzler with respect to claim 25.

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[26] Kanzler *et al.*, "Molecular Single Cell Analysis Demonstrates the Derivation of Peripheral Blood-Derived Cell Line (L1236) From the Hodgkin/Reed-Sternberg Cells of a Hodgkin's Lymphoma Patient", *Blood*, 87: 3429-3436 (1996); **Exhibit PA-8.**

J. SNQ No. 10: Claim 26 is obvious under 35 U.S.C. § 103(a) over Li in view of Gravel

Li has been discussed above. Gravel was published on April 15, 1998 and is prior art to the '706 patent under 35 U.S.C. § 102(b).[27] Gravel is newly cited in the present request.

Li and Gravel raise a new question of patentability as to claim 26 for at least two reasons: (i) Gravel was not considered during original prosecution of the '706 patent and Li, although cited, was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Li in view of Gravel renders claim 26 obvious was not before the original Examiner, and (iii) Li and Gravel present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Li and Gravel raise a substantial question of patentability at least because (i) Li teaches all of the steps of base claim 1, (ii) Gravel teaches the additional recitations of claim 26, and (iii) Li and Gravel are readily combinable to yield the subject matter of claim 26. A detailed explanation of why Li's and Gravel's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Li and Gravel with respect to claim 26.

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[27] Gravel *et al.*, "Single-cell analysis of the t(14;18)(q32;q21) chromosomal translocation in Hodgkin's disease demonstrates the absence of this translocation in neoplastic Hodgkin and Reed-Sternberg cells", *Blood* 91(8):2866-74 (Apr 15, 1998); **Exhibit PA-9.**

K. SNQ No. 11: Claim 28 and 29 are obvious under 35 U.S.C. § 103(a) over Li in view of Marcucci

Li has been discussed above. Marcucci was published on February 15, 1998 and is prior art to the '706 patent under 35 U.S.C. § 102(b).[28] Marcucci is newly cited in the present request.

Li and Marcucci raise a new question of patentability as to claims 28 & 29 for at least two reasons: (i) Marcucci was not considered during original prosecution of the '706 patent and Li, although cited, was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Li in view of Marcucci renders claims 28 & 29 obvious was not before the original Examiner, and (iii) Li and Marcucci present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Li and Marcucci raise a substantial question of patentability at least because (i) Li teaches all of the steps of base claim 1, (ii) Marcucci teaches the additional recitations of claims 28 & 29, and (iii) Li and Marcucci are readily combinable to yield the subject matter of claims 28 & 29. A detailed explanation of why Li's and Marcucci's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Li and Marcucci with respect to claims 28 & 29.

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[28] Marcucci *et al.*, "Detection of Unique ALLÃ•(MLL) Fusion Transcripts in Normal Human Bone Marrow and Blood: Distinct Origin of Normal versus Leukemic ALL1 Fusion Transcripts", *Cancer Res*, 58:790-793. (February 15, 1998); **Exhibit PA-10.**

L. SNQ No. 12: Claim 30 is obvious under 35 U.S.C. § 103(a) over Li in view of Flint

Li has been discussed above. Flint was published on April 1, 1997 and is prior art to the '706 patent under 35 U.S.C. § 102(b).[29] Flint is newly cited in the present request.

Li and Flint raise a new question of patentability as to claim 30 for at least two reasons: (i) Flint was not considered during original prosecution of the '706 patent and Li, although cited, was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Li in view of Flint renders claim 30 obvious was not before the original Examiner, and (iii) Li and Flint present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Li and Flint raise a substantial question of patentability at least because (i) Li teaches all of the steps of base claim 1, (ii) Flint teaches the additional recitations of claim 30, and (iii) Li and Flint are readily combinable to yield the subject matter of claim 30. A detailed explanation of why Li's and Flint's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Li and Flint with respect to claim 30.

M. SNQ No. 13: Claim 31 is obvious under 35 U.S.C. § 103(a) over Li in view of Ponten

Li has been discussed above. Ponten was published on September 1997 and is prior art to the '706 patent under 35 U.S.C. § 102(b).[30] Ponten is newly cited in the present request.

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[29] Flint *et al.*, "NR2A Subunit Expression Shortens NMDA Receptor Synaptic Currents in Developing Neocortex", *J. Neurosci.*, 17(7):2469–2476 (April 1, 1997); **Exhibit PA-11.**

Li and Ponten raise a new question of patentability as to claim 31 for at least two reasons: (i) Ponten was not considered during original prosecution of the '706 patent and Li, although cited, was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Li in view of Ponten renders claim 31 obvious was not before the original Examiner, and (iii) Li and Ponten present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Li and Ponten raise a substantial question of patentability at least because (i) Li teaches all of the steps of base claim 1, (ii) Ponten teaches the additional recitations of claim 31, and (iii) Li and Ponten are readily combinable to yield the subject matter of claim 31. A detailed explanation of why Li's and Ponten's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Li and Ponten with respect to claim 31.

N. SNQ No. 14: Claims 38, 39, 46 & 51 are anticipated by Zhang under 35 U.S.C. § 102(b)

Zhang was published on July 1, 1992, and is thus prior art to the '706 patent under 35 U.S.C. § 102(b). Under the broadest reasonable interpretation of the claims, Zhang discloses methods that meet all of the limitations of the methods of claims 38, 39, 46 & 51.

SNQ No. 14 based on Zhang is new for at least two reasons: (i) Although Zhang was cited by the applicants during original prosecution of the '706 patent, it was not

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[30] Ponten *et al.*, "Genomic analysis of single cells from human basal cell cancer using laser-assisted capture microscopy", *Mutation Research Genomics* 382, 45–55 (1997); **Exhibit PA-12.**

discussed or relied on by the USPTO; and (ii) the explanation presented herein of how Zhang anticipates claims 38, 39, 46 & 51 presented herein was not before the original Examiner.

SNQ No. 14 based on Zhang is substantial at least because Zhang teaches all aspects of claims 38, 39, 46 & 51 and squarely anticipates these claims under their broadest reasonable interpretation. In contrast, none of the art that was discussed or relied on during the original prosecution of the '706 patent was found to anticipate the claims.

Thus, a substantial new question of patentability is raised by Zhang with respect to claims 38, 39, 46 & 51.

O. SNQ No. 15: Claims 40-43, 47, 48, 59 and 64 are obvious under 35 U.S.C. § 103(a) over Zhang in view of Li

Zhang and Li have been discussed above, and both are prior art to the '706 patent under 35 U.S.C. § 102(b).

Zhang and Li raise a new question of patentability as to claims 40-43, 47, 48, 59 and 64 for at least two reasons: (i) Although Zhang and Li were cited by the applicants during original prosecution of the '706 patent, neither reference was discussed or relied on by the USPTO; (ii) the explanation presented herein of how Zhang in view of Li renders claims 40-43, 47, 48, 59 and 64 obvious was not before the original Examiner, and (iii) Zhang and Li present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Zhang and Li raise a substantial question of patentability at least because (i) Zhang teaches all of the steps of base claim 38, (ii) Li teaches the additional recitations of claims 40-43, 47, 48, 59 and 64, and (iii) Zhang and Li are readily combinable to yield

the subject matter of claims 40-43, 47, 48, 59 and 64. A detailed explanation of why Zhang's and Li's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Zhang and Li with respect to claims 40-43, 47, 48, 59 and 64.

P. SNQ No. 16: Claim 44 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Kalinina

Zhang and Kalinina have been discussed above and both are prior art to the '706 patent under 35 U.S.C. § 102(b). Kalinina is newly cited in the present request.

Zhang and Kalinina raise a new question of patentability as to claim 44 for at least two reasons: (i) Kalinina was not considered during original prosecution of the '706 patent and Zhang, although cited, was not discussed or relied on was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Zhang in view of Kalinina renders claim 44 obvious was not before the original Examiner, and (iii) Zhang and Kalinina present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Zhang and Kalinina raise a substantial question of patentability at least because (i) Zhang teaches all of the steps of base claim 38, (ii) Kalinina teaches the additional recitations of claim 44, and (iii) Zhang and Kalinina are readily combinable to yield the subject matter of claim 44. A detailed explanation of why Zhang's and Kalinina's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Zhang and Kalinina with respect to claim 44.

Q. SNQ No. 17: Claim 52 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Chou

Zhang and Chou have been discussed above and both are prior art to the '706 patent under 35 U.S.C. § 102(b). Because Chou was not submitted in an IDS to the examiner, although it is referenced in the '706 specification, Chou is newly cited in the present request.

Zhang and Chou raise a new question of patentability as to claim 52 for at least two reasons: (i) Chou was not considered during original prosecution of the '706 patent and Zhang, although cited, was not discussed or relied on was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Zhang in view of Chou renders claim 52 obvious was not before the original Examiner, and (iii) Zhang and Chou present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Zhang and Chou raise a substantial question of patentability at least because (i) Zhang teaches all of the steps of base claim 38, (ii) Chou teaches the additional recitations of claim 52, and (iii) Zhang and Chou are readily combinable to yield the subject matter of claim 52. A detailed explanation of why Zhang's and Chou's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Zhang and Chou with respect to claim 52.

R. SNQ No. 18: Claims 53-55 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Burg

Zhang and Burg have been discussed above and both are prior art to the '706 patent under 35 U.S.C. § 102(b). Burg is newly cited in the present request.

Zhang and Burg raise a new question of patentability as to claims 53-55 for at least two reasons: (i) Burg was not considered during original prosecution of the '706 patent and Zhang, although cited, was not discussed or relied on was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Zhang in view of Burg renders claims 53-55 obvious was not before the original Examiner, and (iii) Zhang and Burg present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Zhang and Burg raise a substantial question of patentability at least because (i) Zhang teaches all of the steps of base claim 38, (ii) Burg teaches the additional recitations of claims 53-55, and (iii) Zhang and Burg are readily combinable to yield the subject matter of claims 53-55. A detailed explanation of why Zhang's and Burg's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Zhang and Burg with respect to claims 53-55.

S. SNQ No. 19: Claim 56 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Trumper

Zhang and Trumper have been discussed above and both are prior art to the '706 patent under 35 U.S.C. § 102(b). Trumper is newly cited in the present request.

Zhang and Trumper raise a new question of patentability as to claim 56 for at least two reasons: (i) Trumper was not considered during original prosecution of the '706 patent and Zhang, although cited, was not discussed or relied on was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Zhang in view of Trumper renders claim 56 obvious was not before the original Examiner, and (iii) Zhang and Trumper present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Zhang and Trumper raise a substantial question of patentability at least because (i) Zhang teaches all of the steps of base claim 38, (ii) Trumper teaches the additional recitations of claim 56, and (iii) Zhang and Trumper are readily combinable to yield the subject matter of claim 56. A detailed explanation of why Zhang's and Trumper's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Zhang and Trumper with respect to claim 56.

T. SNQ No. 20: Claim 57 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Kanzler

Zhang and Kanzler have been discussed above and both are prior art to the '706 patent under 35 U.S.C. § 102(b). Kanzler is newly cited in the present request.

Zhang and Kanzler raise a new question of patentability as to claim 57 for at least two reasons: (i) Kanzler was not considered during original prosecution of the '706 patent and Zhang, although cited, was not discussed or relied on was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Zhang in view of Kanzler renders claim 57 obvious was not before the original Examiner, and (iii) Zhang and

Kanzler present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Zhang and Kanzler raise a substantial question of patentability at least because (i) Zhang teaches all of the steps of base claim 38, (ii) Kanzler teaches the additional recitations of claim 57, and (iii) Zhang and Kanzler are readily combinable to yield the subject matter of claim 57. A detailed explanation of why Zhang's and Kanzler's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Zhang and Kanzler with respect to claim 57.

U. SNQ No. 21: Claim 58 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Gravel

Zhang and Gravel have been discussed above and both are prior art to the '706 patent under 35 U.S.C. § 102(b). Gravel is newly cited in the present request.

Zhang and Gravel raise a new question of patentability as to claim 58 for at least two reasons: (i) Gravel was not considered during original prosecution of the '706 patent and Zhang, although cited, was not discussed or relied on was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Zhang in view of Gravel renders claim 58 obvious was not before the original Examiner, and (iii) Zhang and Gravel present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Zhang and Gravel raise a substantial question of patentability at least because (i) Zhang teaches all of the steps of base claim 38, (ii) Gravel teaches the additional recitations of claim 58, and (iii) Zhang and Gravel are readily combinable to yield the

subject matter of claim 58. A detailed explanation of why Zhang's and Gravel's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Zhang and Gravel with respect to claim 58.

V. SNQ No. 22: Claims 60 and 61 are obvious under 35 U.S.C. § 103(a) over Zhang in view of Marcucci

Zhang and Marcucci have been discussed above and both are prior art to the '706 patent under 35 U.S.C. § 102(b). Marcucci is newly cited in the present request.

Zhang and Marcucci raise a new question of patentability as to claims 60 & 61 for at least two reasons: (i) Marcucci was not considered during original prosecution of the '706 patent and Zhang, although cited, was not discussed or relied on was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Zhang in view of Marcucci renders claims 60 & 61 obvious was not before the original Examiner, and (iii) Zhang and Marcucci present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Zhang and Marcucci raise a substantial question of patentability at least because (i) Zhang teaches all of the steps of base claim 38, (ii) Marcucci teaches the additional recitations of claims 60 & 61, and (iii) Zhang and Marcucci are readily combinable to yield the subject matter of claims 60 & 61. A detailed explanation of why Zhang's and Marcucci's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Zhang and Marcucci with respect to claims 60 & 61.

W. SNQ No. 23: Claim 62 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Flint

Zhang and Flint have been discussed above and both are prior art to the '706 patent under 35 U.S.C. § 102(b). Flint is newly cited in the present request.

Zhang and Flint raise a new question of patentability as to claim 62 for at least two reasons: (i) Flint was not considered during original prosecution of the '706 patent and Zhang, although cited, was not discussed or relied on was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Zhang in view of Flint renders claim 62 obvious was not before the original Examiner, and (iii) Zhang and Flint present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Zhang and Flint raise a substantial question of patentability at least because (i) Zhang teaches all of the steps of base claim 38, (ii) Flint teaches the additional recitations of claim 62, and (iii) Zhang and Flint are readily combinable to yield the subject matter of claim 62. A detailed explanation of why Zhang's and Flint's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Zhang and Flint with respect to claim 62.

X. SNQ No. 24: Claim 63 is obvious under 35 U.S.C. § 103(a) over Zhang in view of Ponten

Zhang and Ponten have been discussed above and both are prior art to the '706 patent under 35 U.S.C. § 102(b). Ponten is newly cited in the present request.

Zhang and Ponten raise a new question of patentability as to claim 63 for at least two reasons: (i) Ponten was not considered during original prosecution of the '706 patent and Zhang, although cited, was not discussed or relied on was not discussed or relied on by the USPTO; (ii) the explanation presented herein of how Zhang in view of Ponten renders claim 63 obvious was not before the original Examiner, and (iii) Zhang and Ponten present non-cumulative technological teachings that were not discussed on record during the prosecution of the '706 patent.

Zhang and Ponten raise a substantial question of patentability at least because (i) Zhang teaches all of the steps of base claim 38, (ii) Ponten teaches the additional recitations of claim 63, and (iii) Zhang and Ponten are readily combinable to yield the subject matter of claim 63. A detailed explanation of why Zhang's and Ponten's combined teachings would have rendered the claims obvious is presented in the next section applying the art to the claims.

Thus, a substantial new question of patentability is raised by Zhang and Ponten with respect to claim 63.

VI. MANNER OF APPLYING THE CITED PRIOR ART AND PROPOSED REJECTIONS

A. Proposed Rejection No. 1: Li anticipates claims 1-3, 7-9, 14-16, 19, 21, 22, 27 & 32 under 35 U.S.C. § 102(b)

1. Short introductory overview of relevant portions of Li's disclosure

Independent claim 1 and its dependent claims 2-3, 7-9, 14-16, 19, 21, 22, 27 & 32 are anticipated by Li[31] under 35 U.S.C. § 102(b). To provide a quick orientation to the Examiner, this section presents an introductory high-level overview of Li's experiments, the steps of the claims, and how Li's experiments map onto each of these steps. A more detailed application of Li's teachings to each claimed step, showing the details of how Li performed each step with specific cites to Li's relevant disclosure is presented in the next section.

Li performed three separate experiments, each of which anticipates independent claims 1 and 38 and various dependent claims. These three experiments can be briefly summarized as follows:

- ***Experiment 1: Analysis of LDLr locus in sperm:***[32] Li amplified assay samples containing single haploid sperm cells from a heterozygous donor carrying two distinguishable alleles LDLr1 and LDLr2 at the LDLr locus and compared the number of sperm containing each allele against each other to determine the composition of the starting sample with respect to this particular locus.

- ***Experiment 2: Analysis of the LDLr and the HLA DQ-α loci in sperm*** [33] Li amplified assay samples containing single haploid sperm cells from a

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[31] Li *et al., Amplification and analysis of DNA sequences in single human sperm and diploid cells.* Nature. 29;335(6189):414-7 (1988), which forms prior art to the '706 patent under 35 U.S.C. § 102(b) (**Exhibit PA-1**).

[32] This experiment is described in Li on page 415, Section titled "Analysis in single human sperm," paragraph bridging the left and right cols.

[33] This experiment is described in Li on page 415, Section titled "Independent assortment of chromosomes," paragraph bridging pages 415-416, and page 416, left col.

heterozygous donor carrying two distinguishable alleles LDLr1 and LDLr2 at the LDLr locus, and two distinguishable alleles DQA1 and DQA2 at the HLA DQ-α locus. Li compared the number of sperm containing each allele at each individual locus. In addition, Li also analyzed of the number of samples for each observed combination of alleles between the two different loci.

- ***Experiment 3: Analysis of the ß-globin locus in single diploid cells***:[34] Li amplified assay samples containing single diploid cells obtained from a mixture of two different clonal subpopulations of cells. The first subpopulation was homozygous wild-type at the globin locus, while the second subpopulation was homozygous mutant at this locus.

In each of these experiments, Li performed and disclosed each of the principal steps of the claims of the '706 patent. Generally, the claims of the '706 patent are directed to a method requiring four steps: (1) diluting or otherwise forming a set of assay samples containing template molecules from a biological sample; (2) amplifying the template molecules in the assay samples; (3) analyzing the amplified molecules to determine a first number of assay samples that contain one sequence and a second number of assay samples that contain a different sequence; and (4) comparing those numbers to ascertain a ratio that reflects the composition of the biological sample.

Indeed, the PTO has already found that substantially similar claims of a related pending application are not patentable over Li. In particular, in the October 10, 2012 non-final Office Action in App. No. 13/071,105 ("the '105 application), which is a continuation application of the '706 patent, the PTO rejected nearly all of the pending claims because Li anticipates and/or renders them obvious.[35] The similarities between the main steps of independent claims 1 and 38 of the '706 patent and pending claim 49 of the '105 application are compared side-by-side in the chart below.

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[34] This experiment is described in Li on page 414, paragraph bridging the left and right cols.

[35] Relevant portions of the '105 file history are provided as Exhibit 5.

'706 claim 1	'706 claim 38	'105 claim 49 (as of 6/11/12)[36]
1. A method for determining the ratio of a selected genetic sequence in a population of genetic sequences, comprising the steps of:	38. A method for determining the ratio of a selected genetic sequence in a population of genetic sequences, comprising the steps of:	A method for detecting a genetic sequence in a mixed population of nucleic acid sequences, comprising:
diluting nucleic acid template molecules in a biological sample to form a set comprising a plurality of assay samples;		distributing or diluting a mixed population of nucleic acid sequences into at least ten assay samples such that said at least ten assay samples each comprises less than ten template molecules;
amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set;	amplifying template molecules within a set comprising a plurality of assay samples to form a population of amplified molecules in each of the assay samples of the set;	amplifying the template molecules in the assay samples, wherein an assay sample with a single template molecule forms homogeneous amplification products in the assay sample;
analyzing the amplified molecules in the assay samples of the set	analyzing the amplified molecules in the assay samples of the set	determining nucleic acid sequence of amplification products from an assay sample with homogeneous amplification products.
to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence;	to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence,	
	wherein at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence;	
comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample.	comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample.	

[36] See '105 file history, Response to Restriction Requirement filed June 11, 2012, claim 49, at page 8 (Exhibit 5).

In each of his three experiments, Li performed the four steps of independent claim 1 of the '706 patent, as follows.

a) Diluting and/or set-forming step

- The first step involves "*diluting ... template molecules in a biological sample*" to form "*a set comprising a plurality of assay samples*" starting from a biological sample.

- In multiple experiments, Li started by diluting a suspension of template-containing haploid or diploid cells, thereby "*diluting ... template molecules in a biological sample.*" Li then generated a plurality of single-cell assay samples by transferring single isolated cells each into separate tubes, thereby forming "*a set comprising a plurality of assay samples*" starting from a biological sample.

- ***The PTO has already found that Li discloses "distributing or diluting a mixed population of nucleic acid sequences into at least ten assay samples,"*** in the pending '105 continuation of the '706 patent.[37] Under the reasoning, Li anticipates forming "*a set comprising a plurality of assay samples*"

b) Amplifying step

- The second step involves "*amplifying the template molecules within the assay samples.*"

- Li subjected each of his single-cell assay samples to amplification by a polymerase chain reaction (PCR) using primers directed to one or more genetic loci of interest, thereby "*amplifying the template molecules within the assay samples.*" Amplification at a given locus would generate a single amplification product of a single allele from a haploid sperm cell or two different amplification products corresponding to two different alleles from a diploid cell (or alternatively a single amplification product if allele-specific primers are used).

- ***The PTO has already found that Li discloses "amplifying the template molecules in the assay samples"*** as recited in the pending claims of the '105 continuation the '706 patent.[38]

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[37] October 10, 2012 Non-Final Office Action in the '105 application, at page 3 (Exhibit 5).
[38] October 10, 2012 Non-Final Office Action in the '105 application, at page 4 (Exhibit 5).

c) Analyzing/determining step

- The third step involves "*analyzing the amplified molecules ... to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence*."

- Li determined the allelic identity of each amplification product from each single-cell sample by hybridizing the products with allele-specific oligonucleotides (ASOs), thereby "*analyzing the amplified molecules.*"[39] Li then counted the number of samples showing amplification of a first allele at a locus of interest, and also the number showing amplification of a second allele at the same locus, thereby determining a "*first number of samples which contain the selected genetic sequence* and a *second number of samples which contain a reference genetic sequence*" in the form of the second allele at the same locus.

- ***The PTO has already found that Li discloses "determining nucleic acid sequence of amplification products from an assay sample***," as recited in the claims of the pending '105 continuation the '706 patent.[40] Under the same reasoning, Li discloses "*analyzing the amplified molecules.*" Since the '105 claims did not recite determining a first and second number of assay samples, the PTO did not reach this issue.

d) Comparing step

- The fourth step involves "*comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample*."

- Li compared the number of single-cell assay samples showing amplification of the first allele against the number of assay samples showing amplification of the second allele, thereby "*comparing the first number to the second number to ascertain a ratio.*"[41] This ratio reflected the relative frequencies of each allele in the initial biological sample, and thus "*reflect[ed] the composition of the biological sample*."

Each of Li's three experiments also anticipates various dependent claims.

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[39] See descriptions of Experiments 1, 2 and 3 below for details.

[40] October 10, 2012 Non-Final Office Action in the '105 prosecution history, at page 4 (Exhibit 5).

[41] See descriptions of Experiments 1, 2 and 3 below for details.

2. Detailed application of Li to independent claim 1

This section discusses in detail how, under the broadest reasonable interpretation of the claims, Li discloses methods that meet each and every limitation of independent claim 1.

i) Li discloses "A method for determining the ratio of a selected genetic sequence in a population of genetic sequences"

This language forms the preamble of claim 1. Under the PTO's standards for patentability, a preamble which merely recites an "intended use" does not limit the claim in any way.[42]

But even if it were limiting (which it is not), Li discloses "*determining the ratio of a selected genetic sequence in a population of genetic sequences*" under the broadest reasonable interpretation. Requester notes as a threshold matter that, as written, the preamble broadly uses the word "ratio" in a manner synonymous with "amount" or perhaps "relative amount" as opposed to using "ratio" in a strict mathematical sense. In addition, based on the language and structure of the body of the claim, "*a method for determining the ratio of a selected genetic selection in a population of genetic sequences*" as recited in the preamble more specifically involves "*comparing the first number [of assay samples] to the second number [of assay samples] to ascertain a ratio which reflects the composition of the biological sample*" as recited in the final limitation of claim 1.

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[42] *Rowe v. Dror*, 112 F.3d 473, 478, 42 USPQ2d 1550, 1553 (Fed. Cir. 1997) (preamble's recitation of an intended use is not a limitation). Solely for the purposes of this reexamination, Requester will proceed on the premise that the preamble is not limiting in any way, or at least does not impose an additional limitation over what is recited in the body of the claim.

Accordingly, under the broadest reasonable interpretation (and solely for the purposes of this reexamination), a direct explicit numerical comparison between a first number of assay samples with a selected sequence and a second number of assay samples with a reference sequence,[43] constitutes "*comparing the first number to the second number to ascertain a ratio*" as recited in the last step of claim 1 and "*determining the ratio of a selected genetic sequence in a population of genetic sequences"* as recited in the preamble. In other words, an explicit comparison of the two numbers, even without any explicit mention of a "ratio" between the two numbers, is a disclosure of "*determining the ratio of a selected genetic sequence in a population of genetic sequences.*"

Li not only made exactly such comparisons in each of his three experiments, thereby determining or ascertaining ratios as set forth in claim 1, he expressly characterized his comparisons as "ratios" that were reflective of the composition of the biological sample.

In **Experiment 1** identified in the overview section, Li "analysed the LDLr genotypes in 80 individual sperm" assay samples[44] and counted the number of samples containing each allele. Li compared these two numbers, noting that "[t]wenty-two [samples] carried one allele; 21 the other,"[45] in an equal ratio – noting that "[t]he distribution of the two amplified alleles obeyed Mendels' law of independent segregation," which results in an approximate 1:1 ratio between the two alleles. Li explained later that Mendelian segregation requires that the number of samples

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[43] Specifically, comparing a first number of assay samples containing a selected sequence with a second number of assay samples containing a reference sequence.

[44] Li, page 415, paragraph bridging left and right cols.

[45] Li, page 415, paragraph bridging left and right cols.

containing each allele should be the same, in an "expected 1:1 ratio."[46] By comparing these two numbers in this manner, Li thereby performed a step of "*determining the ratio of a selected genetic sequence in a population of genetic sequences.*"

Li later explicitly noted that Mendelian segregation predicts that the number of samples containing each allele should be in an "expected 1:1 ratio."[47] By comparing these two numbers in this manner to confirm that they were approximately equal in a 1:1 ratio, Li thereby "*determin[ed] the ratio of a selected genetic sequence in a population of genetic sequences"* as recited in the preamble.

Li also performed the same analysis on two loci together, DQA (also called HLA DQ-α) and LDLr, in **Experiment 2** (results displayed in Table 1 and Fig. 3). In particular, Li determined the frequencies of each of two alleles at both of the loci in a set of 150 different single-sperm assay samples,[48] 114 of which were amplifiable for one or more alleles by Li's methods.[49] Under the broadest reasonable interpretation, either of the two alleles at a single locus corresponds to the "*selected genetic sequence*" of the preamble.

With respect to the LDLr locus, Li noted that the number of samples containing the first and second allele should be approximately equal, in an "expected 1:1 ratio."[50] Li confirmed that actual results conformed to the expected ratio: "96 could be typed at the LDLr locus with 45 having LDLr1 and 51 having LDLr2," leading Li to conclude that

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[46] Li, page 416, left col., second paragraph.
[47] Li, page 416, left col., second paragraph.
[48] Li, page 415, right col., last paragraph and Table 1.
[49] Li, page 416, left col., second paragraph.
[50] Li, page 416, left col., second paragraph.

"[t]he two alleles segregate in the expected 1:1 ratio."[51] By comparing these two numbers in this manner, Li thereby "*determinin[ed] the ratio of a selected genetic sequence in a population of genetic sequences*" as recited in the preamble.

With respect to the DQA locus, Li noted that "eighty-eight [assay samples] could be typed at the DQA locus: 53 had the DQA1 allele, 35 the DQA2."[52] Li noted that the "segregation of the DQA alleles with the expected 1:1 ratio is at the borderline of statistical significance,"[53] thereby "*determining the ratio of a selected genetic sequence in a population of genetic sequences*" as recited in the preamble.

In **Experiment 3**, shown in Fig. 1, Li again determined "*the ratio of a selected genetic sequence in a population of genetic sequences*" in diploid tissue culture cells where some of the cells were homozygous for the β-globin sickle-cell mutation β^S and some cells were homozygous for the normal β^A allele. From that starting tissue culture sample, Li created a set of 37 single-cell samples from this mixture.[54] Li then determined the number of single-diploid-cell samples containing the β^A and β^S globin alleles by PCR amplification followed by hybridization to allele-specific oligonucleotide probes that selectively hybridized to either one of the two β globin alleles investigated. Under the broadest reasonable interpretation, either of these β globin alleles forms the "*selected genetic sequence*" of the preamble. 84% of the single-cell samples showed hybridization to a probe, thereby demonstrating the presence of the corresponding allele within the sample – specifically, "19 [samples hybridized] with the β^A probe and 12 with the β^S

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[51] Li, page 416, left col., second paragraph.
[52] Li, page 416, left col., second paragraph.
[53] Li, page 416, left col., second paragraph.
[54] Li, page 414, right col., first paragraph.

probe. . . . No sample hybridized with both probes."[55] By noting that 19 samples contained the β^A allele and 12 contained the β^S allele,[56] *i.e.*, a 19:12 ratio, Li thereby "*determin[ed] the ratio of a selected genetic sequence in a population of genetic sequences.*"

ii) *Li discloses "diluting nucleic acid template molecules in a biological sample to form a set comprising a plurality of assay samples"*

Claim 1 of the '706 patent recites a step of "*diluting nucleic acid template molecules in a biological sample.*" Li discloses such a step. In particular, for **Experiments 1 and 2**, Li purified sperm by diluting a biological sample in the form of 0.5 ml of semen by adding 3 ml of a sucrose solution to the sample, and further diluted this sample by applying it to a 10.5 ml volume of a sucrose gradient and spinning the sample through the gradient.[57] From the purified sperm sample (the concentration of which was 1 x10^5 sperm/ml)[58], Li then created a set comprising "80 individual sperm"[59] assay samples from a sample for Experiment 1 and a set comprising a "total of 150 individual sperm" assay samples for Experiment 2.[60] Such samples are more dilute than the purified sample as they contain only one sperm in a volume of 20 µl.[61] By diluting sperm cells, where each sperm contains template molecules in the form of a haploid

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[55] Li, page 414, right col., first paragraph.
[56] Li, page 414, right col., first paragraph.
[57] Li, Fig. 2, legend ("0.5 ml semen was mixed with 3 ml of 40% sucrose. The mixture was applied to the top of a sucrose step gradient made by adding 3.5 ml 90%, 70% and 50% (w/ v) sucrose successively to a 15-ml graduated plastic tube (Falcon 2095). The sample was spun at 14,500g for two hours at room temperature. 0.5 ml of the interface between 70% and 90% sucrose was collected and applied to an identical sucrose gradient. This was repeated twice more.")
[58] Li, page 415, legend to Figure 2.
[59] Li, page 415, left col., bottom paragraph.
[60] Li, page 415, right col., last paragraph.
[61] Li, page 415, legend to Figures 1 and 2.

genome, Li thereby "*dilut[ed] nucleic acid template molecules in a biological sample*" as recited in claim 1. Further, the dilutions resulted in a set of 80 and a set of 150 single-sperm samples which constitute "*a set comprising a plurality of assay samples.*"

For **Experiment 3**, Li started with a co-cultivation of "cells homozygous for β^A and cells homozygous for β^S in the same tissue culture flask. . . ."[62] Under the broadest reasonable interpretation, this starting tissue-cultured sample of cells is "a biological sample" as recited in claim 1. Solely for the purposes of this reexamination, Requester will accordingly proceed under the premise that Li's cell sample constitutes "a biological sample" under the broadest reasonable interpretation. Li discloses that this cell sample was subjected to "washing three times,"[63] which necessarily involves addition of washing solution, thereby diluting the templates in the cell sample (*i.e.*, "*biological sample*"). The concentration of the cell sample at that step was 1-3 x 10^5 cells/ml.[64] Next, Li selected single cells from the washed cell suspension, where "[i]ndividual cells were drawn into a thin plastic pipette during observation under a phase-contrast microscope. Each individual cell was delivered into a PCR tube containing a lysis solution and, after incubation, PCR buffer [and other components]."[65] Each cell contains template molecules for the subsequent PCR. Each single-cell sample was in a volume of 20 µl,[66] which is more dilute than 1-3 x 10^5 cells/ml. Thus, Li diluted template molecules as required by this claim limitation.

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62 Li, page 414, left col., top paragraph.
63 Li, Fig. 1, legend.
64 Li, page 415, legend to Figure 1.
65 Li, page 415, legend to Figure 1.
66 Li, page 415, legend to Figures 1 and 2.

For **Experiment 3**, Li also formed a set comprising a plurality of assay samples. In particular Li created and analyzed 37 single-diploid-cell samples.[67] Under the broadest reasonable construction, a set of 37 single-cell samples constitutes "*a set comprising a plurality of assay samples*."

Furthermore, as discussed in the overview section (Section VI.A.1), the claims of a related pending continuation of the '706 patent (the '105 application) have a similar requirement of "*distributing ... nucleic acid sequences into at least ten assay samples*."[68] The PTO recently found that this limitation in '105 claim 49 is anticipated by Li. In particular, the PTO found that:

> The mixed population of nucleic acid sequences was "a semen sample": page 415, column 1, last [partial] paragraph: "Sperm were purified from a semen sample ..." Each sperm would contain 23 chromosomes, and some sperm would comprise maternally derived chromosomes, while others would comprise paternally derived chromosomes. In addition, some sperm would comprise Y chromosomes, whereas others would comprise X chromosomes. Furthermore, the semen sample was obtained from an individual known to be heterozygous at a locus of the LDLr gene (page 415, column 1, last [full] paragraph). Li took single sperm and placed them into separate tubes for amplification (page 415, column 1, last [partial] paragraph). By doing so, Li distributed the mixed population into at least ten (in this case, 80) "assay samples" such that each of the assay samples comprised less than ten (in this case, 1) template molecules; the template, in this case, is the LDLr gene.[69]

Under the same reasoning, Li anticipates the broader recitation of "*form[ing] a set comprising a plurality of assay samples*" containing nucleic acid template molecules as recited in '706 claim 1.

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[67] Li, page 414, right col., first paragraph.

[68] *See* '105 file history, Response to Restriction Requirement filed June 11, 2012, at page 8 (Exhibit 5).

[69] October 10, 2012 Non-Final Office Action in the '105 prosecution history, at page 3 (Exhibit 5).

iii) *Li discloses "amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set"*

Li discloses "*amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set*" as recited in claim 1.

In **Experiment 1** (LDLr locus only),[70] Li subjected his 80 sperm assay samples to amplification with a single set of "two primers … for PCR of the LDLr locus".[71]

In **Experiment 2** (LDLr and DQA loci together),[72] Li "attempted to amplify simultaneously DNA sequences at two different loci on non-homologous chromosomes in a single sperm" in 180 assay samples, using a primary multiplex amplification reaction of "20 amplification cycles in the presence of both primer pairs" for two different loci, DQA and LDLr.[73]

In **Experiment 3** (β-globin locus), Li subjected his single-diploid-cell assay samples to amplification with "a [single] set of PCR primers that amplify the informative region of the globin gene," using "50 cycles of amplification."[74]

In these experiments, the chromosomal DNA of the single cells in the assay samples acted as "*template molecules*" that were amplified by PCR, resulting in the generation of a "*population of amplified molecules in the assay samples of the set.*"

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[70] Li, page 415, paragraph bridging left and right cols.

[71] Li, page 415, paragraph bridging left and right cols. ("We adapted the detection of an LDLr polymorphism to PCR … with the PCR primers … shown in the Fig. 2 legend") and Fig. 2, legend (providing "sequence of the two primers used for PCR of the LDLr locus").

[72] Li, page 415, paragraph bridging left and right cols.

[73] Li, paragraph bridging pages 415 and 416 (We attempted to amplify simultaneously DNA sequences at two different loci on non-homologous chromosomes in a single sperm. … we performed only the first 20 amplification cycles in the presence of both primer pairs").

[74] Li, page 414, right col., first paragraph ("after incubation, PCR buffer containing deoxyribonucleotides, Taq DNA polymerase and a set of PCR primers that amplify the informative region of the globin gene was added. After DNA denaturation 50 cycles of amplification were performed").

Therefore, Li discloses "*amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set*" as recited in claim 1. Moreover, the PTO has already found that Li discloses "*amplifying the template molecules in the assay samples*" as recited in the pending claims of the '105 continuation of the '706 patent.[75]

iv) *Li discloses "analyzing the amplified molecules in the assay samples of the set"*

Under the broadest reasonable interpretation, Li's experiments included a step of "*analyzing the amplified molecules in the assay samples of the set*" as recited in claim 1.

In Li's **Experiment 1** (*i.e.*, LDLr locus only),[76] Li "analysed the LDLr genotypes in 80 individual sperm" to determine which LDLr allele was present in each individual sperm sample.[77] Specifically, Li "adapted the detection of an LDLr polymorphism … to PCR and ASO [*i.e.*, allele-specific oligonucleotide probe] analysis,"[78] by way of dot blots, where the probes in particular were a first allele-specific oligonucleotide probe (ASO) "for the LDLrl allele" and the second ASO probe specific for the LDLr2 allele.[79] Through this process of hybridizing the amplified molecules obtained from a PCR reaction to allele-specific probes in dot-blot format on a membrane, Li "*analyz[ed] the amplified molecules in the assay samples of the set.*"

Li's **Experiment 2** used a similar hybridization assay to determine the allelic identity of his primary amplification products (thereby "*analyzing the amplified*

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[75] October 10, 2012 Non-Final Office Action in the '105 prosecution history, at page 4 (Exhibit 5).

[76] Li, page 415, paragraph bridging left and right cols.

[77] Li, page 415, left col., last paragraph.

[78] Li, page 415, legend to Fig. 1.

[79] Li, Figure 2 legend.

molecules in the assay samples of the set"). In particular, Li analyzed "*the amplified molecules in the assay samples*" in the form of amplification products of a first "primary" amplification reaction, by first subjecting aliquots of his primary amplification products to secondary amplification with either LDLr or DQA primers and hybridizing "part of each secondary [amplification] reaction … to either of the two ASOs for that locus," [80] where such hybridization was ultimately informative of the allelic identity of the "*amplified molecules in the assay samples*" generated by the primary amplification reaction. Under the broadest reasonable interpretation, analyzing can include a multi-step process where one of the steps is a second amplification reaction. The '706 patent does not limit the broadest reasonable interpretation in any way. In fact, the '706 specification allows the use of any analytical technique of choice: "[a]lthough the working examples demonstrate the use of molecular beacon probes as the means of analysis of the amplified dilution samples, other techniques can be used as well."[81] Thus, under the broadest reasonable construction, Li's second round of PCR in Experiment 2 is part of the analysis step.

In **Experiment 3**, Li "*analyz[ed] the amplified molecules in the assay samples of the set*" by subjecting aliquots of amplified products to hybridization "separately with the β^A and β^S probes after fixation to nylon membranes."[82]

Moreover, as discussed in the overview section, the PTO has already found that Li discloses the step of "*amplifying the template molecules in the assay samples, wherein an assay sample with a single template molecule from homogenous amplification*

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[80] Li, paragraph bridging pages 415-416.
[81] '706 patent, col. 7, lines 45-51.
[82] Li, page 414, right col., first paragraph.

products in the assay sample" in the claims of the pending '105 continuation of the '706 patent is disclosed by Li.[83] Specifically, the examiner stated: "Li amplified each of the assay samples containing individual sperm; page 415, column 1, last [partial] paragraph: "Individual sperm were drawn into a fine plastic needle under microscopic observation and delivered to a tube for lysis and amplifications." Since each sample containing one sperm would contain one template molecule (*i.e.* LDLr gene), the amplification product from such a sample would inherently be homogeneous."[84] Accordingly, because claim 1 of the '706 patent and the pending claim of the '105 application both contain an identical recitation of "*analyzing the amplified molecules in the assay samples of the set,*" Li discloses this precise limitation.

v) *Li's analysis was done "to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence"*

Li's experiments included a step "*to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence*" as recited in claim 1. Under the broadest reasonable interpretation, (1) a "*number of assay samples which contain [a particular] sequence*" can be a subset of the original set of assay samples in which each member of the subset contains that particular sequence, and (2) a "*selected genetic sequence*" can be any one allele at a locus and a "*reference genetic sequence*" can be the other allele at the same locus.

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[83] October 10, 2012 Non-Final Office Action in the '105 prosecution history, at page 3 (Exhibit 5).

[84] *Id.* at 4.

In Li's **Experiment 1** (LDLr locus only), Li noted that "[t]wenty-two [single-sperm assay samples] carried one [LDLr] allele," [85] thereby *"determin[ing] a first number of assay samples which contain the selected genetic sequence*." Li also noted that "21 [assay samples contained the] the other" allele, [86] thereby "*determin[ing] a second number of assay samples which contain a reference genetic sequence.*"

In Li's **Experiment 2** (LDLr and DQA loci together), Li stated that "96 [assay samples] could be typed at the LDLr locus with 45 having LDLr1,"[87] thereby *"determin[ing] a first number of assay samples which contain the selected genetic sequence*." Li also found that 51 assay samples contained LDLr2, [88] thereby "*determin[ing] a second number of assay samples which contain a reference genetic sequence.*" Similarly, in the same experiment, Li noted that "eighty-eight [assay samples] could be typed at the DQA locus: 53 had the DQA1 allele, 35 the DQA2,"[89] thereby also teaching *"determin[ing] a first number of assay samples which contain the selected genetic sequence*" and "*determin[ing] a second number of assay samples which contain a reference genetic sequence.*"

Thus in **Experiment 1**, either one of the LDLr alleles constituted the "*selected genetic sequence,*" whereas the other remaining allele constituted the "*reference genetics sequence*." In the DQA locus analysis of **Experiment 2**, either one of the DQA alleles constituted the "*selected genetic sequence,*" whereas the other remaining allele

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[85] Li, page 415, paragraph bridging left and right cols. ("In a series of experiments we analysed the LDLr genotypes in 80 individual sperm … Twenty-two [sperm] carried one allele; 21 the other.")

[86] Li, page 416, left col., first paragraph.

[87] Li, page 416, left col., first paragraph.

[88] Li, page 416, left col., first paragraph.

[89] Li, page 416, left col., first paragraph.

constituted the "*reference genetics sequence.*" In the LDLr locus analysis of **Experiment 2**, either one of the DQA alleles constituted the "*selected genetic sequence,*" whereas the other remaining allele constituted the "*reference genetics sequence.*" Similarly, in **Experiment 3**, either one of the β^A and β^S globin alleles constituted the "*selected genetic sequence,*" whereas the other remaining allele constituted the "*reference genetics sequence*."

Li therefore *"determine[d] a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence*" as recited in claim 1.

Moreover, as discussed in the overview section, the PTO has already found that Li discloses the step of "*determin[ing] nucleic acid sequence of amplification products from an assay sample with homogenous amplification products*" in the claims of the pending '105 continuation of the '706 patent. In particular, the examiner stated: "Li amplified a known sequence: a 254 bp fragment of the LDLr gene (page 415, column 1, last [full] paragraph). The only missing sequence information was which single nucleotide polymorphism was present in each of the homogeneous amplified products, which Li determined using allele-specific oligonucleotides (see figure 2 and legend.) In this way, Li determined "nucleic acid sequence of amplification products from an assay sample with homogeneous amplification products."[90]

[90] October 10, 2012 Non-Final Office Action in the '105 prosecution history, at page 4 (Exhibit 5).

vi) *Li discloses "comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample."*

Li discloses "*comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample*" under the broadest reasonable interpretation. Requester notes as a threshold matter that the claim broadly uses the word "ratio" in a manner synonymous with "amount" or perhaps "relative amount" as opposed to a formal mathematical determination of a "ratio." In addition, based on the language and structure of the body of the claim, "*a method for determining the ratio of a selected genetic selection in a population of genetic sequences*" as recited in the preamble more specifically involves "*comparing the first number [of assay samples] to the second number [of assay samples] to ascertain a ratio which reflects the composition of the biological sample*" as recited in the final limitation of claim 1

Accordingly, under the broadest reasonable interpretation (and solely for the purposes of this reexamination), a direct and explicit numerical comparison between a first number of assay samples with a selected sequence and a second number of assay samples with a reference sequence,[91] constitutes "comparing the first number to the second number to ascertain a ratio" as recited in the last step of claim 1.

Li not only made exactly such comparisons in each of his three experiments, thereby determining or ascertaining ratios as set forth in claim 1, he expressly characterized his comparisons as "ratios" that were reflective of the composition of the biological sample.

[91] Specifically, comparing a first number of assay samples containing a selected sequence with a second number of assay samples containing a reference sequence.

In **Experiment 1** identified in the overview section, Li "analysed the LDLr genotypes in 80 individual sperm" assay samples[92] and counted the number of samples containing each allele. Li compared these two numbers, noting that "[t]wenty-two [samples] carried one allele; 21 the other,"[93] in an equal ratio – noting that "[t]he distribution of the two amplified alleles obeyed Mendels' law of independent segregation," which results in an approximate 1:1 ratio between the two alleles. Li explained later that Mendelian segregation requires that the number of samples containing each allele should be the same, in an "expected 1:1 ratio."[94] By comparing these two numbers in this manner, Li thereby performed a step of "*comparing the first number to the second number to ascertain a ratio.*"

Experiment 2 identified in the overview section involved the same analysis in more extensive form. In this experiment, Li analyzed alleles at two separate loci, HLA DQ-α and LDLr, in a set of 150 different single-sperm assay samples.[95] Among other things, Li compared the frequencies of both alleles at the LDLr locus and noted that "96 [assay samples] could be typed at the LDLr locus with 45 having LDLr1 and 51 having LDLr2,"[96] at approximately equal frequencies as expected. Li concluded that the "two alleles segregate in the expected 1: 1 ratio,"[97] thereby "*comparing the first number to the second number to ascertain a ratio.*"

From the same **Experiment 2**, Li similarly compared the frequencies of both alleles at the DQA locus and noted that "eighty-eight [assay samples] could be typed at

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92 Li, page 415, paragraph bridging left and right cols.
93 Li, page 415, paragraph bridging left and right cols.
94 Li, page 416, left col., second paragraph.
95 Li, page 415, right col., last paragraph and Table 1.
96 Li, page 416, left col., second paragraph.
97 Li, page 416, left col., second paragraph.

the DQA locus: 53 had the DQA1 allele, 35 the DQA2"[98] at approximately equal frequencies as expected. Li concluded that the "segregation of the DQA alleles with the expected 1:1 ratio is at the borderline of statistical significance,"[99] thereby "*comparing the first number to the second number to ascertain a ratio.*" An allelic frequency ratio of 1:1 "*reflects the composition of the biological sample*" in various ways, *e.g.*, by reflecting a normal segregation of alleles in the sample.

In **Experiment 3**, Li took a biological sample in the form of a tissue-cultured diploid cell sample containing cells homozygous for the β^A globin allele and cells homozygous for the β^S globin allele, and created a set of 37 single-cell samples, determined the number of single-diploid-cell samples containing the β^A and β^S globin allele and compared these two numbers by noting that 19 samples contained the β^A allele and 12 contained the β^S allele,[100] *i.e.*, a 19:12 ratio. By comparing these two numbers in this manner, Li thereby performed a step of "*comparing the first number to the second number to ascertain a ratio*" under the broadest reasonable interpretation. The ratio of 19:12 "*reflects the composition of the biological sample*" in various ways, *e.g.*, by reflecting the proportion of the two different cell lines in the original co-cultivated mixture.

Therefore, Li teaches the "*comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample*" as recited in claim 1.

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[98] Li, page 416, left col., second paragraph.

[99] Li, page 416, left col., second paragraph.

[100] Li, page 414, right col., first paragraph ("Out of the 37 cells analysed, 84% hybridized with only one of the two allele-specific probes; 19 with the β^A probe and 12 with the β^S probe … No sample hybridized with both probes").

3. Detailed explanation of the pertinency and manner of applying Li to claim 2

Dependent claim 2 claims the method of claim 1 "*wherein the step of diluting is performed until at least one-tenth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required for the step of analyzing to determine the presence of the selected genetic sequence.*"

This language of claim 2 is insolubly vague and ambiguous - for example, it is completely unclear what is meant by "*total genetic sequences required for the step of analyzing to determine the presence of the selected genetic sequence.*" Although Requester believes it fails to meet the requirements of Section 112, ¶ 2, such a determination not within the scope of this reexamination. Although the specification does not use the phrase "total genetic sequences," the specification instead explains that "it would be desirable that at least 1/50 of the diluted samples have a ***detectable proportion*** of analyte," such that "[a]t least 1/10, 1/5, 3/10, 2/5, 1/2, 3/5, 7/10, 4/5, or 9/10 of the diluted samples may have a ***detectable proportion*** of analyte,"[101] and apparerently equates "analyte" with "selected genetic sequence."[102] Solely for the purposes of this reexamination, Requester proceeds on the assumption that claim 2 in effect requires that at least one-tenth of the assay samples must contain a detectable proportion of the selected sequence of interest compared to other sequences present in the same assay sample, such that the selected sequence is present in an amount that is detectable by the particular detection method being used. Consequently, a showing that

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[101] '706 patent, col. 4, lines 19-23, emphasis added.

[102] '706 patent, col. 3, line 66-Col. 4, line 2; col. 4, lines 13-22; col. 5, line 44-col. 6, line 2.

the selected sequence of interest was ***actually detected*** in at least one-tenth of the assay samples is sufficient to anticipate this recitation of claim 2.

Under this premise, all three experiments of Li each anticipate claim 2.

In **Experiment 1**, Li detected a "*selected genetic sequence*" in the form of a first LDLr allele in 22 of 80 single-sperm assay samples,[103] thereby demonstrating that about 28% of his samples (*i.e.*, "*at least one-tenth of the assay samples in the set*") contained a detectable proportion of this selected genetic sequence.

In **Experiment 2**, Li detected a "*selected genetic sequence*" in the form of the LDLr1 allele in 45 of 150 single-sperm assay samples,[104] thereby demonstrating that about 30% of his samples (*i.e.*, "*at least one-tenth of the assay samples in the set*") contained a detectable proportion of this selected genetic sequence.

In **Experiment 3**, Li detected a "*selected genetic sequence*" in the form of the β^A globin allele in 19 of 37 single-cell assay samples,[105] thereby demonstrating that about 51% of his samples (*i.e.*, "*at least one-tenth of the assay samples in the set*") contained a detectable proportion of this selected genetic sequence.

By demonstrating in these experiments that his selected genetic sequence was actually detected in more than one-tenth of his assay samples, Li anticipates dependent claim 2 in addition to base claim 1.

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[103] Li, page 415, paragraph bridging left and right cols.
[104] Li, page 416, left col., second paragraph; and page 415, right col., last paragraph
[105] Li, page 416, left col., second paragraph; and page 415, right col., last paragraph

4. Detailed explanation of the pertinency and manner of applying Li to claim 3

Dependent claim 3 recites the method of claim 1, wherein "*the step of diluting is performed until between 0.1 and 0.9 of the assay samples yield an amplification product when subjected to a polymerase chain reaction.*"

Although this claim does not actually require the assay samples to be actually subjected to a PCR reaction (or that a PCR reaction is part of the amplifying step of the base claim), Li did perform PCR during the amplifying step of base claim 1 in all three of his experiments (see discussion of the base claim above).

Li found in **Experiment 1** that of 80 single-sperm assay samples, 55% showed the presence of a PCR product as determined by probe hybridization.[106] Because 0.55 of the assay samples yielded an amplification product after PCR, and because 0.55 is within the claimed range, this demonstrates that in Experiment 1, "*between 0.1 and 0.9 of the assay samples yield an amplification product when subjected to a polymerase chain reaction.*" Moreover, the Examiner of the related '105 application has pointed to this same teaching of Li as disclosing the corresponding feature in pending claim 51 in the '105 application.[107]

Li found in **Experiment 2** that of 150 single sperm assay samples, 82% showed the presence of an amplification product as determined by allele-specific probe hybridization.[108] Because 0.82 of the assay samples yielded an amplification product after PCR, and because 0.82 is within the claimed range, this demonstrates that in

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[106] Li, page 415, paragraph bridging left and right cols. ("we analysed the LDLr genotypes in 80 individual sperm: … Altogether 55% of the sperm gave a hybridization signal").

[107] October 10, 2012 Office Action, page 4.

[108] Li, page 415, right col., last paragraph. ("A total of 150 individual sperm were analysed … we did detect [probe] hybridization signals in 123 samples (82%)").

Experiment 2, "*between 0.1 and 0.9 of the assay samples yield an amplification product when subjected to a polymerase chain reaction.*"

Li found in **Experiment 3** that of 37 the single cell assay samples analyzed, 84% showed the presence of an amplification product as determined by allele-specific probe hybridization.[109] Because 0.84 is within the claimed range and because 0.84 of the assay samples yielded an amplification product after PCR, this demonstrates that "*between 0.1 and 0.9 of the assay samples yield an amplification product when subjected to a polymerase chain reaction.*"Li thereby anticipates claim 3 in addition to anticipating base claim 1.

5. Detailed explanation of the pertinency and manner of applying Li to claims 7-9

Dependent claims 7-9 recite the method of claim 1 "*wherein the number of assay samples within the set is greater than"* 10, 50 or 100 assay samples, as recited in claims 7-9 respectively. These claims are anticipated by Li.

In particular, in **Experiment 2**, Li made and analyzed a set of 150 single-sperm assay samples.[110] Because 150 is always greater than 10, 50, or 100 assay samples, Li anticipates claims 7-9 in addition to base claim 1.

6. Detailed explanation of the pertinency and manner of applying Li to claim 14

Dependent claim 14 recites the method of claim 1, "*wherein the step of analyzing employs gel electrophoresis*." Under the broadest reasonable interpretation, Li anticipates claim 14.

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[109] Li, page 414, right col., first paragraph.
[110] Li, page 415, right col., last paragraph.

As described in the summary above, Li discloses features in **Experiments 1, 2 and 3** that correspond to the "analyzing step" of claim 1. Li goes on to describe other possible ways of analysis. Li contemplates that his single-cell PCR can be performed "in conjunction with gel electrophoresis procedures for very large DNA fragments and chromosome-walking data" to measure the frequency of recombination between genetic markers.[111]

Accordingly, Li anticipates claim 14 in addition to base claim 1.

7. Detailed explanation of the pertinency and manner of applying Li to claims 15-16

Dependent claim 15-16 recites the method of claim 1, "*wherein the step of analyzing employs hybridization to at least*" one (claim 15) or two (claim 16) "*nucleic acid probes.*"

Under the broadest reasonable interpretation, Li anticipates claims 15-16 in addition to base claim 1. For example, in **Experiments 1, 2 and 3** Li determined the allelic identity of the LDLr allelic amplification products in his single-sperm assay samples by hybridizing to two probes in the form of two allele-specific oligonucleotides (ASOs).

Specifically, in **Experiment 1** Li used "ASO [*i.e., allele-specific oligonucleotide*] for the LDLrl allele[which] had the sequence 5'AGGATATGGTCCTCTTCCA3' whereas the LDLr2 ASO had the sequence 5'TGGAAGAGAACCATATCCT3' ").

[111] Li at page 416, right column.

Similarly, in **Experiment 2** "part of each secondary reaction was hybridized to either of the two ASOs for that locus."[112]

For **Experiment 3**, "dot blot analysis of 20 μl samples of the PCR reaction were carried out using β^S and β^A allele specific probes."[113]

Accordingly, Li discloses the method of claim 1 "*wherein the step of analyzing employs hybridization to at least*" one (claim 15) or two (claim 16) "*nucleic acid probes.*" Li therefore anticipates claims 15 and 16 in addition to base claim 1.

8. Detailed explanation of the pertinency and manner of applying Li to claim 19

Dependent claim 19 recites the method of claim 1, "*wherein the step of amplifying employs a single pair of primers.*"

Under the broadest reasonable interpretation, Li anticipates claim 19 in addition to base claim 1. For example, Li amplified the LDLr locus in single sperm samples using a single set of LDLr primers in **Experiment 1**. As stated in the legend to Figure 2, for the LDLr locus, Li used one pair of primers "5'AGTGCCAACCGCCTCACAGG3' and 5'CCTCTCACACCAGTTCACTC3'." [114]

Li likewise amplified the globin locus in single diploid-cell samples using a single set of primers in **Experiment 2**, stating ("Each individual cell was delivered into a PCR tube containing … PCR buffer … and ***a set of PCR primers*** that amplify the informative region of the globin gene … 50 cycles of amplification were performed".[115]

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[112] Li, page 415, right col., last paragraph.
[113] Li, Fig. 1 legend, respectively.
[114] Li, Fig. 2, legend.
[115] Li, page 414, right col., top paragraph.

Accordingly, Li discloses that "*the step of amplifying employs a single pair of primers*." Li anticipates claim 19 in addition to base claim 1.

9. Detailed explanation of the pertinency and manner of applying Li to claims 21 & 22

Dependent claim 21 recites the method of claim 1 "*wherein the step of amplifying employs at least 40 cycles of heating and cooling*." Dependent claim 22 recites the method of claim 1 "*wherein the step of amplifying employs at least 50 cycles of heating and cooling*." Li anticipates both these claims in addition to base claim 1.

In **Experiment 3** (β-globin locus), Li subjected his single-diploid-cell assay samples to amplification with single pair of β-globin primers such that "PCR product was produced in 50 cycles" of heating and cooling between three temperatures (95°C, 54°C and 72°C). [116]

In **Experiment 1** (LDLr locus only),[117] Li subjected his 80 sperm assay samples to identical amplification reactions as in Experiment 3, with a different set of primers to the LDLr locus.[118] Because the amplification reactions were identical to that of Experiment 3, it necessarily follows that identical cycling conditions, *i.e.*, 50 cycles of heating and cooling between three temperatures, were used.

By employing 50 cycles of heating and cooling, Li anticipates claims 21 & 22 in addition to base claim 1.

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[116] Li, page 414, right col., first paragraph, and Fig. 1, legend ("each cycle of PCR consisted of incubation at 95 °C for 15 s, 15 s incubation at 54 °C and a 1 min incubation at 72 °C… fifty cycles of PCR, dot blot analysis of 20 µl samples of the PCR reaction were carried out").

[117] Li, page 415, paragraph bridging left and right cols.

[118] Li, page 415, Fig. 2, legend (explaining that "PCR and dot blot analysis were performed as in Fig. 1 except that the final washes of the filters hybridized with the LDLr probes").

10. Detailed explanation of the pertinency and manner of applying Li to claim 27

Dependent claim 27 recites the method of claim 1, "*wherein the selected genetic sequence is a wild-type allele.*"

Under the broadest reasonable interpretation, Li anticipates claim 27 in addition to base claim 1. In particular, **Experiment 3** of Li counted single-diploid-cell assay samples containing "normal" (*i.e.*, wild-type) β-globin allele, and the number of samples containing a mutant allele. The wild-type allele is a "*selected genetic sequence*" under the broadest reasonable interpretation. Thus, Li anticipates claim 27.

11. Detailed explanation of the pertinency and manner of applying Li to claim 32

Dependent claim 32 recites the method of claim 1, "*wherein the selected genetic sequence and the reference genetic sequence are on distinct chromosomes.*"

Under the broadest reasonable interpretation, Li anticipates claim 32 in addition to base claim 1. For example, in **Experiment 1** identified in the overview section, Li "analysed the LDLr genotypes in 80 individual haploid sperm" assay samples[119] and counted the number of samples containing each allele at the LDLr locus (where each allele is located on one of two homologous "*distinct chromosomes*" of a chromosome pair). Under the broadest reasonable interpretation, any one of these two alleles acts as the "*selected genetic sequence*" whereas the other allele acts as the "*reference genetic sequence*" of claim 32. Both alleles are necessarily located on two "*distinct chromosomes*" of the same chromosome pair, instead of being located on a single chromosome. Thus, Li anticipates claim 32 under the broadest reasonable interpretation.

[119] Li, page 415, paragraph bridging left and right cols.

B. Proposed Rejection No. 2: Li renders claims 4 and 5 obvious under 35 U.S.C. § 103(a) in view of Zhang

Dependent claims 4 and 5 recite the method of claim 1, "*wherein the step of diluting is performed until all of the assay samples yield an amplification product when subjected to a polymerase chain reaction and each assay sample contains less than*" 10 (claim 4) or 100 (claim 5) "*nucleic acid template molecules containing the reference genetic sequence*." These claims are rendered obvious by the combination of Li and Zhang.

Li anticipates claim 1 as detailed above. Also in Li's and Zhang's experiments "*each assay sample contain[ed] less than 10 [or 100] nucleic acid template molecules containing the reference genetic sequence*," as recited in claims 4 and 5 respectively. In addition, Zhang teaches single-cell amplification methods like Li's in which "*diluting is performed until all of the assay samples yield an amplification product when subjected to a polymerase chain reaction*" as also required by claims 4 and 5.

More specifically, in all of Li's and Zhang's experiments, "*each assay sample contain[ed] less than 10 [or 100] nucleic acid template molecules containing the reference genetic sequence*," as recited in claims 4 and 5 respectively. Both Li and Zhang generated single-cell samples by micromanipulating individual sperm or diploid cells into different tubes. A single haploid sperm cell sample contains a single template molecule, whereas a diploid cell sample generally contains two template molecules under the broadest reasonable interpretation.

Taking for example Li's analysis of the LDLr locus in single-sperm samples in **Experiments 1 and 2**, each haploid single-sperm sample was expected to contain either a single LDLr1 allele or a single LDLr2 allele. Treating the LDLr2 allele as the "*reference*

genetic sequence," the LDLr1-containing samples would contain zero LDLr2 copies (*i.e.*, "*less than 10 [or 100] nucleic acid template molecules containing the reference genetic sequence*"), and the LDLr2-containing sperm samples would contain one LDLr2 copy (*i.e.*, "*less than 10 [or 100] nucleic acid template molecules containing the reference genetic sequence*"). Although Li observed a small number of samples (nine in total) apparently contained two bands,[120] even these samples contained at most two copies of the LDLr2 "*reference genetic sequence.*" Thus "*each assay sample contain[ed] less than 10 [or 100] nucleic acid template molecules containing the reference genetic sequence*" in Li's experiments, as recited in claims 4 and 5 respectively. ***The PTO already reached the conclusion that Li teaches the generation of assay samples each containing less than ten template molecules*** in pending claim 49 of the pending '105 application.[121]

Similarly, in **Experiment 3**, each homozygous diploid single cell sample was expected contain either a homozygous cell that contained two copies of the β^A or the β^S allele. Treating the β^A allele as the "*reference genetic sequence*," the β^S-containing samples would contain zero β^A copies (*i.e.*, "*less than 10 [or 100] nucleic acid template molecules containing the reference genetic sequence*"), and the β^A-containing sperm samples would contain two β^A copies (*i.e.*, "*less than 10 [or 100] nucleic acid template molecules containing the reference genetic sequence*").[122] Li did not observe any amplified samples that "hybridized with both probes, indicating that that a single cell only was introduced into each tube."[123] Thus "*each assay sample contain[ed] less than*

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[120] Li, sentence bridging pages 415-416.
[121] October 10, 2012 Office Action in the '105 application, page 3 (Exhibit 5).
[122] Li, paragraph bridging pages 414-415.
[123] Li, paragraph bridging pages 415-416.

10 [or 100] nucleic acid template molecules containing the reference genetic sequence" in Li's **Experiment 3**, as recited in claims 4 and 5 respectively.

In addition, Li at least renders obvious a method "*wherein the step of diluting is performed until all of the assay samples yield an amplification product when subjected to a polymerase chain reaction.*" A single-cell assay samples will at least in theory generate a primary amplification product of the LDLr locus, although in practice Li demonstrated amplification in up to 84% instead of 100% of his samples.[124] Li clearly recognized the desirability of getting *each* sample to amplify, and taught this could be done by "[i]mproving the absolute rate of successful amplification of single sperm" by various methods, including "improving lysis procedures" and improving delivery of single cells into the assay samples.[125] Thus, it would have been obvious over Li to ensure that amplification occurred in each assay sample - and a later study by Zhang *et al.*[126] (from the same laboratory in which Li did his work[127]) did exactly that. In particular, Zhang noted that in previous single-sperm studies, including that of Li, a "single cell [sample] can be analyzed only once and independent confirmation of the genotype of any one cell is impossible."[128] Zhang overcame this perceived limitation by subjecting his single-sperm samples to a random genome-wide amplification called primer-extension

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[124] Li, page 414, right col., top paragraph.

[125] Li, page 416, paragraph bridging left and right cols.

[126] Zhang *et al., Whole genome amplification from a single cell: implications for genetic analysis*. PNAS USA, 89(13):5847-51 (1992), forming prior art under 35 U.S.C. § 102(b) to the '706 patent. (Exhibit PA-2).

[127] In particular, the laboratory of Norman Arnheim at the University of Southern California.

[128] Zhang, page 5847, left col., first paragraph.

preamplification (PEP), followed by PCR. [129] In particular, Zhang subjected 18 single-sperm assay samples to amplification by PEP[130] and then subjected an aliquot of each PEP-amplified assay sample to locus-specific PCR.[131] Zhang observed a PCR amplification product in each and every assay sample.[132] For example, Zhang observed a PCR amplification product of the *LDLR* and *APOC2* loci in all 18 samples.[133] Thus, Zhang demonstrated that "*all of the assay samples yield[ed] an amplification product when subjected to a polymerase chain reaction*," as recited in claims 4 and 5.

Obviousness: Reasons to Combine

Although a reason to combine Li with Zhang is not required, an apparent reason to combine the known elements may be evidenced by the teachings of the references themselves, issues in the technical area, or the skill in the art. *KSR*, 550 U.S. at 418. Here, reasons to combine are directly provided by the references themselves. Li initially demonstrated the feasibility of single-cell PCR, wherein "*the step of diluting is performed until ... each assay sample contains less than*" 10 (claim 4) or 100 (claim 5) "*nucleic acid template molecules containing the reference genetic sequence*." Li also suggested that such assays should be designed to ensure that "*all of the assay samples yield an amplification product when subjected to a polymerase chain* reaction," and Zhang actually demonstrated such results using refined single-cell amplification methods. Thus,

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[129] Zhang, abstract (explaining that PEP is a "method for amplifying a large fraction of the DNA sequences present in a single haploid cell … using a mixture of 15-base random oligonucleotides").

[130] Zhang, page 5848, second-last paragraph.

[131] In particular, Zhang's locus-sfx analysis involved a secondary reamplification and probe hybridization, discussed in detail in the application of Zhang to claim 38.

[132] Zhang, page 5848, second-last paragraph, and Table 2.

[133] Zhang, page 5849, Table 2.

one of ordinary skill would have had ample reason to combine the teachings of Li and Zhang.

The combination of Li and Zhang thus renders claims 4 and 5 obvious.

C. Proposed Rejection No. 3: Li renders claim 6 obvious under 35 U.S.C. § 103(a) in view of Jeffreys

Dependent claim 6 recites the method of claim 1, "*wherein the biological sample is cell-free*." Li performed the method of claim 1 on biological samples in the form of haploid or diploid cell suspensions. Although Li did not analyze cell-free biological samples, it would have been obvious to do so in view of Jeffreys.[134]

Jeffreys demonstrated single-cell amplification of hypervariable minisatellites as well as cell-free single-molecule amplification. Jeffreys cited to Li's methods[135] as the groundwork for his single-cell amplification procedures. In his experiments, Jeffreys not only used single-cell samples as his assay samples, but also used cell-free assay samples containing equivalent amounts of genomic DNA as individual cells.[136]

In particular, Jeffreys took a diluted biological sample of cell-free human genomic DNA to create a "*set comprising a plurality of assay samples*" in the form of 16 assay samples containing 6 picograms of DNA, equivalent to a single diploid genome.[137] Jeffreys next amplified the minisatellite locus pMS51 in these assay samples, thereby

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[134] Jeffreys *et al.*, *Amplification of human niinisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells*, Nucl. Acids. Res., vol 16, no. 23, pages 10953-10971 (1988) (Exhibit PA-3). Jeffreys forms prior art to the '706 patent under 35 U.S.C. § 102(b).

[135] In particular, Jeffreys notes that PCR and Taq polymerase have "allowed typing of … polymorphisms to be extended to … individual somatic cells and sperm." Jeffreys, sentence bridging pages 10953- 10954.

[136] Jeffreys, pages 10954-10956.

[137] Jeffreys, Fig. 4A, showing the amplification results on 16 assay samples each containing 6 pg genomic DNA, and paragraph bridging pages 10960- 10961, explaining that "6 and 60 pg (*i.e.*, picogram) aliquots of human DNA, [are] equivalent to 1 and 10 cells respectively."

"*amplifying the template molecules within the assay samples to form a population of amplified molecules in the assay samples of the set*," as taught by Li, and recited in base claim 1. Jeffreys then "*analyz[ed] the amplified molecules in the assay samples of the set"* and noted that "amplification products of one or both alleles were also seen in some of the 6pg [assay] samples, indicating that single target molecules can be faithfully amplified."[138] Jeffreys finally "*determin[ed] a ... number of assay samples*" that amplified each allele to estimate the sensitivity of his PCR reactions and concluded that "on average 0.46 successful amplification events per 6pg DNA sample were actually observed, compared with 1 event predicted [per assay sample]."[139]

Jeffreys made clear that cell-free DNA samples and cell samples were interchangeable alternatives for a starting biological sample. In addition to his experiments in which he started with 6 or 60 pg of cell-free DNA, in Fig. 7, Jeffreys compared single-cell amplification with cell-free amplification. Jeffreys noted that similar results were obtained in both cases: in "single cell PCR reactions in which at least some loci have amplified, approximately 75% of alleles present could be detected following PCR," which result "agrees with the efficiency of single molecule amplification determined from PCR analysis of 6pg samples of human DNA (Fig. 4)."[140]

The only described method of generating a set of cell-free DNA samples disclosed in the '706 patent involves distribution of a dilute DNA solution into a plurality of assay samples, which results in a set of assay samples, most of which contain zero, one

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[138] Jeffreys, pagen 10960-61, sentence bridging pages.
[139] Jeffreys, page 10961, first paragraph.
[140] Jeffreys, page 10966, third paragraph.

or more molecules of DNA.[141] For the purposes of nucleic acid amplification and analysis, cell-free samples containing a one or two template molecules are equivalent to haploid or diploid single-cell samples, respectively. A single haploid cell contains only a single template sequence, and a single diploid cell contains two template sequences, where all other genomic DNA sequences and molecules are not "template" molecules and are therefore irrelevant. Jeffreys clearly recognized this analogy – as mentioned, Jeffreys confirmed cell-free DNA samples were equivalent to single-cell samples such as Li's because he obtained comparable amplification results from both types of samples. Specifically, Jeffreys found that in single cell PCR "approximately 75% of alleles present could be detected … This estimate agrees with the efficiency of single molecule amplification determined from PCR analysis of 6pg samples of human DNA (Fig. 4)."[142] Thus, Jeffreys explicitly recognized that cell-free biological samples and cellular biological samples were equivalent starting points in the method of claim 1.

It would thus have been obvious to generate Li's set of assay samples from cell-free DNA as taught by Jeffreys, for at least the following reasons.

Obviousness: Known Elements and Predictable Result

Under 35 U.S.C. § 103, where a claim "'simply rearranges old elements with each performing the same function it had been known to perform' and yields no more than what one would expect from such an arrangement, the combination is obvious." *KSR Intl. Co. v. Teleflex, Inc.*, 550 U.S. 398, 417 (2007), quoting *Sakraida v. Ag. Pro., Inc.*,

[141] In particular, such random distribution is governed by the Poisson distribution, which demonstrates that a random-distribution method is incapable of generating a "pure" set of samples which each contain one single DNA molecule. In practice, only a minority of assay samples will contain one template molecule, whereas other assay samples will contain no molecules and still other assay samples will contain two or more template molecules.

[142] Jeffreys, page 10966, third paragraph.

425 U.S. 273, 282 (1976). Li performed the methods of base claim 1 by creating a set of single-cell assay samples for amplification and analysis. As discussed above, Jeffreys teaches that single-cell assay samples are equivalent to cell-free assay samples containing the same amount of genomic DNA. It would have been *prima facie* obvious to one of ordinary skill to use Li's methods on Jeffreys' cell-free biological samples. Thus, claim 6 would have been *prima facie* obvious to one of ordinary skill in the art at the time of invention.

Obviousness: Reasons to Combine

Although a reason to use biological samples in the form of Jeffreys's cell-free DNA samples in Li's typing methods is not required, an apparent reason to combine the known elements as claimed may be evidenced by the teachings of the references themselves, issues in the technical area, or the skill in the art. *KSR*, 550 U.S. at 418. Here, those of ordinary skill would have found ample reasons to combine. Jeffreys confirmed that he got comparable results in genotyping methods like Li's whether starting with a cellular sample or cell-free DNA – specifically, in single cell PCR "approximately 75% of alleles present could be detected … This estimate agrees with the efficiency of single molecule amplification determined from PCR analysis of 6pg samples of human DNA (Fig. 4)."[143] Like Jeffreys, Li also intended his methods for use in genetic typing.[144] In addition, Jeffreys' cell-free single-molecule dilution methods were quicker and easier and scaling up to a large number of assay samples required no extra effort (besides pipetting a larger number of aliquots into tubes). Li's analysis used a large number of assay samples (specifically, 150 samples in Experiment 2), and Li's

[143] Jeffreys, page 10966, third paragraph.
[144] Li, page 416, right col., "Discussion."

micromanipulation methods used to generate single-cell assay samples were much more laborious (generating only 500 assay samples in one week).[145] Thus, one of ordinary skill would have had ample reason to combine the teachings of Li with Jeffreys.

D. Proposed Rejection No. 4: Li renders claims 10-11 obvious under 35 U.S.C. § 103(a)

Dependent claims 10 and 11 recite the method of claim 1 "*wherein the number of assay samples within the set is greater than 500 [(claim 10) or 1000 (claim 11)] assay samples*" as recited in claim 10 and 11, respectively.

Li at least renders the use of 500 or 1000 assay samples obvious. Li expressly teaches the desirability of performing single-cell PCR on 500 samples per week.[146] Further increasing the number of samples would have been a trivial change. Such an increase would have been obvious to a POSITA for at least the reason that it would have involved a simple substitution of one known element for another (*i.e.*, a small number of assay samples substituted for a larger number of assay samples) resulting in a predictable result (an increase in the number of single sperm assay samples). The '706 claims therefore embody a merely predictable use of prior-art elements.

In addition, ***the PTO has already found that it would be obvious over Li to use a set of 500 or 1000 assay samples in Li's analysis***.[147] In particular, the PTO stated that:

> Li expressly suggested analyzing 500 assay samples (page 416, last paragraph), and that it would have been prima facie obvious … to distribute 500, or even 1000 individual sperm [samples] and assay according to Li's technique. One would have been

[145] Li, page 416, right col., last paragraph ("we can envisage typing as many as 500 meiotic products in a week.")

[146] Li, page 416, right col.

[147] October 10, 2012 Non-Final Office Action in the '105 prosecution history, at pages 7-8 (Exhibit 5).

> motivated to do so because Li stated (page 416, first paragraph of "Discussion"): A significant advantage of the approach described here is that a large number of meiotic products can be examined from a single individual allowing determination of the recombination frequency … Li's express contemplate[ion] [sic] of 500 individual meiotic events certainly renders claim 63 obvious, and, by simple extrapolation, … [other claims] which merely require more assay samples (*i.e.*, 1000).

Thus, dependent claims 10-11 are *prima facie* obvious over Li.

E. Proposed Rejection No. 5: Li renders claim 12 obvious under 35 U.S.C. § 103(a) in view of Kalinina

Dependent claim 12 recites the method of claim 1 "*wherein the step of amplifying and the step of analyzing are performed on assay samples in the same receptacle*." At least under the broadest reasonable interpretation of the claim, Li renders claim 12 obvious in view of Kalinina.[148]

Under the broadest reasonable interpretation of the claims, Requester proceeds on the premise that claim 1 is broader than dependent claim 12 in that the amplifying and/or analyzing steps need not be performed in a structured container (*i.e.*, "receptacle"). Accordingly, Requester proceeds on the premise that claim 12 is narrower than claim 1 in requiring the assay samples to be contained in a receptacle.

As described above in relation to claim 1, Li discloses a method corresponding to all of the steps of claim 1, including amplification of single template molecules in single-sperm samples.

Kalinina performed amplification of single-template molecules just as Li did, where amplification and analysis are both performed "*in the same receptacle*" as required

[148] Kalinina *et al.*, NAR 25, 1999-2004 (1997)), is prior art to the '706 patent under 35 U.S.C. § 102(b), and is newly cited in this request. (Exhibit PA-4).

by claim 12. In short, Kalinina performed PCR reactions in microcapillary-tube receptacles[149] containing dual-labelled TaqMan® probes[150]. The assembled device containing multiple assay samples in individual receptacles was subjected to amplification by thermocycling and analysis by fluorescence detection:

> This sample holding device [in Figure 1] was then attached with Scotch® tape to the sample holder of a Rapidcycler air oven . . . and cycled through 92 C for 5 s, 54 C for 5s, 72 C for 15 for 40 cycles; this cycling protocol takes ~ 30 min in the Rapidcycler. . . Fluorescence of samples in glass capillaries was measured with a Zeiss axiovert 410 laser scanning microscope using a 20X-0.5N objection, 15 mW external argon laser… [151]

The analysis method used in Kalinina is the well-known TaqMan® assay, in which dual-labelled TaqMan® probes are included within the amplification reaction mixture during the amplification procedure itself, and hybridize in real-time to a cognate amplification product as it is being generated. In Kalinina's TaqMan® assay, PCR amplification of a sequence of interest is performed in the presence of an oligonucleotide probe labeled with a fluorescent reporter and a quencher molecule.[152] As amplification progresses, the dual-labeled probe hybridizes to the target sequence and the reporter molecule will be cleaved from the probe by Taq polymerase, resulting in an increase in fluorescence of the reporter.[153] The TaqMan® probe assay has the advantages of being more sensitive than conventional probe assays, and better able to "detect PCR product derived from single template molecules,"[154] such as Li's amplification products.

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[149] Kalinina, page 2000, and Figure 1, "Schematic diagram of microcapillary PCR sample holding device."
[150] Kalinina, page 2000 (PCR apparatus describing capillary PCR).
[151] Kalinina, pages 2000 (PCR apparatus and Fluorescence detection).
[152] *Id.*
[153] *Id.*
[154] Kalinina at page 2003.

Moreover, both the amplification and analysis (*e.g.*, determination of the allelic identity of the PCR product) are performed in the same receptacle, which format Kalinina teaches can reduce "carry-over contamination."[155]

Furthermore, Kalinina indicates that his methods are analogous to Li's in being designed to "detect single starting template molecules."[156]

It would have thus been obvious to include TaqMan® probes in Li's amplification reactions in order to analyze Li's amplification products in real time within the same receptacle, for at least the following reasons.

Obviousness: Known Elements and Predictable Result

Under 35 U.S.C. § 103, where a claim "'simply rearranges old elements with each performing the same function it had been known to perform' and yields no more than what one would expect from such an arrangement, the combination is obvious." *KSR Intl. Co. v. Teleflex, Inc.*, 550 U.S. 398, 417 (2007), quoting *Sakraida v. Ag. Pro., Inc.*, 425 U.S. 273, 282 (1976).

Kalinina taught the suitability of TaqMan® probes for use in single-molecule PCR assays such as Li's. Both Li and Kalinina both amplified and analyzed amplification products from a single template molecule. However, Li amplified his single-cell samples in a receptacle and then analyzed the amplification products outside the receptacle. As a result, Li's amplification and analysis steps required separate reagents and a separate apparatus. In contrast, Kalinina taught that TaqMan® probes allowed both the amplification and analysis of the amplified products to occur "*in the same receptacle*"—in particular, a microcapillary sample holding device — as recited in claim 12.

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[155] Kalinina at page 1999.
[156] Kalinina, Abstract.

It would have been obvious to the skilled person to have used TaqMan® probes as taught by Kalinina when amplifying and analyzing single cells as taught by Li. Both Li and Kalinina disclose the genetic analysis of very small quantities of starting genetic material, such as a single cell or single template. However, the TaqMan® assay, as discussed above, was a well-developed commercial assay with significant advantages over Li, including the ability to perform both the amplification and the analysis in a single reaction container or receptacle. Furthermore, the '706 patent acknowledges that TaqMan® probes were commercially available by the priority date of the '706 patent, and that a skilled person would have been able to routinely implement the assay in Li's system to obtain predictable results.[157] Thus, claim 12 would have been *prima facie* obvious to one of ordinary skill in the art at the time of invention.

Obviousness: Reasons to Combine

Although a reason to use Kalinina's TaqMan® assay to generate Li's single-genome assay samples is not required, an apparent reason to combine the known elements as claimed may be evidenced by the teachings of the references themselves, issues in the technical area, or the skill in the art. *KSR*, 550 U.S. at 418. Here, strong reasons to combine are directly provided by the references themselves.

Li specifically mentions that "elimination of all sources of possible contamination is critical to the success of these experiments."[158] Kalinina explicitly teaches the many advantages of TaqMan® assays, including that the "assay involves fluorescence

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[157] '706 patent, col. 7, lines 45-52 ("Although the working examples demonstrate the use of molecular beacon probes as the means of analysis … other techniques can be used as well. These include … hybridization with other types of probes, including TaqManTM (dual-labeled fluorogenic) probes (Perkin Elmer Corp./Applied Biosystems, Foster City, Calif.),").

[158] Li at page 415, column 1, line 4.

measurements that can be performed without opening the PCR tube," and, as a result, "the risk of carry-over contamination is greatly reduced."[159] In addition, Kalinina details use of an improved PCR technique that would eliminate the need for the dot blot analysis of Li altogether by allowing for amplification and analysis in a single device. Kalinina also points out that the cycling protocol, including amplification and analysis, "takes ~30 min in the Rapidcyler."[160] It would have been *prima facie* obvious to one of ordinary skill in the art to modify the PCR method taught by Li to use the TaqMan® assays described in Kalinina to perform the amplification and detection/analysis of DNA sequences in single human sperm and diploid cells, in a single tube or receptacle with predictable results.

Obviousness: Known Technique to Improve Known Method

KSR and the MPEP provide that where a known technique has been used to improve a base method ready for improvement, a POSITA would be capable of applying the known improvement to the base method.[161] Both Li and Kalinina are directed to the use of PCR methods for molecular analysis of target nucleic acids. The base methods of Li used PCR techniques for amplification followed by dot blots for analysis. Li indicates that "elimination of all sources of possible contamination is critical to the success of these experiments."[162] Kalinina recognizes that advances in PCR techniques, specifically"'Taqman' fluorescence energy transfer assays" provide scientists with the opportunity to perform PCR amplification and analysis simultaneously and that "[b]ecause this assay involves fluorescence measurements that can be performed without

[159] Kalinina at page 1999.
[160] Kalinina, page 2000, right column.
[161] *See* MPEP at §2143(C).
[162] Li at page 415, column 1, line 4.

opening the PCR tube, the risk of carry-over contamination is greatly reduced."[163] It would have been obvious to one of skill in the art to use the single tube assay techniques in Kalinina to improve the base assays of Li with predictable results.

For at least these reasons, the combination of the teachings of Kalinina applied to the teachings of Li renders claim 12 obvious.

F. Proposed Rejection No. 6: Li renders claim 20 obvious in view of Chou under 35 U.S.C. § 103(a)

Dependent claim 20 recites the method of claim 1, "*wherein the step of amplifying employs a polymerase which is activated only after heating.*"

Li performed the method of base claim 1, using conventional Taq polymerase. Chou[164] teaches that non-specific amplification and mis-priming during PCR can be avoided or at least reduced by using a "hot start" PCR in which a reagent such as the polymerase is withheld from fluid contact with the rest of the reaction mixture by a layer of solid wax until the reaction tube temperature has reached 60- 80° C.[165] Thus, Chou teaches a step of "*amplifying [which] employs a polymerase which is activated only after heating.*" In fact, the '706 patent cites to Chou as teaching a polymerase which is activated only after heating.[166]

Obviousness: Reasons to Combine

It would have been obvious to use Chou's hot-start polymerase in Li's amplification reactions.

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[163] Kalinina at page 1999.

[164] Chou *et al.*, *Nucleic Acids Res.*, 20(7): 1717–1723 (April 11, 1992). Chou forms prior art to the '706 patent under 35 U.S.C. § 102(b). (Exhibit PA-5).

[165] Chou, abstract.

[166] '706 patent, col. 10, lines 13-17. However, Chou was not cited in an IDS and thus not of record during original prosecution of the '706 patent.

Although a reason to combine Chou's and Li's teachings is not required, an apparent reason to combine the known elements as claimed may be evidenced by the teachings of the references themselves, issues in the technical area, or the skill in the art. *KSR*, 550 U.S. at 418. Here, reasons to combine are directly provided by the references themselves. Chou explicitly teaches the many advantages of a hot-start polymerase over the conventional polymerase of Li. Chou teaches that mispriming and spurious amplification can be reduced by his hot-start polymerase, and such problems are especially prominent in low-copy-number samples such as Li's (in particular, "PCR amplification of low-copy-number targets is vulnerable to interference by the amplified extension of primer pairs annealed to non-target nucleic acid sequences in the test sample ('mispriming') and by the amplified extension of two primers across one another's sequence without significant intervening sequence ('primer dimerization')."[167] Thus, one of ordinary skill would have had ample reason to use Chou's hot-start polymerase in Li's amplifications.

Under the broadest reasonable interpretation, Li therefore renders claim 20 obvious in view of Chou.

G. Proposed Rejection No. 7: Li renders claim 23 obvious in view of Burg under 35 U.S.C. § 103(a)

Dependent claim 23 recites the method of claim 1, wherein "*the step of amplifying employs at least 60 cycles of heating and cooling.*" While Li anticipates base claim 1, Li does not expressly disclose a method that includes an amplification that employs "at least 60 cycles of heating and cooling."

[167] Chou, page 1717, left col., first paragraph.

As discussed above with respect to claims 21 and 22, Li performed single-cell amplification using "fifty cycles" of heating and cooling between three temperatures (95°C, 54°C and 72°C) with a single set of primers.[168] In addition, Burg[169] discloses a single-cell amplification procedure that employs at least 60 cycles of heating and cooling between three temperatures (93°C, 55°C and 72°C).[170] Both Li and Burg amplified a specific target sequence from a single cell. Moreover, both Li and Burg also analyzed the amplified products by detection of the immobilized product with a labeled probe in a dot-blot or slot-blot format.

For at least the reasons below, claim 23 would have been rendered obvious over Li in view of Burg under the broadest reasonable interpretation of the claim.

Obviousness: Reasons to Combine

Although a reason to use 60 cycles of PCR as taught by Burg in Li's single-cell amplification methods is not required, strong reasons to combine are provided by the references themselves. Both Li and Burg performed single-cell amplification. In particular, Burg describes the amplification of a target sequence (B1 gene) from a single cell sample of *T. gondii*.[171] Using 60 cycles of thermocycling, Burg found that the "signal from a single cell was easily detected" and also found "detection of a single cell to

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[168] Li, page 415, Fig. 2, legend (explaining that "PCR and dot blot analysis were performed as in Fig. 1 except that the final washes of the filters hybridized with the LDLr probes").

[169] Burg, *et al.*, "Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction." *J. Clin. Microbiol.* 27, 1787-1792 (1989). Burg is prior art to the '706 patent under 35 U.S.C. § 102(b). (Exhibit PA-6).

[170] Burg, page 1790, paragraph bridging left and right cols., and Fig. 4, legend.

[171] Burg, page 1790, paragraph bridging left and right cols., and Fig. 4, legend.

be highly reproducible,"[172] thus teaching that 60 cycles are desirable in single-cell amplification.

Obviousness: Known Technique to Improve Known Method

KSR and the MPEP provides that where a known technique has been used to improve a base methods ready for improvement, a POSITA would be capable of applying the known improvement to the base method.[173] Li and Burg are both directed to single-cell PCR. Burg teaches that amplifying the target sequence from a single cell for sixty (60) cycles gave a good yield of amplification product that was "easily detected" and "highly reproducible."[174] Thus a skilled person would have been motivated to improve the method of Li by using 60 cycles of amplification in an attempt to achieve such reproducible and detectable signal.

Moreover, modifying the method of Li would have only required increasing the number of amplification cycles from the fifty cycles of Li to at least the sixty cycles of Burg. Such a modification would have been readily performed by a POSITA, and the expected effects of such an increase, *i.e.* an increase in the amount of amplified product, would have been well-known and predictable to a skilled person.

Therefore, Li in view of Burg render obvious claim 23, including wherein the amplification step "*employs at least 60 cycles of heating and cooling*" as recited in the claim.

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[172] Burg, page 1790, paragraph bridging left and right cols., and Fig. 4, legend.
[173] *See* MPEP at § 2143(C).
[174] Burg, page 1790, paragraph bridging left and right cols., and Fig. 4, legend.

H. Proposed Rejection No. 8: Li renders claim 24 obvious in view of Trumper under 35 U.S.C. § 103(a)

Dependent claim 24 recites the method of claim 1, "*wherein the biological sample is selected from the group consisting of stool, blood, and lymph nodes.*"

Under the broadest reasonable interpretation, Li renders claim 24 obvious in view of Trumper.[175] Li anticipates base claim 1, whereas Trumper used single-cell PCR as pioneered by Li on a cell from a lymph node sample as specified by dependent claim 24.

In particular, Li anticipates claim 1 by performing single-cell PCR on individual cells in separate wells ("assay samples") and determining a ratio by comparing a first number of assay samples containing a selected sequence and a second number of assay samples containing a reference sequence.

Trumper also performed Li's single-cell PCR method substantially as recited in claim 1. Specifically, Trumper isolated single H&RS [*i.e.*, Hodgkin and Reed-Sternberg] cells, where "single cells were drawn into a glass micropipette," and then the single cells were "ejected into a 500 μL Eppendorf tube containing 4 μL of lysis buffer."[176] The lysed cells were reverse-transcribed and subjected to PCR amplification.[177] Thus, Trumper performed the "*diluting*" and "*amplifying*" steps of claim 1. Trumper also disclosed that the amplified molecules were analyzed to determine both presence and amount of a reference housekeeping gene (*e.g.*, actin) and both the presence and amount

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[175] Trumper *et al.*, *Single-Cell Analysis of Hodgkin and Reed-Sternberg Cells: Molecular Heterogeneity of Gene Expression and p53 Mutations*, Blood, 81: 3097-3115 (1993), forming prior art to the '706 patent under 35 U.S.C. § 102(b). (Exhibit PA-7).

[176] Trumper, page 3098-3099, Section titled "Preparation of HD Lymph Nodes"

[177] Trumper, page 3099, right col., last paragraph, describing the generation of cDNA by reverse transciption, and page 3100, second paragraph, indicating that "the tailed cDNA" was used as template for PCR amplification.

of 22 genes of interest, including c-myc.[178] Under the broadest reasonable interpretation, any single one of Trumper's genes of interest constitutes a "*selected sequence*" while any single one of Trumper's reference housekeeping genes constitutes a "*reference*" sequence. Therefore, Trumper also performed the "*analyzing*" step of claim 1 by determining a first "*number of assay samples which contain the selected genetic sequence*" in the form of any one of the 22 genes of interest, and a second "*number of assay samples which contain a reference genetic sequence*" in the form of a housekeeping gene (*e.g.,* actin). Trumper also presented a detailed numerical analysis of the results.[179]

Finally, Trumper meets the added requirements of claim 24 by isolating single cells from a biological sample in the form of a "*lymph node*," as recited in claim 24. In particular, Trumper explains that "single-cell suspensions were prepared from fresh, HD-implicated lymph nodes."[180]

Obviousness: Reasons to Combine

Although a reason to perform Li's single-cell genotyping analysis on Trumper's lymph node samples is not necessarily required, reasons to combine are directly provided by Li himself. Li explicitly recognized the usefulness of modifying his PCR methods to use RT-PCR instead, in order to detect gene expression (and thereby detect the presence of the gene itself).[181] Li also demonstrated the feasibility of amplifying and genotyping single diploid cells as found in lymph node samples, and noted that "the ability to study

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[178] Trumper, Tables 4-7.

[179] Trumper, page 3104, right col.

[180] Trumper, page 3098, indicating that a "single-cell suspensions were prepared from fresh, HD-implicated lymph nodes" and "[i]ndividual H&RS cells were identified" and samples "containing a single cell" was transferred to a tube.

[181] Li, page 417, right col., third paragraph, stating that "analysis of messenger RNAs in single cells would be possible if efficient reverse transcription could be carried out before PCR was initiated."

DNA sequences in individual diploid cells will make it possible to study cell-to-cell variation in developmental processes."[182] Trumper did precisely as Li suggested by studying cell-to-cell variations in expression of cancer cells from lymph node samples. It would have been *prima facie* obvious to one of ordinary skill to use Li's single-cell PCR methods on clinically relevant biological samples such as lymph nodes in blood-cancer patients as taught by Trumper.

Thus, claim 24 would have been *prima facie* obvious at the time of invention.

I. Proposed Rejection No. 9: Li renders claim 25 obvious in view of Kanzler under 35 U.S.C. § 103(a)

Dependent claim 25 recites the method of claim 1 "*wherein the biological sample is blood or bone marrow of a leukemia or lymphoma patient who has received anti-cancer therapy.*"

Under the broadest reasonable interpretation, Li renders claim 25 obvious in view of Kanzler.[183] Li anticipates base claim 1, whereas Kanzler used single-cell PCR as pioneered by Li on the types of biological samples that are specified by dependent claim 25.

In particular, Li anticipates claim 1 by performing single-cell PCR on individual cells in separate wells ("assay samples") and determining a ratio by comparing a first number of assay samples containing a selected sequence and a second number of assay samples containing a reference sequence.

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[182] Li, page 417, right col., third paragraph.

[183] Kanzler *et al.*, *Molecular Single Cell Analysis Demonstrates the Derivation of Peripheral Blood-Derived Cell Line (L1236) From the Hodgkin/Reed-Sternberg Cells of a Hodgkin's Lymphoma Patient*, Blood, 87: 3429-3436 (1996), forming prior art to the '706 patent under 35 U.S.C. § 102(b). (Exhibit PA-8).

Kanzler also performed Li's single-cell PCR method substantially as recited in claim 1. Specifically, Kanzler isolated single malignant B cells designated Hodgkin/Reed-Sternberg cells from a Hodgkin's lymphoma patient: "Single cells were isolated from frozen sections of a bone marrow specimen of an HD patient by micromanipulation as previously described" by Kuppers *et al.* (Exhibit 6).[184] Kuppers in turn explains that cell "sections were incubated with 5 mg/ml collagenase H (Boehringer, Mannheim) in PBS" buffer before micromanipulation, and "aspirated" with a micropipette and transferred to a tube with buffer, thereby indicating that the biological sample was diluted in the process of making single-cell assay samples.[185] Kanzler also subjected the individual cells to single-cell PCR,[186] thereby performing the "*diluting*" and "*amplifying*" steps of claim 1. Kanzler also determined a first and second "*number of assay samples which contain the selected genetic sequence and ... a selected genetic sequence*" in the form of rearranged V_H3 and $V_\kappa3$ sequences found in a related cell line L1236,[187] thereby performing the "*analyzing*" step of claim 1. Kanzler also analyzed the numbers of cells carrying the selected sequence of interest to determine information about the composition of the biological sample. Kanzler noted for example that "amplification of at least one of three V gene rearrangements carried by the cell line from

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[184] Kanzler, page 3429, right col., third paragraph, indicating that "Single cells were isolated from frozen sections of a bone marrow specimen of an HD patient by micromanipulation as previously described" by Kuppers *et al.* (*i.e.*, Kuppers *et al.*, The EMBO Journal vol.12 no.13 pp.4955-4967, 1993, Exhibit 6).

[185] Kuppers *et al.*, The EMBO Journal vol.12 no.13 pp.4955-4967, 1993, paragraph bridging pages 4965-4966 (Exhibit 6).

[186] Kanzler, page 3429, right col., third & fourth paragraphs, referring to "single cell PCR" of 10 H-RS cells.

[187] Kanzler, abstract.

11 of 20 H-RS cells … demonstrate that H-RS cells in this patient represent a clonal population.".[188]

In addition, Kanzler meets the added requirements of claim 25. Kanzler used a biological sample in the form of "*bone marrow... of a ... lymphoma patient*" as recited in claim 25. In particular, "[s]ingle cells were isolated from frozen sections of a bone marrow specimen of an HD [*i.e.*, Hodgkin's disease] patient.[189]"

In addition, the patient in question had earlier "*received anti-cancer therapy*" as also recited in claim 25. Kanzler explains that the bone marrow sample used in the study was obtained in April 1994 and cites to an article by Wolf *et al.* for further details on the history of the same patient.[190] The Wolf article indicates that the same patient was treated with radiotherapy in 1991 and with chemotherapy in 1993, before Kanzler obtained his blood marrow sample in 1994.[191]

Obviousness: Reasons to Combine

Li pioneered the method of single-cell PCR, and Kanzler applied single-cell PCR to blood from a treated lymphoma patient. Although a reason to use Li's single-cell PCR on Kanzler's lymphoma blood samples is not necessarily required, reasons to combine are directly provided by Li himself. Li demonstrated the feasibility of amplifying and genotyping single diploid cells as found in bone marrow samples, and noted that "the ability to study DNA sequences in individual diploid cells will make it possible to study cell-to-cell variation in developmental processes involving DNA rearrangements or other

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[188] Kanzler, page 3434, right col., third paragraph.
[189] Kanzler, page 3429, right col., third paragraph.
[190] Kanzler, page 3429, bottom paragraph.
[191] Wolf *et al.*, Blood. 87: 3418-3428 (1996), paragraph bridging pages 3418-3419 (Exhibit 7).

genetic alterations."[192] Kanzler did precisely as Li suggested by studying DNA rearrangements in diploid lymphoma cells, where prevalence of DNA rearrangements and other genetic alterations is well recognized. It would have been *prima facie* obvious to one of ordinary skill to use Li's single-cell PCR methods on clinically relevant biological samples such as lymph nodes from a treated lymphoma patient as taught by Kanzler.

Thus, claim 25 would have been *prima facie* obvious over Li in view of Kanzler.

J. Proposed Rejection No. 10: Li renders claim 26 obvious in view of Gravel under 35 U.S.C. § 103(a)

Dependent claim 26 recites the method of claim 1, "*wherein the selected genetic sequence is a translocated allele.*"

Under the broadest reasonable interpretation, Li renders claim 26 obvious in view of Gravel.[193] Li anticipates base claim 1, whereas Gravel performed single-cell PCR as pioneered by Li using a translocated allele as his selected sequence. In particular, Gravel used a translocated allele in the form of a t(14;18)(q32;q21) translocation, which Gravel also referred to as the" bcl-2/JH rearrangement" since the translocation placed "the bcl-2 gene of the 18q21 chromosomal region under the transcriptional control of the Ig heavy chain gene (IgH) region."[194]

In particular, Li anticipates claim 1 by performing single-cell PCR on individual cells in separate wells ("assay samples") and determining a ratio by comparing a first

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[192] Li, page 417, right col., third paragraph.

[193] Gravel *et al.*, *Single-cell analysis of the t(14;18)(q32;q21) chromosomal translocation in Hodgkin's disease demonstrates the absence of this translocation in neoplastic Hodgkin and Reed-Sternberg cells.* Blood. 91(8):2866-74 (Apr 15, 1998), forming prior art to the '706 patent under 35 U.S.C. § 102(b). (Exhibit PA-9).

[194] Gravel, abstract and page 2866, left col.

number of assay samples containing a selected sequence and a second number of assay samples containing a reference sequence.

Gravel also performed Li's single-cell PCR method substantially as recited in claim 1. Specifically, Gravel isolated single malignant B cells designated Hodgkin/Reed-Sternberg cells from a Hodgkin's lymphoma patient[195] and subjected the individual cells to single-cell PCR,[196] thereby performing the "diluting" and "amplifying" steps of claim 1. Gravel also determined a "*first number of assay samples*" containing a selected sequence in the form of a t(14;18) translocation sequence, and a "*second number of assay samples*" containing a reference sequence in the form of the c-raf-1 gene,[197] thereby performing the "analyzing" step of claim 1. Gravel also explicitly compared the first and second numbers of assay samples to determine information about the composition of the biological sample.[198]

In addition, Gravel meets the added requirements of claim 26 by using "*a translocated allele*" in the form of a t(14;18)(q32;q21) translocation[199] as his "*selected genetic sequence.*"

Obviousness: Reasons to Combine

Li pioneered the method of single-cell PCR, and Gravel applied Li's single-cell PCR to cells from a treated lymphoma patient using a translocated allele as his selected

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[195] Gravel, page 2867, right col., second paragraph, indicating that "stained sections were overlaid with phosphate buffered saline (PBS)" and "[s]ingle cells were picked up … and then transferred by aspiration," thereby diluting the biological sample to form a set comprising a plurality of assay samples.

[196] Gravel, page 2869, right col., bottom paragraph.

[197] Gravel, page 2869, right col., bottom paragraph.

[198] Gravel, page 2869, right col., bottom paragraph

[199] In particular, Gravel used a translocated allele in the form of a t(14;18)(q32;q21) translocation. Gravel, abstract and page 2866, left col.

sequence. Although a reason to use a translocated allele as a selected sequence in Li's single-cell PCR is not necessarily required for obviousness, reasons to do so are directly provided by Li himself. Li demonstrated the feasibility of his single-cell PCR methods on diploid cells such as those used by Gravel. Li noted that "the ability to study DNA sequences in individual diploid cells will make it possible to study cell-to-cell variation in developmental processes involving DNA *rearrangements* or other genetic alterations."[200] Gravel explains that his translocated allele is a type of genetic "rearrangement."[201] Thus, Gravel did precisely as Li suggested by studying DNA translocation rearrangements in cancer cells, where prevalence of DNA rearrangements and other genetic alterations is well recognized. It would have been *prima facie* obvious to one of ordinary skill to use a translocated allele as taught by Gravel as the selected sequence in Li's single-cell PCR methods.

Thus, claim 26 would have been *prima facie* obvious at the time of invention over Li in view of Gravel.

K. Proposed Rejection No. 11: Li renders claims 28 & 29 obvious in view of Marcucci under 35 U.S.C. § 103(a)

1. Detailed explanation of the pertinency and manner of applying Li and Marcucci to claim 28

Dependent claim 28 recites the method of claim 1, "*wherein the selected genetic sequence is within an amplicon which is amplified during neoplastic development.*"

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[200] Li, page 417, right col., third paragraph.

[201] In particular, Gravel referred to the t(14;18)(q32;q21) translocation as the" bcl-2/JH rearrangement" since the translocation placed "the bcl-2 gene of the 18q21 chromosomal region under the transcriptional control of the Ig heavy chain gene (IgH) region." Gravel, Abstract and page 2866, left col.

Under the broadest reasonable interpretation, Li renders claim 28 obvious in view of Marcucci.[202] Li anticipates base claim 1, whereas Marcucci analyzed biological cancer samples for the presence of "*an amplicon which is amplified during neoplastic development*" as recited in claim 28.

In particular, Li anticipates claim 1 by performing single-cell PCR on individual cells in separate wells ("assay samples") and determining a ratio by comparing a first number of assay samples containing a selected sequence and a second number of assay samples containing a reference sequence.

In addition, Marcucci analyzed biological samples by PCR to determine the presence of a "*selected genetic sequence*" in the form of an "*ALL1* [gene] rearrangement" which is expressed as an aberrant mRNA in the form of an "*ALL1* fusion transcript."[203] It had been previously discovered that this rearrangement involves a "partial tandem duplication (PTD)" of a portion of the *ALL1* gene, noting that the "partial tandem duplication (PTD) of ALLÃ• (MLL) is one of the more common molecular abnormalities in adult *de novo* acute myeloid leukemia (AML) and carries a poor prognosis."[204] As explained in detail below, such a duplication is "*an amplicon which is amplified during neoplastic development*" as recited by claim 28, under the broadest reasonable interpretation.

More specifically, Marcucci's goal was to "determine if the *ALL1* fusion transcript is specific for leukemic blasts or instead can be found with any frequency in normal

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[202] Marcucci *et al.*, *Detection of Unique ALLÃ•(MLL) Fusion Transcripts in Normal Human Bone Marrow and Blood: Distinct Origin of Normal versus Leukemic ALL1 Fusion Transcripts.* Cancer Res, 58:790-793. (February 15, 1998), forming prior art under 35 U.S.C. § 102(b). (Exhibit PA-10).

[203] Marcucci, Abstract.

[204] Marcucci, Abstract,

cells."[205] Upon finding by RT-PCR analysis on RNA samples that 10 of 60 apparently normal individuals appeared to express a fusion mRNA transcript, Marcucci followed up with a genomic PCR analysis on the genomic DNA of eight such individuals to check for the presence of an actual rearrangement of the *ALL1* gene as a PTD amplicon.[206] In his genomic PCR assay, Marcucci used "primers specific for the exons involved in the fusion"[207] to check for the PTD amplicon as his "*selected genetic sequence*." In the same assay, Marcucci also amplified a β-actin gene [208] as a "*reference genetic sequence*." Marcucci found that all eight normal samples did not amplify the PTD amplicon at the genomic level, despite the apparent expression of a fusion transcript at the mRNA level.[209] In particular, "[a]ll eight normal donor samples that were positive by RT-PCR failed to show a genomic fusion" by genomic PCR, although "[i]ntegrity of the DNA for all the samples was verified by the [successful] amplification of the β-actin gene."[210] Thus, Marcucci compared the number of samples containing his "*selected genetic sequence*" and his "*reference genetic sequence*."

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[205] Marcucci, Abstract.

[206] Marcucci, Abstract ("we analyzed … [RNA] samples from 60 normal donors by nested RT-PCR. Ten of 60 samples … contained a unique transcript showing a fusion of two *ALL1* exons that was consistent with the PTD of *ALL1*. However, a corresponding genomic rearrangement or a unique genomic fusion of *ALL1* could not be demonstrated by Southern analysis or DNA PCR, respectively.") *See also* page 791, left col., bottom paragraph ("[RNA] samples from 60 healthy normal donors were analyzed for the PTD of *ALL1* by nested RT-PCR. Ten of 60 samples … amplified a transcript showing a unique fusion of two *ALL1* exons.")

[207] Marcucci, page 791, left col., "DNA Analysis" Section, first paragraph.

[208] Marcucci, page 791, left col., "DNA Analysis" Section, first paragraph ("DNA integrity was demonstrated by successful β-actin amplification in each sample.")

[209] Marcucci, Abstract ("Ten of 60 [RNA] samples … contained a unique [*ALL1* fusion] transcript … that was consistent with the PTD of *ALL1*. However, a corresponding … unique genomic fusion of *ALL1* could not be demonstrated by … DNA PCR"). *See also* page 791, left col., "DNA Analysis" Section, first paragraph ("no evidence of fusion at the genomic level could be found following DNA PCR amplification across the putative introns involved in the unique fusion").

[210] Marcucci, Fig. 4, legend.

In addition, Marcucci meets all additional limitations recited in the body of claim 28 itself.

First, Marcucci's partial tandem duplication (PTD) of the *ALL1* gene sequence is an "*amplicon*" as recited in claim 28 under the broadest reasonable interpretation, at least because (1) the art recognized duplication of any particular portion of a chromosome as a type of genetic amplification,[211] (2) claim 29 does not limit the scope of "amplicon" in any way, and (3) the '706 specification does not give "amplicon" a special definition contrary to the art.[212]

Second, Marcucci's PTD amplicon is "*amplified during neoplastic development,*" as required by claim 28. In particular, Marcucci explains that his PTD amplicon "is one of the more common molecular abnormalities in adult de novo acute myeloid leukemia (AML) and carries a poor prognosis."[213]

Obviousness: Reasons to Combine

It would have been *prima facie* obvious to one of ordinary skill to use a "*selected genetic sequence ... within an amplicon which is amplified during neoplastic development"* in the form of a PTD sequence in the ALL1 oncogene, as taught by Marcucci, in Li's single-cell PCR methods. Marcucci amplified a "*selected genetic sequence*" (*i.e.*, a PTD amplicon) and a "*reference genetic sequence*" (*i.e.*, β-actin gene)

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[211] Mandahl *et al.*, Int. J. Cancer: 67,632-635 (1996), (Exhibit 8), at Abstract (explaining that genetic "duplication of 12q sequences may be a sufficient level of amplification" to cause cancerous change in cells), and at page 633, right col., second paragraph, last sentence, describing " low-level amplification, resulting in 1.5 to 2.5 times the normal copy number").

[212] The '706 patent only uses amplicon once in the specification, and that is in Table 1, where "Gene amplifications" is listed as a potential application of digital PCR, of which one non-limiting example is to "Determine presence or extent of amplification" using a first probe to a "sequence within [an] amplicon" and a second probe to a "sequence from another part of [the] same chromosome arm." As mentioned, the '706 patent indicates that this is a non-limiting example of analyzing gene amplifications using the claimed methods.

[213] Marcucci, Abstract, first sentence.

directly on his biological cell sample instead of dividing his biological sample into a plurality of single-cell assay samples as Li did. However, Li explicitly taught the benefits of using his single-cell format to study differences between individual cells due to "cell-to-cell variation in developmental processes involving DNA rearrangements,"[214] which bulk amplification is incapable of assessing. Marcucci teaches that his PTD amplicon sequence is precisely the kind of "rearrangement" that Li recognized as a suitable target – specifically, Marcucci noted that the "PTD of ALL1 is identified in leukemic blasts at the genomic level by ALL1 rearrangement upon Southern analysis."[215] Marcucci's specific goal was to "determine if the ALL1 fusion transcript is specific for leukemic blasts or instead can be found with any frequency in normal cells."[216] Marcucci designed his PCR assays to be sensitive enough to detect the PTD amplicon "when … present in 1% of the cells in the processed sample,"[217] because his biological samples (*i.e.*, lymph node biopsies) would normally contain at least some normal cells as well as leukemic cells, potentially obfuscating his data. This problem would have been addressed by Li's single-cell PCR format.

It would thus have been *prima facie* obvious to one of ordinary skill to used Marcucci's "*selected genetic sequence*" (*i.e.*, a PTD amplicon) and his "*reference genetic sequence*" (*i.e.*, β-actin gene), in Li's single-cell PCR methods.

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[214] Li, page 417, right col., third paragraph.
[215] Marcucci, Abstract.
[216] Marcucci, Abstract.
[217] Marcucci, page 791, left col., second paragraph.

2. Detailed explanation of the pertinency and manner of applying Li and Marcucci to claim 28

Dependent claim 29 recites the method of claim 1, "*wherein the selected genetic sequence is a rare exon sequence.*"

Under the broadest reasonable interpretation, Li renders claim 29 obvious in view of Marcucci.[218] Li anticipates base claim 1, whereas Marcucci analyzed biological cancer samples for the presence of a "*selected genetic sequence [that] is a rare exon sequence* " as recited by claim 29.

In particular, Li anticipates claim 1 by performing single-cell PCR on individual cells in separate wells ("assay samples") and determining a ratio by comparing a first number of assay samples containing a selected sequence and a second number of assay samples containing a reference sequence.

In addition, Marcucci analyzed single cells from B-lymphoma patients containing an *ALL1* gene rearrangement by RT-PCR, in order to study cell-to-cell expression of the unique *ALL1* mRNA "fusion transcript"[219] encoded by the rearranged gene, which as explained below is a "*rare exon sequence.*" Marcucci's goal was to "determine if the *ALL1* fusion transcript is specific for leukemic blasts or instead can be found with any frequency in normal cells."[220] Marcucci detected an *ALL1* fusion transcript in the RNA of 10 out of 60 apparently normal individuals by RT-PCR.[221] Marcucci ensured that his

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[218] Marcucci *et al.*, *Detection of Unique ALLÃ•(MLL) Fusion Transcripts in Normal Human Bone Marrow and Blood: Distinct Origin of Normal versus Leukemic ALL1 Fusion Transcripts.* Cancer Res, 58:790-793. (February 15, 1998), forming prior art under 35 U.S.C. § 102(b). (Exhibit PA-10).

[219] Marcucci, Abstract.

[220] Marcucci, Abstract.

[221] Marcucci, Abstract ("we analyzed … [RNA] samples from 60 normal donors by nested RT-PCR. Ten of 60 samples … contained a unique transcript showing a fusion of two *ALL1* exons that was consistent with the PTD of *ALL1*.") *See also* page 791, left col., bottom paragraph

RT-PCR assay would selectively amplify only *ALL1* fusion transcripts instead of normal *ALL1* mRNA by using a primer pair specific for the fusion transcript.[222] Marcucci also ensured that each RNA sample was amplifiable by amplifying the β-actin gene as a reference control ("*reference genetic sequence*") in each sample.[223] Despite observing amplification of the actin reference in each and every sample, Marcucci found that only "[t]en of 60 [RNA] samples … amplified a transcript showing a unique fusion of two *ALL1* exons,"[224] Thus, Marcucci compared the number of samples containing the *ALL1* fusion transcript (a "*selected genetic sequence*") with the number of samples containing the actin transcript (a "*reference genetic sequence*") although the comparison was not explicitly presented as a ratio.[225]

In addition, Marcucci meets the added requirements of claim 29 in that his "*selected genetic sequence is a rare exon sequence*." Claim 29 does not limit the scope of "*rare exon*" in any way, and the '706 specification does not give "*rare exon*" a special definition contrary to the art. Marcucci's "*selected genetic sequence*" in the form of an *ALL1* fusion transcript was "a unique transcript showing a fusion of two *ALL1* exons."[226] In Fig. 3B, Marcucci also provided an exemplary sequence one of his observed "fusion

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("[RNA] samples from 60 healthy normal donors were analyzed for the PTD of *ALL1* by nested RT-PCR. Ten of 60 samples … amplified a transcript showing a unique fusion of two *ALL1* exons.")

[222] Marcucci, Fig. 2, providing a "schematic illustration of … unique exon fusion transcripts detected by nested RT-PCR" and also showing the positions of the "primers that amplify the PTD of ALL1," so that "[e] ach transcript is consistent with a PTD of the *ALL1* gene."

[223] Marcucci, page 790, right col., Section on "RT-PCR Analysis," explaining that "each RNA sample was also amplified for β-actin transcript. *See also* page 792, left col., "Poly(A)+ RNA Analysis" section (Each poly(A)+ RNA sample was successfully amplified for the β-actin transcript.)" *See also* page 792, right col., top paragraph ("Integrity of the RNA was verified by successful amplification of the β-actin transcript.")

[224] Marcucci, page 791, left col., last sentence.

[225] Marcucci, page 791, left col., last sentence.

[226] Marcucci, Abstract.

transcripts in which the 3' exon involved in the fusion is spliced, not at the consensus spliced site of the 5' exon but rather in the middle of the sequence, resulting in a frameshift of the ORF."[227] Because Marcucci's *ALL1* fusion transcript contains an aberrant fusion exon that is not found in most normal individuals, it is a "*rare exon sequence*" as recited in claim 29 under the broadest reasonable interpretation. Thus, Marcucci's fusion transcript is a "*selected genetic sequence [which] is a rare exon sequence*" as required by claim 29.

Obviousness: Reasons to Combine

It would have been *prima facie* obvious to one of ordinary skill to use a "*selected genetic sequence ... within an amplicon which is amplified during neoplastic development"* in the form of a PTD sequence in the ALL1 oncogene, as taught by Marcucci, in Li's single-cell PCR methods. Marcucci amplified a "*selected genetic sequence*" (*i.e.*, a PTD amplicon) and a "*reference genetic sequence*" (*i.e.*, β-actin gene) directly on his biological cell sample instead of dividing his biological sample into a plurality of single-cell assay samples as Li did. However, Li explicitly taught the benefits of using his single-cell format to study differences between individual cells due to "cell-to-cell variation in developmental processes involving DNA rearrangements,"[228] which a bulk amplification procedure like Marcucci's would be incapable of detecting. Marcucci teaches that his PTD amplicon sequence is precisely the kind of "rearrangement" that Li recognized as a suitable target – specifically, Marcucci noted that the "PTD of ALL1 is identified in leukemic blasts at the genomic level by ALL1 *rearrangement*."[229]

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[227] Marcucci, Fig 3B, legend.
[228] Li, page 417, right col., third paragraph.
[229] Marcucci, Abstract.

Marcucci's specific goal was to "determine if the ALL1 fusion transcript is specific for leukemic blasts or instead can be found with any frequency in normal cells."[230] Marcucci designed his PCR assays to be sensitive enough to detect the PTD amplicon "when … present in 1% of the cells in the processed sample,"[231] because his biological samples would normally contain both normal and leukemic cells, potentially obfuscating his data. This problem would have been addressed by Li's single-cell PCR format.

It would thus have been *prima facie* obvious to one of ordinary skill to used Marcucci's "*selected genetic sequence*" (*i.e.*, a PTD amplicon) and his "*reference genetic sequence*" (*i.e.*, β-actin gene), in Li's single-cell PCR methods.

L. Proposed Rejection No. 12: Li renders claim 30 obvious in view of Flint under 35 U.S.C. § 103(a)

Dependent claim 30 recites the method of claim 1, "*wherein the nucleic acid template molecules comprise cDNA of RNA transcripts and the selected genetic sequence is present on a cDNA of a first transcript and the reference genetic sequence is present on a cDNA of a second transcript.*"

Under the broadest reasonable interpretation, Li renders claim 30 obvious in view of Flint.[232] Li anticipates base claim 1, whereas Flint used single-cell RT-PCR as explicitly suggested by Li, in order to amplify a selected and reference transcript as recited by claim 30.

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[230] Marcucci, Abstract.

[231] Marcucci, page 791, left col., second paragraph.

[232] Flint *et al. NR2A Subunit Expression Shortens NMDA Receptor Synaptic Currents in Developing Neocortex.* J. Neurosci., 17(7):2469–2476 (April 1, 1997), forming prior art to the '706 patent under 35 U.S.C. § 102(b). (Exhibit PA-11).

In particular, Li anticipates claim 1 by performing single-cell PCR on individual cells ("assay samples") and determining a ratio by comparing a first number of assay samples containing a selected sequence and a second number of assay samples containing a reference sequence.

In addition, Flint performed single-cell RT-PCR, a method explicitly suggested by Li, in order to generate and amplify cDNA of RNA transcripts in individual cells. Specifically, Flint performed "single-cell RT-PCR … to detect NR2A–D subunit expression" in rat brains taken at different "postnatal ages P3/4 or P8/9."[233] Flint extracted the cytoplasmic contents (including the mRNA) of individual neurons and transferred the contents of each individual neuron cell to a PCR reaction tube, thereby generating a plurality of single-genome assay samples from a biological sample.[234] Flint then "*amplified the template molecules*" by RT-PCR, in which "[r]everse-transcription of single-cell mRNA was followed by a PCR designed to coamplify all four NR2 subunits."[235] Flint then "*analyzed the amplified molecules*"[236] to determine the "*number of assay samples*" containing mRNA of each different subunit, as well as the levels of

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[233] Flint, page 2470, right col., last paragraph ("we used single-cell RT-PCR … to detect NR2A–D subunit expression in physiologically characterized neurons").

[234] Flint, page 2470, right col., last paragraph ("After characterization of [neuron cell behavior] … cytoplasmic harvest was performed" followed by RT-PCR on "single-cell mRNA"). *See also* page 2471, Fig. 1 legend, showing amplification "products obtained by RT-PCR for NR2A–D subunits on cytoplasmic material harvested from physiologically characterized neurons.")

[235] Flint, page 2470, right col., last paragraph. *See also* same page, left col., last paragraph ("Coamplification of NR2A–D subunits was performed by nested hot-start PCR" using a single pair of primers in a primary PCR reaction).

[236] Flint, page 2470, paragraph bridging left and right cols., (samples were analyzed by first re-amplifying one "microliter of the first-round PCR product … in a second PCR" with a single pair of hemi-nested primers, and performing "dot-blot hybridization" in which "PCR products of expected size … were … extracted… [and a] serial dilution of each PCR product was dotted onto four different nitrocellulose membranes, each containing … one of the four "NR2 standards" … obtained by cloning PCR fragments (244 bp) of each NR2 subunit ").

each mRNA. Flint analyzed the single-cell samples to determine a "*first number of assay samples*" containing a "*selected genetic sequence*" in the form of NR2B mRNA, and a "*second number of assay samples*" containing a "*reference genetic sequence*" in the form of NR2C mRNA, subsequently "*comparing*" these two numbers to determine the "*composition of the biological sample*." Specifically, Flint observed that "the NR2B subunit was expressed above the estimated background level … in almost every cell … In contrast, very few cells expressed significant relative amounts of … NR2C."[237] In particular, "NR2C was encountered in only one cell at P3/4 [age group] and one cell at P8/9 [age group]."[238]

In addition, Flint meets the added requirements of claim 30. Because Flint performed "reverse-transcription of single-cell mRNA … followed by PCR,"[239] Flint used "*nucleic acid template molecules compris[ing] cDNA of RNA transcripts.*"

In addition, Flint's "*selected genetic sequence is present on a cDNA of a first transcript*" whereas the "*reference genetic sequence is present on a cDNA of a second transcript*." As discussed, Flint assayed for cDNA of NR2C as his "*selected genetic sequence,*" and for cDNA of NR2B as his "*reference genetic sequence.*"[240]

Obviousness: Reasons to Combine

It would have been obvious to use Flint's single-cell RT-PCR analysis in which two different cDNAs are used as the "*selected genetic sequence*" and "*reference genetic sequence*," in Li's own single-cell methods of analysis. Reasons to combine are directly provided by Li himself. Although Li himself used PCR instead of RT-PCR, Li explicitly

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[237] Flint, page 2472, right col., section on "Single-cell expression of NR2 subunit mRNA."
[238] Flint, page 2472, right col., section on "Single-cell expression of NR2 subunit mRNA."
[239] Flint, page 2470, right col., last paragraph.
[240] Flint, page 2472, right col., section on "Single-cell expression of NR2 subunit mRNA."

recognized the usefulness of using single-cell RT-PCR as Flint did, in order to detect and analyze mRNA expression in single diploid cells: "[A] nalysis of messenger RNAs in single cells would be possible if efficient reverse transcription could be carried out before PCR was initiated.".[241] Li also noted that "the ability to study DNA sequences in individual diploid cells will make it possible to study cell-to-cell variation in developmental processes."[242] Flint did precisely as Li suggested by studying cell-to-cell variations in expression between different neurons by converting mRNA into cDNA for subsequent amplification. In view of Flint, it would have been *prima facie* obvious to one of ordinary skill to use Li's methods in an RT-PCR format, using a first cDNA as a "*selected genetic sequence*" of interest, and a second cDNA as a "*reference genetic sequence.*"

Thus, claim 30 would have been *prima facie* obvious at the time of invention over Li in view of Flint.

M. Proposed Rejection No. 13: Li renders claim 31 obvious in view of Ponten under 35 U.S.C. § 103(a)

Dependent claim 31 recites the method of claim 1, "*wherein the selected genetic sequence comprises a first mutation and the reference genetic sequence comprises a second mutation.*"

Under the broadest reasonable interpretation, Li renders claim 31 obvious in view of Ponten.[243] Li anticipates base claim 1, whereas Ponten used single-cell RT-PCR as

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[241] Li, page 417, right col., third paragraph.
[242] Li, page 417, right col., third paragraph.
[243] Ponten *et al.*, *Genomic analysis of single cells from human basal cell cancer using laser-assisted capture microscopy*. Mutation Research Genomics 382, 45–55 (1997). (Exhibit PA-11).

explicitly suggested by Li, in order to amplify a selected and reference sequence each comprising a different mutation as recited by claim 30.

In particular, Li anticipates claim 1 by performing single-cell PCR on individual cells ("assay samples") and determining a ratio by comparing a first number of assay samples containing a selected sequence and a second number of assay samples containing a reference sequence.

In addition, Ponten performed single-cell PCR, a method explicitly suggested by Li, in order to perform "mutational analysis of genomic DNA … on single somatic cells."[244] Specifically, Ponten isolated "[e]ighty-nine single tumor cells" from a *biological sample* in the form of a "stained tissue section" of a "human basal cell cancer BCC" thereby generating a plurality of single-cell assay samples from a biological sample.[245] Ponten then "*amplified the template molecules*" by PCR, in which "[e]xons 4–9, of the human p53 gene, and the HLA-DQB1 locus were amplified in a multiplex/nested configuration."[246] Ponten then "*analyzed the amplified molecules*" in the single-cell samples by performing a secondary (nested) amplification and DNA sequencing.[247] Ponten checked each cell for a "*selected genetic sequence*" in the form of a first p53 allele mutated in exon 7 (codon 245), and a "*reference genetic sequence*" in the form of second p53 allele mutated in exon 8 (codon 266).[248] Ponten then determined

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[244] Ponten, Abstract. *See also* page 46, right col., second paragraph ("Single cells were picked up, with the aid of the micromanipulator, on the tip of a small glass capillary … The tip of the capillary, with the attached cell, was broken off against the bottom of a PCR tube.")

[245] Ponten, page 45, Abstract.

[246] Ponten, page 46, Section 2.3.

[247] Ponten, page 46, Section 2.3, and page 44, Section 2.3.

[248] Ponten, page 49, paragraph bridging left and right cols., explaining that 'crude' microdissection … had [already] uncovered two point mutations in the tumor (Fig. 2). One was in exon 7 codon 245. and the other in exon 8 codon 266 … Cloning of this fragment revealed that

a "*first number of assay samples*" containing the "*selected* " exon 7-mutated p53 allele and a "*second number of assay samples*" containing the "*reference*" exon 8-mutated p53 allele, and compared the two numbers. In particular, out of 44 single-cell samples which yielded an amplification product of exon 7 and/or exon 8, Ponten found that "[t]wo mutations were dominant, codon 245 (GGC to GTC), [in] exon 7, mutated in 20 cells, and codon 266 (GGA to GAA), [in] exon 8, mutated in 13 cells."[249]

In addition, Ponten meets the added requirements of claim 30. Ponten's *selected genetic sequence*" (*i.e.*, exon 7-mutated p53) "*comprises a first mutation and the reference genetic sequence*" (*i.e.*, exon 8-mutated p53) "*comprises a second mutation.*"

Obviousness: Reasons to Combine

It would have been obvious to use Ponten's single-cell RT-PCR analysis in which the number of samples containing two different mutant sequences are compared, in Li's own single-cell methods of analysis. Reasons to combine are directly provided by Li himself. Li explicitly recognized the usefulness of single-cell PCR methods, which gave a user "the ability to study DNA sequences in individual diploid cells [which] will make it possible to study cell-to-cell variation."[250] Ponten did precisely as Li suggested for precisely the same reason – specifically, to "resolve important and fundamental questions determining cancer heterogeneity," *i.e.*, cell-to-cell variation in cancer.[251] Ponten explained that "[i]n crude microdissections … mutations [in single cells] would be

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the mutations were situated on different alleles." *See also* Fig. 4, Table 1 and page 50, left col., bottom paragraph, describing the results of the single-cell PCR analysis, finding that "[t]wo mutations were dominant ... Both mutations were identical to those found in the previous crude microdissections."

[249] Ponten, page 50, paragraph bridging left and right cols.

[250] Li, page 417, right col., third paragraph.

[251] Ponten, Abstract, and page 46, left col., last paragraph of Introduction section.

'diluted' and thus not detected … while … [analysis] of single cells would disclose such mutations."[252] Ponten also noted that two separate mutations on either allele commonly take place in cancer progression: "we have microdissected different parts of individual BCC's and found that p53 mutations often affect both alleles and that progression of p53 alterations can take place within a tumor."[253] Accordingly, Ponten renders it obvious that a mutant "*selected genetic sequence*" in the form of a first mutant allele and a mutant "*reference genetic sequence*" in the form of a second mutant allele could often be found in cancer samples.

In view of Ponten, it would have been *prima facie* obvious to one of ordinary skill to use Li's methods on cancer samples such as Ponten's in which "*the selected genetic sequence comprises a first mutation and the reference genetic sequence comprises a second mutation.*"

Thus, claim 31 would have been *prima facie* obvious at the time of invention over Li in view of Ponten.

N. Proposed Rejection No. 14: Zhang anticipates claims 38, 39, 46 & 51 under 35 U.S.C. § 102(b)

1. Short introductory overview of relevant portions of Zhang's disclosure

Independent claim 38 and dependent claims 39, 46 & 51 are anticipated by Zhang.[254] To provide a quick orientation to the Examiner, this section presents an

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[252] Ponten, page 54, second paragraph.

[253] Ponten, page 46, left col., last paragraph of Introduction section.

[254] Zhang *et al., Whole genome amplification from a single cell: implications for genetic analysis*. PNAS USA, 89(13):5847-51 (1992), forming prior art under 35 U.S.C. § 102(b) to the '706 patent. (Exhibit PA-2).

introductory high-level overview of the steps of this claim and broadly maps Zhang's experiments onto each of these steps. A more detailed application of Zhang's teachings to each claimed step, showing the details of how Zhang performed each step with specific cites to Zhang's relevant disclosure is presented in the next section.

Generally, claim 38 of the '706 patent is directed to a method requiring three steps: (1) amplifying template molecules within a set of assay samples to form a population of amplified molecules in each of the assay samples; (2) analyzing the amplified molecules to determine a first number of assay samples that contains one sequence and a second number of assay samples that contains a different sequence; and (3) comparing those numbers to ascertain a ratio that reflects the composition of the biological sample. More specifically with respect to independent claim 38, Zhang performed the three main steps of independent claim 38 of the '706 patent, as follows.

a) Amplifying step

- The first step involves "*amplifying template molecules within a set comprising a plurality of assay samples to form a population of amplified molecules in each of the assay samples of the set.*"

- Zhang made 18 single-sperm samples which were lysed and pre-amplified by primer extension pre-amplification (PEP),[255] thereby forming "*a set comprising a plurality of assay samples*." The templates molecules in each sample were subjected to PCR amplification using locus-specific primers, such as the APOC2 locus,[256] resulting in "*amplifying template molecules within ... assay samples to form a population of amplified molecules in each of the assay samples of the set.*"

b) Analyzing/number-determining step

- The second step involves "*analyzing the amplified molecules ... to determine a first number of assay samples which contain the selected*

[255] Zhang, page 5847, left and right cols., section titled "PEP of Single-Sperm DNA."
[256] Zhang, page 5847, right col., section titled "Specific Gene Analysis."

genetic sequence and a second number of assay samples which contain a reference genetic sequence."

- Zhang determined the allelic identity of the locus-specific PCR amplification products at the APOC2 locus by using gel electrophoresis to measure the size of the PCR product,[257] thereby "*analyzing the amplified molecules.*" For the APOC2 locus, Zhang then counted the number of samples showing presence of one APOC2 allele, and also the number of samples showing presence of the other APOC2 allele, thereby determining a "*first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence.*"

- The second step also recites a complicated "wherein" clause, "*wherein at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence."* The next section explains in detail how Zhang meets this limitation.

c) Comparing step

- The third step involves "*comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample*."

- Zhang compared the number of single-sperm assay samples containing the first APOC2 allele against the number of assay samples containing the second APOC2 allele. Zhang noted that there were 9 samples of each allele (*i.e.*, an equal ratio of both) and that "the segregation pattern of the APOC2 alleles could clearly be seen,"[258] thereby "*comparing the first number to the second number to ascertain a ratio*." This ratio reflected the heterozygous nature of the sperm sample and its donor, and thus "*reflect[ed] the composition of the biological sample*."

[257] Zhang, page 5847, right col., section titled "Specific Gene Analysis."
[258] Zhang, page 5848, right col.

2. Detailed explanation of the pertinency and manner of applying Zhang to independent claim 38

This section discusses in detail how, under the broadest reasonable interpretation of the claims, Zhang discloses methods that meet each and every limitation of independent claim 38.

i) Zhang discloses "A method for determining the ratio of a selected genetic sequence in a population of genetic sequences"

This language forms the preamble of claim 38. Under the PTO's standards for patentability, a preamble which merely recites an "intended use" does not limit the claim in any way.[259]

But even if it were limiting (which it is not), Zhang discloses "*determining the ration of a selected genetic sequence in a population of genetic sequences*" under the broadest reasonable interpretation. Requester notes as a threshold matter that, as written, the preamble broadly uses the word "ratio" in a manner synonymous with "amount" or perhaps "relative amount" as opposed to using "ratio" in a strict mathematical sense. In addition, based on the language and structure of the body of the claim, "*a method for determining the ratio of a selected genetic selection in a population of genetic sequences*" as recited in the preamble more specifically involves "*comparing the first number [of assay samples] to the second number [of assay samples] to ascertain a ratio which reflects the composition of the biological sample*" as recited in the final limitation of claim 38.

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[259] *Rowe v. Dror*, 112 F.3d 473, 478, 42 USPQ2d 1550, 1553 (Fed. Cir. 1997) (preamble's recitation of an intended use is not a limitation). Solely for the purposes of this reexamination, Requester will proceed on the premise that the preamble is not limiting in any way or if the examiner disagrees, that it does not impose an additional limitation over what it cited in the body of the claim.

Accordingly, under the broadest reasonable interpretation (and solely for the purposes of this reexamination), a direct and explicit numerical comparison between a first number of assay samples with a selected sequence and a second number of assay samples with a reference sequence,[260] constitutes "*comparing the first number to the second number to ascertain a ratio*" as recited in the last step of claim 1 and "*determining the ratio of a selected genetic sequence in a population of genetic sequences"* as recited in the preamble. In other words, an explicit comparison of the two numbers of assay samples, even without any explicit mention of a "ratio" between the two numbers, is a disclosure of "*determining the ratio of a selected genetic sequence in a population of genetic sequences*." Zhang ascertained a ratio by making exactly such a comparison. This ratio was also reflective of the composition of the biological sample under the broadest reasonable interpretation, at least by reflecting the proportion of the selected and reference sequence within the biological sample.

In particular, Zhang amplified the sequences at the APOC2 locus in eighteen individual sperm cells derived from a single donor.[261] Zhang counted samples that carried one APOC2 allele and samples that carried the other APOC2 allele: Under the broadest reasonable interpretation, either of these two alleles corresponds to the "*selected genetic sequence*" of the preamble "*in a population of genetic sequences*."

Zhang explicitly compared these two numbers of assay samples: "[a]mong the 18 sperm, 9 contained one APOC2 allele and 9 contained the other." [262] in an approximately 1:1 ratio, remarking that "the segregation pattern of the APOC2 alleles . . . can be clearly

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[260] Specifically, comparing a first number of assay samples containing a selected sequence with a second number of assay samples containing a reference sequence.

[261] Zhang, page 5848.

[262] Zhang, page 5848.

seen," (where Mendelian segregation predicts that the alleles should be distributed in a 1:1 ratio).[263] By comparing these two numbers in this manner, Zhang thereby "*determin[ed] the ratio of a selected genetic sequence in a population of genetic sequences.*"

ii) *Zhang discloses "amplifying template molecules within a set comprising a plurality of assay samples to form a population of amplified molecules in each of the assay samples of the set "*

Under the broadest reasonable meaning of the claim terms, Zhang taught "*amplifying template molecules within a set comprising a plurality of assay samples to form a population of amplified molecules in each of the assay samples of the set*" as recited in claim 38.

Zhang started with 18 single-sperm samples and performed three separate and sequential amplification reactions, generating 18 new assay samples for each successive amplification reaction. In particular, Zhang subjected 18 single-sperm samples to a primary amplification reaction in the form of PEP, thereby generating a set of 18 "primary" PEP-amplified assay samples.[264] Zhang then generated a set of 18 "secondary" assay samples where each secondary assay sample contained an aliquot of the amplification product of a corresponding primary assay sample, and subjected the secondary assay samples to a "secondary" amplification in the form of a locus-specific PCR with outer primers flanking the target locus sequence of interest, thereby generating

[263] Zhang, page 5848.

[264] Zhang, page 5847, right col., last sentence ("Twelve single sperm were sorted by flow cytometry, lysed, and subjected to PEP for 50 primer- extension cycles using the mixture of random primers").

"secondary" amplification product.[265] Finally, Zhang then subjected aliquots of these secondary assay samples to a "tertiary" amplification in the form of a hemi-nested PCR with inner primer, thereby generating a set of 18 "tertiary" nested-PCR assay samples.[266]

It should be noted that in contrast to claim 1, claim 38 does not recite a diluting/distributing step and thus does not require that the "set comprising a plurality of assay samples" is generated directly from the biological sample. As a result, any of Zhang's sets of single-sperm, primary, secondary or tertiary samples reads upon a "*set comprising a plurality of assay samples*" under the broadest reasonable interpretation, and the DNA molecules in these assay samples acts as "*template molecules*" in the next subsequent amplification reaction which generates a "*population of amplified molecules in each of the assay samples of the set.*" For example, the inner-primer PCR-amplified molecules in each of Zhang's secondary assay samples are used as "*template molecules*" in the tertiary samples, where the tertiary amplification reaction with nested primers generates a "*population of amplified molecules*" from the secondary molecules.

In addition, Zhang amplified molecules "*in each of the assay samples of the set.*" In particular, Zhang started with 18 single-sperm samples and ultimately generated a "*set comprising a plurality of assay samples*" in the form of 18 corresponding tertiary assay

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[265] Zhang, page 5847, right col., "Specific Gene Analysis" section ("We tested the aliquots from a single sperm subjected to PEP for the presence of specific DNA sequences by using conditions that are capable of detecting single DNA molecules. A total of 12 loci were studied. We used a hemi-nesting strategy that enhances yield and specificity when starting with one target DNA molecule and allows the product to be detected by ethidium bromide staining (17, 18). The first round of PCR utilizes a pair of primers that flank the target sequence. The second round uses one of the two original primers and a second internal primer. In every case we took 2 μl of first-round product for the second round of PCR").

[266] Zhang, page 5847, right col., "Specific Gene Analysis" section ("We used a hemi-nesting strategy … The first round of PCR utilizes a pair of primers that flank the target sequence. The second round uses one of the two original primers and a second internal primer. In every case we took 2 μl of first-round product for the second round of PCR").

samples, which Zhang subjected to tertiary amplification with inner hemi-nested primers for the APOC2 locus.[267] Table 2 summarizes the results obtained after primary, secondary and tertiary amplification at twelve different loci; col. 5 shows that tertiary amplification products were detected in *each* of the 18 assay samples of the APOC2 set. Similar results were obtained for other loci such as the LDLR and pa loci. Thus, Zhang generated a "*population of amplified molecules in each of the assay samples of the set*."

Therefore, Zhang taught "*amplifying template molecules within a set comprising a plurality of assay samples to form a population of amplified molecules in each of the assay samples of the set*" as recited in claim 38.

iii) *Zhang discloses "analyzing the amplified molecules in the assay samples of the set"*

Under the broadest reasonable meaning of the claim terms, Zhang's experiments included a step of "*analyzing the amplified molecules in the assay samples of the set*" as recited in claim 38.

As summarized above, Zhang's tertiary amplification (a PCR amplification reaction using inner nested primers on secondary amplification product as template) "*form[ed] a population of amplified molecules in each*" of the 18 assay samples of the APOC2 set of assay samples, as recited in claim 38..[268] Subsequently, Zhang analyzed the tertiary amplification products of each tertiary assay sample by gel electrophoresis

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[267] Zhang, page 5847, right col., "Specific Gene Analysis" section ("We tested the aliquots from a single sperm subjected to PEP for the presence of specific DNA sequences by using conditions that are capable of detecting single DNA molecules. A total of 12 loci were studied. We used a hemi-nesting strategy that enhances yield and specificity when starting with one target DNA molecule and allows the product to be detected by ethidium bromide staining (17, 18). The first round of PCR utilizes a pair of primers that flank the target sequence. The second round uses one of the two original primers and a second internal primer. In every case we took 2 μl of first-round product for the second round of PCR").

[268] Zhang, page 5847, right col., "Specific Gene Analysis"

and "ethidium bromide staining"[269] to determine the allelic identity of the APOC2 allele originally present in the starting single-sperm sample which the primary, secondary and tertiary assay samples were derived from. In particular, Zhang differentiated the two APOC2 alleles by a difference in band sizes visible on the gel.[270] Under the broadest reasonable interpretation, Zhang's ethidium bromide staining and gel electrophoresis corresponds to "*analyzing the amplified molecules in the assay samples of the set*" as recited in claim 38.

iv) *Zhang's analysis was done "to determine a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence"*

Zhang *"determine[d] a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence*" as recited in claim 38.

For the analysis of the APOC2 locus described above, Zhang determined the allelic identity of the tertiary amplification product in each assay sample of his set of 18 tertiary assay samples. Zhang counted the number of samples showing the presence of one APOC2 allele, and also the number of samples showing the presence of the other APOC2 allele. Either of these two alleles corresponds to the "*selected genetic sequence*"

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[269] Zhang, page 5847, right col., "Specific Gene Analysis" section ("We tested the aliquots from a single sperm subjected to PEP for the presence of specific DNA sequences by using conditions that are capable of detecting single DNA molecules. A total of 12 loci were studied. We used a hemi-nesting strategy that enhances yield and specificity when starting with one target DNA molecule and allows the product to be detected by ethidium bromide staining (17, 18). The first round of PCR utilizes a pair of primers that flank the target sequence. The second round uses one of the two original primers and a second internal primer. In every case we took 2 µl of first-round product for the second round of PCR").

[270] Zhang, page 5848, right col., second paragraph ("The PCR systems for these loci were designed so that the allelic state at APOC2 (the number ofCA repeats) … could be determined from the size of the PCR product alone"). *See also* Fig. 3 and Fig. 3 legend.

while the other APOC2 allele corresponds to the "*reference genetic sequence*." Zhang noted that nine assay samples carried one APOC2 allele, thereby *"determin[ing] a first number of assay samples which contain the selected genetic sequence*." Zhang also noted that nine assay samples contained the other APOC2 allele, thereby "*determin[ing] a second number of assay samples which contain a reference genetic sequence.*"[271]

Zhang thus *"determine[d] a first number of assay samples which contain the selected genetic sequence and a second number of assay samples which contain a reference genetic sequence*" as recited in claim 38.

v) *Zhang discloses "wherein at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence."*

Under the broadest reasonable meaning of the claim terms, in Zhang's set of 18 assay samples "*at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence*" as recited in claim 38.

This language of claim 38 is insolubly vague and ambiguous - for example, it is completely unclear what is meant by "*total genetic sequences required for the step of analyzing to determine the presence of the selected genetic sequence*." Although Requester believes it fails to meet the requirements of Section 112, ¶ 2, such a

[271] Zhang, page 5848, right col. ("Among the 18 sperm, 9 contained one APOC2 allele and 9 contained the other. Similarly, among the 17 samples that were positive for STS, 9 carried the X chromosome and 8 carried the Y chromosome. Fig. 3 shows the genotype of 9 of the 18 sperm. The segregation pattern of the APOC2 alleles and the X and Y chromosomes can be clearly seen. The independent assortment of the sex chromosomes from chromosome 19 is also observed.")

determination not within the scope of this reexamination. Although the specification does not use the phrase "total genetic sequences," the specification instead explains that "it would be desirable that at least 1/50 of the diluted samples have a ***detectable proportion*** of analyte," such that "[a]t least 1/10, 1/5, 3/10, 2/5, 1/2, 3/5, 7/10, 4/5, or 9/10 of the diluted samples may have a ***detectable proportion*** of analyte,"[272] and apparently equates "analyte" with "selected genetic sequence."[273] Solely for the purposes of this reexamination, and at least for this claim to be found valid, Requester will proceed on the premise that the unclear language of claim 38 indicates that at least one-fiftieth of the assay samples must contain sufficient proportion of the selected sequence of interest compared to other sequences present in the same assay sample, such that it is present in an amount that is detectable by the particular detection method being used. Consequently, a showing that the selected sequence of interest was ***actually detected*** in at least one-tenth of the assay samples is sufficient to anticipate this recitation of claim 38.

Under this premise, Zhang anticipates this recitation of claim 38. As discussed, Zhang subjected eighteen tertiary assay samples corresponding to individual sperm to tertiary amplification and observed a "*selected genetic sequence*" in the form of a first APOC2 allele in nine of eighteen assay samples,[274] thereby demonstrating that exactly half of his assay samples (*i.e.*, "*at least one-fiftieth of the assay samples in the set*") contained a sufficient proportion of the selected sequence that it was present in detectable amounts. Zhang thereby demonstrated that "*at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of*

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[272] '706 patent, col. 4, lines 19-23, emphasis added.
[273] '706 patent, col. 3, line 66-Col. 4, line 2; col. 4, lines 13-22; col. 5, line 44-col. 6, line 2.
[274] Zhang, page 5848, right col..

selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence" as recited in claim 38.

vi) *Zhang discloses "comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample."*

Under the broadest reasonable meaning of the claim terms, Zhang teaches "*comparing the first number [of assay samples] to the second number to ascertain a ratio which reflects the composition of the biological sample*" as recited in claim 38.

As described in the overview above, Zhang amplified the template molecules and analyzed the amplified molecules in a plurality of assay samples (specifically, eighteen assay samples derived from eighteen individual sperm cells from a single donor).[275] Of these eighteen assay samples, Zhang counted nine samples that carried one APOC2 allele ("*the first number of assay samples*") and nine samples that carried the other APOC2 allele ("*the second number of assay samples*"). Zhang then compared the respective first and second numbers of assay samples for the APOC2 locus and determined that these numbers were equal to each other, in a 1:1 ratio. Zhang remarked that "the segregation pattern of the APOC2 alleles . . . can be clearly seen,"[276] where Mendelian segregation predicts that the alleles should be distributed in a 1:1 ratio. Zhang's observed 1:1 ratio thus "*reflect[ed] the composition of the biological sample*" by indicating that the sample showed a Mendelian segregation pattern. Therefore, by comparing these two numbers of assay samples in this manner and determining the 1:1 ratio, Zhang thereby performed a step of "*comparing the first number to the second number to ascertain a ratio which reflects the composition of the biological sample*."

[275] Zhang, page 5848, right column.
[276] Zhang, page 5848, right column.

3. Detailed explanation of the pertinency and manner of applying Zhang to claim 39

Dependent claim 39 recites the method of claim 38 "*wherein the number of assay samples within the set is greater than 10*." For example, Zhang amplified and analyzed a set of eighteen (18) single-sperm assay samples.[277] Because 18 assay samples is always greater than 10 assay samples, Zhang anticipates claim 39 under the broadest reasonable interpretation.

4. Detailed explanation of the pertinency and manner of applying Zhang to claim 46

Dependent claim 46 recites the method of claim 38 "*wherein the step of analyzing employs gel electrophoresis*." Zhang analyzed the tertiary amplification products of each tertiary assay sample by gel electrophoresis and "ethidium bromide staining"[278] to determine the allelic identity of the APOC2 allele originally present in the starting single-sperm sample which each tertiary assay sample were derived from. In particular, Zhang differentiated the two APOC2 alleles by a difference in band sizes visible on the gel.[279] Zhang thereby "*analyz[ed] the amplified molecules in the assay samples of the set.*" Zhang thus anticipates claim 46 under the broadest reasonable interpretation.

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[277] Zhang, page 5848, right column.

[278] Zhang, page 5847, right col., "Specific Gene Analysis" section ("We tested the aliquots from a single sperm subjected to PEP for the presence of specific DNA sequences by using conditions that are capable of detecting single DNA molecules. A total of 12 loci were studied. We used a hemi-nesting strategy that enhances yield and specificity when starting with one target DNA molecule and allows the product to be detected by ethidium bromide staining (17, 18). The first round of PCR utilizes a pair of primers that flank the target sequence. The second round uses one of the two original primers and a second internal primer. In every case we took 2 µl of first-round product for the second round of PCR").

[279] Zhang, page 5848, right col., second paragraph ("The PCR systems for these loci were designed so that the allelic state at APOC2 (the number ofCA repeats) … could be determined from the size of the PCR product alone"). *See also* Fig. 3 and Fig. 3 legend.

5. Detailed explanation of the pertinency and manner of applying Zhang to claim 51

Dependent claim 51 recites the method of claim 38, "*wherein the step of amplifying employs a single pair of primers.*"

Under the broadest reasonable interpretation, Zhang anticipates claim 51. As discussed for base claim 38, Zhang performed a step of "*amplifying template molecules within a set comprising a plurality of assay samples to form a population of amplified molecules in each of the assay samples of the set*" by subjecting his set of 18 tertiary assay samples to tertiary amplification using "one of the two original primers and a second internal primer."[280] These two primers constitute "*a single pair of primers*" as recited by claim 51.

O. Proposed Rejection No. 15: Zhang renders claims 40-43, 47-48, 59 & 64 obvious in view of Li under 35 U.S.C. § 103(a)

1. Detailed explanation of the pertinency and manner of applying Zhang and Li to claims 40-43

Dependent claims 40-43 depend from independent claim 38, "*wherein the number of assay samples within the set is greater than*" 50 (claim 40), 100 (claim 41), 500 (claim 42) or 1000 (claim 43). Under the broadest reasonable interpretation, Zhang renders claims 40-43 obvious in view of Li.[281] Zhang anticipates base claim 38, where Li discloses or suggests a number of assay samples that is greater than 50, greater than 100, greater than 500, and greater than 1000 as recited in claims 40-43 respectively.

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[280] Zhang at page 5847, right column

[281] Li *et al.*, *Amplification and analysis of DNA sequences in single human sperm and diploid cells.* Nature. 29;335(6189):414-7 (1988), which forms prior art to the '706 patent under 35 U.S.C. § 102(b) (Exhibit PA-1).

As discussed above with respect to claims 7-9, in **Experiment 2** Li made and analyzed a set of 150 single-sperm assay samples.[282] Both Zhang and Li relate to methods of genotyping isolated, individual haploid single sperm cells, as discussed in this request. Moreover, Zhang describes using its technique for a "multipoint mapping strategy" in which "the large number of sperm that can be examined would also allow the recombination fraction to be estimated with great accuracy."[283] Therefore, it would have been obvious for the skilled person to increase the number of single sperm assay samples in Zhang in order to obtain such increased accuracy.

Obviousness: Known Elements and Predictable Result

Under 35 U.S.C. § 103, where a claim "'simply rearranges old elements with each performing the same function it had been known to perform' and yields no more than what one would expect from such an arrangement, the combination is obvious." *KSR Intl. Co. v. Teleflex, Inc.*, 550 U.S. 398, 417 (2007), *quoting Sakraida v. Ag. Pro., Inc.,* 425 U.S. 273, 282 (1976).

It would have been *prima facie* obvious to detect allelic loss at the D11S904 locus, as taught by Zhang, using a large number of samples as suggested by Li. When considering obviousness of a combination of known elements, the operative question is "whether the improvement is more than the predictable use of prior art elements according to their established functions." *KSR*, 550 U.S. at 398; MPEP § 2141. Here, the methods of Zhang and Li perform the same functions when operating together as each does separately, forming nothing more than a combination of well-known procedures in

[282] Li, page 415, right col., last paragraph.
[283] Zhang, page 5850, paragraph bridging left and right columns.

accordance with their intended functions. Such a modification of Zhang would have been obvious to a POSITA for at least the reason that it would have involved a simple substitution of one known element for another (*i.e.*, a small number of assay samples of Zhang substituted with a larger number of assay samples of Li) resulting in a predictable result (an increased number of single sperm assay samples). The '706 claims therefore embody a merely predictable use of prior-art elements.

Because the 150 assay samples of Li is greater than 50 or 100 assay samples, Zhang combined with Li in this manner would have rendered obvious at least claims 40 and 41, respectively, under the broadest reasonable interpretation.

Similarly, Zhang in view of Li would have also rendered obvious claims 42 and 43, which recite wherein the number of assay samples within the set is greater than 500 (claim 42) or 1000 (claim 42). ***The PTO has already found that it would be obvious over Li to use a set of 500 or 1000 assay samples in Li's analysis***.[284] In particular, the PTO found that:

> Li expressly suggested analyzing 500 assay samples (page 416, last paragraph), and that it would have been prima facie obvious … to distribute 500, or even 1000 individual sperm [samples] and assay according to Li's technique. One would have been motivated to do so because Li stated (page 416, first paragraph of "Discussion"): A significant advantage of the approach described here is that a large number of meiotic products can be examined from a single individual allowing determination of the recombination frequency … Li's express contemplate[ion] [sic?] of 500 individual meiotic events certainly renders claim 63 obvious, and, by simple extrapolation, … [other claims] which merely require more assay samples (*i.e.*, 1000).

[284] October 10, 2012 Non-Final Office Action in the '105 prosecution history, at pages 7-8 (Exhibit 5).

Therefore, because Li would have rendered obvious a method involving over 500 and over 1000 samples, the proposed combination of Zhang and Li discussed above would have also rendered obvious claims 42 and 43 for at least the reasons set forth by the PTO and the reasons discussed above with respect to claims 40 and 41. Thus, dependent claims 42 and 43 are *prima facie* obvious over Zhang in view of Li.

2. Detailed explanation of the pertinency and manner of applying Zhang and Li to claims 47 & 48

Dependent claims 47 and 48 depends from independent claim 38, "*wherein the step of analyzing employs hybridization to at least*" one (claim 47) or two (claim 48) "*nucleic acid probes.*" Under the broadest reasonable interpretation, Zhang renders claims 47 and 48 obvious in view of Li.[285] Zhang anticipates base claim 38, where Li discloses a step of analyzing employs hybridization to at least one (claim 47) or two (claim 48) nucleic acid probes respectively.

As discussed above with respect to claims 15 and 16, in **Experiments 1, 2 and 3** Li determined the allelic identity of the LDLr allelic amplification products in his single-sperm assay samples by hybridizing to two probes in the form of two allele-specific oligonucleotides (ASOs).[286] Both Zhang and Li relate to the genotyping of haploid single sperm cells, as discussed in this request. Therefore, it would have been obvious for the

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[285] Li *et al.*, *Amplification and analysis of DNA sequences in single human sperm and diploid cells.* Nature. 29;335(6189):414-7 (1988), which forms prior art to the '706 patent under 35 U.S.C. § 102(b) (Exhibit PA-1).

[286] For Experiment 1, *see* Li, Fig. 2, legend (explaining that the "ASO [*i.e., allele-specific oligonucleotide*] for the LDLrl allele had the sequence 5'AGGATATGGTCCTCTTCCA3' whereas the LDLr2 ASO had the sequence 5'TGGAAGAGAACCATATCCT3'"); for Experiment 2 *see* Li, page 415, right col., bottom paragraph ("part of each secondary reaction was hybridized to either of the two ASOs for that locus"); for Experiment 3, *see* Li, Fig. 1 legend ("dot blot analysis of 20 µl samples of the PCR reaction were carried out using β^S and β^A allele specific probes").

skilled person to substitute the gel electrophoresis analysis to identify the APOC2 allele in Zhang with the dot blot hybridization of Li. Indeed, a POSITA would have been motivated to use the dot blot analysis of Li over the gel electrophoresis of Zhang as the latter merely relies on "the size of the PCR product alone."[287] In contrast, Li's method of analysis is more precise as it relies on sequence-specific hybridization of two probes in the form of two allele-specific oligonucleotides (ASOs).[288]

For at least this reason, under the broadest reasonable interpretation Zhang in view of Li would have rendered obvious claims 47 and 48.

3. Detailed explanation of the pertinency and manner of applying Zhang and Li to claim 59

Dependent claim 59 recites the method of claim 38, "*wherein the selected genetic sequence is a wild-type allele.*"

Under the broadest reasonable interpretation, Zhang renders claim 59 obvious in view of Li.[289] Zhang anticipates base claim 38, where Li counted single-diploid-cell assay samples containing "normal" (*i.e.*, wild-type) β-globin allele, and the number of samples containing a mutant allele. The wild-type allele is a "selected genetic sequence" under the broadest reasonable interpretation.

Obviousness: Reasons to Combine

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[287] Zhang at page 5848, right column.

[288] For Experiment 1, *see* Li, Fig. 2, legend (explaining that the "ASO [*i.e., allele-specific oligonucleotide*] for the LDLrl allele had the sequence 5'AGGATATGGTCCTCTTCCA3' whereas the LDLr2 ASO had the sequence 5'TGGAAGAGAACCATATCCT3'"); for Experiment 2 *see* Li, page 415, right col., bottom paragraph ("part of each secondary reaction was hybridized to either of the two ASOs for that locus"); for Experiment 3, *see* Li, Fig. 1 legend ("dot blot analysis of 20 µl samples of the PCR reaction were carried out using β^S and β^A allele specific probes").

[289] Li *et al.*, *Amplification and analysis of DNA sequences in single human sperm and diploid cells.* Nature. 29;335(6189):414-7 (1988), which forms prior art to the '706 patent under 35 U.S.C. § 102(b) (Exhibit PA-1).

Although a reason to use a wild-type allele as a reference sequence in Zhang's genotyping analysis is not necessarily required for obviousness, reasons to do so would have been known in the art. Both Zhang and Li relate to the genotyping of single haploid cells, as discussed in this request. It would have been *prima facie* obvious to one of ordinary skill to use a wild-type allele as taught by Li as the selected sequence in Zhang's single-cell amplification and analysis methods.

For at least this reason, under the broadest reasonable interpretation Zhang in view of Li would have rendered obvious claim 59.

4. Detailed explanation of the pertinency and manner of applying Zhang and Li to claim 64

Dependent claim 64 recites the method of claim 38, "*wherein the selected genetic sequence and the reference genetic sequence are on distinct chromosomes.*"

Under the broadest reasonable interpretation, Zhang renders claim 64 obvious in view of Li.[290] Zhang anticipates base claim 38, where Li discloses corresponding steps in **Experiment 1** that identified in the overview section, Li "analysed the LDLr genotypes in 80 individual haploid sperm" assay samples[291] and counted the number of samples containing each allele (where each allele is located on one of two "*distinct chromosomes*" as recited in claim 64. Under the broadest reasonable interpretation, any one of these two alleles acts as the "*selected genetic sequence*" whereas the other allele acts as the "*reference genetic sequence*" of claim 64. Both alleles are necessarily located on two

[290] Li *et al.*, *Amplification and analysis of DNA sequences in single human sperm and diploid cells.* Nature. 29;335(6189):414-7 (1988), which forms prior art to the '706 patent under 35 U.S.C. § 102(b) (Exhibit PA-1).

[291] Li, page 415, paragraph bridging left and right cols.

"*distinct chromosomes*" of the same chromosome pair, instead of being located on a single chromosome.

Obviousness: Reasons to Combine

Although a reason to use a LDLr alleles as a selected sequence in Zhang's genotyping analysis is not necessarily required for obviousness, strong reasons to do so would have been known in the art. Both Zhang and Li relate to the genotyping of single haploid cells, as discussed in this request. It would have been *prima facie* obvious to one of ordinary skill to investigate the LDLr alleles as taught by Li as the selected sequence in Zhang's single-cell amplification and analysis methods.

For at least this reason, under the broadest reasonable interpretation Zhang in view of Li would have rendered obvious claim 64.

P. Proposed Rejection No. 16: Zhang renders claim 44 obvious in view of Kalinina under 35 U.S.C. § 103(a)

Dependent claim 44 recites the method of claim 38 "*wherein the step of amplifying and the step of analyzing are performed on assay samples in the same receptacle*."[292] At least under the broadest reasonable interpretation of the claim, Zhang renders claim 44 obvious in view of Kalinina.[293]

As described above in relation to claim 38, Zhang discloses methods corresponding to all of the steps of claim 38, , including amplification of single template molecules in single-sperm samples.

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[292] Under the broadest reasonable interpretation of the claims, Requester proceeds on the premise that claim 44 is narrower than claim 38 in requiring the assay samples to be contained in a (single) receptacle.

[293] Kalinina *et al.*, NAR 25, 1999-2004 (1997)), is prior art to the '706 patent under 35 U.S.C. § 102(b), and is newly cited in this request. (Exhibit PA-4).

Kalinina describes amplification of single-template molecules just as Zhang did, where amplification and analysis are both performed "*in the same receptacle*" as required by claim 44. The analysis method used in Kalinina is the well-known TaqMan® assay, in which dual-labeled TaqMan® probes are included within the amplification reaction mixture during the PCR amplification procedure, and these probes hybridize in real-time to a cognate amplification product as it is being generated.

Kalinina indicates that his methods are analogous to Zhang's in being designed to "detect single starting template molecules."[294] In Kalinina's TaqMan® assay, PCR amplification of a sequence of interest is performed in the presence of a TaqMan® oligonucleotide probe labeled with a fluorescent reporter and a quencher molecule.[295] As PCR amplification progresses, the dual-labeled probe will hybridize to the target sequence and the reporter molecule will be cleaved from the probe by Taq polymerase, resulting in an increase in fluorescence of the reporter.[296] The TaqMan® probe assay has the advantages of being more sensitive than conventional probe assays, and better able to "detect PCR product derived from single template molecules,"[297] such as Zhang's amplification products. Moreover, both the amplification and analysis (*e.g.*, determination of the allelic identity of the PCR product) can be performed in the same receptacle, which format Kalinina teaches can reduce "carry-over contamination."[298]

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[294] Kalinina, Abstract.
[295] *Id.*
[296] *Id.*
[297] Kalinina at page 2003.
[298] Kalinina at page 1999.

Therefore, it would have been obvious to include TaqMan® probes in Zhang's amplification reactions in order to analyze Zhang's amplification products in real time within the same receptacle, for at least the following reasons.

Obviousness: Known Elements and Predictable Result

Under 35 U.S.C. § 103, where a claim "'simply rearranges old elements with each performing the same function it had been known to perform' and yields no more than what one would expect from such an arrangement, the combination is obvious." *KSR Intl. Co. v. Teleflex, Inc.*, 550 U.S. 398, 417 (2007), quoting *Sakraida v. Ag. Pro., Inc.*, 425 U.S. 273, 282 (1976).

Kalinina taught the suitability of TaqMan® probes for use in single-molecule PCR assays such as Zhang's. Both Zhang and Kalinina both amplified and analyzed amplification products from a single template molecule. However, Zhang amplified his single-cell samples in a receptacle and then analyzed the amplification products outside the receptacle. As a result, Zhang's amplification and analysis steps required separate reagents and apparatus. In contrast, Kalinina taught that TaqMan® probes allowed both the amplification and analysis of the amplified products to occur "*in the same receptacle*" as recited in claim 44.

Therefore, it would have been obvious to the skilled person to have used TaqMan® probes as taught by Kalinina when amplifying and analyzing single cells as taught by Zhang. Both Zhang and Kalinina disclose the genetic analysis of very small quantities of starting genetic material, such as a single cell or single template. It would have been obvious to have used TaqMan® probes as taught by Kalinina in a single-cell amplification procedure as taught by Zhang. Kalinina's TaqMan® assays were designed

for exactly the kind of analysis such as Zhang's single-locus (APOC2) analysis that this request is applying against the claims. Analysis of multiple loci within a single cell, also disclosed in Zhang, is not required by the claimed methods, and is thus irrelevant. In any event, it would also have been obvious to perform multi-locus analysis by using Kalinina's TaqMan® assays in a multiplexed format on Zhang's single-cell samples if a skilled worker wished to analyze multiple loci within a single cell.

In addition, the TaqMan® assay, as discussed above, was a well-developed commercial assay with significant advantages over Zhang, including the ability to perform both the amplification and the analysis in a single reaction container or receptacle. The '706 patent acknowledges that TaqMan® probes were commercially available by the priority date of the '706 patent, such that a skilled person would have been able to routinely implement a TaqMan® assay in Zhang's system to obtain predictable results.[299] Thus, claim 44 would have been *prima facie* obvious to one of ordinary skill in the art at the time of invention.

Obviousness: Reasons to Combine

Although a reason to use Kalinina's TaqMan® assay in Zhang's single-cell assay samples is not required, an apparent reason to combine the known elements as claimed may be evidenced by the teachings of the references themselves, issues in the technical area, or the skill in the art. *KSR*, 550 U.S. at 418. Here, strong reasons to combine are directly provided by the references themselves.

[299] '706 patent, col. 7, lines 45-52 ("Although the working examples demonstrate the use of molecular beacon probes as the means of analysis … other techniques can be used as well. These include … hybridization with other types of probes, including TaqManTM (dual-labeled fluorogenic) probes (Perkin Elmer Corp./Applied Biosystems, Foster City, Calif.),").

Kalinina explicitly teaches the many advantages of TaqMan® assays, including that the "assay involves fluorescence measurements that can be performed without opening the PCR tube," and, as a result, "the risk of carry-over contamination is greatly reduced."[300] In particular, Kalinina details use of an improved PCR technique that would eliminate the need for the gel electrophoretic analysis of Zhang altogether by allowing for amplification and analysis in a single tube. It would have been *prima facie* obvious to one of ordinary skill in the art to modify the amplification and analysis steps taught by Zhang to use the TaqMan® single-tube PCR assays described in Kalinina to perform the amplification and detection/analysis of DNA sequences in single human sperm and diploid cells, in a single tube or receptacle with predictable results.

Obviousness: Known Technique to Improve Known Method

KSR and the MPEP provide that where a known technique has been used to improve a base methods ready for improvement, a POSITA would be capable of applying the known improvement to the base method.[301] Both Zhang and Kalinina are directed to the use of methods for molecular analysis of target nucleic acids. The base methods of Zhang used PCR techniques for amplification followed by gel electrophoresis for analysis. Kalinina recognizes that advances in PCR techniques, specifically "Taqman' fluorescence energy transfer assays" provide scientists with the opportunity to perform PCR amplification and analysis in PCR tubes without opening the tube and that "[b]ecause this assay involves fluorescence measurements that can be performed without opening the PCR tube, the risk of carry-over contamination is greatly reduced."[302] It

[300] Kalinina at page 1999.
[301] *See* MPEP at §2143(C).
[302] Kalinina at page 1999.

would have been obvious to one of skill in the art to use the single tube assay techniques in Kalinina to improve the base assays of Zhang with predictable results.

For at least these reasons, the combination of the teachings of Kalinina applied to the teachings of Zhang renders claim 44 obvious.

Q. Proposed Rejection No. 17: Zhang renders claim 52 obvious in view of Chou under 35 U.S.C. § 103(a)

Dependent claim 52 recites the method of claim 38, "*wherein the step of amplifying employs a polymerase which is activated only after heating.*"

Zhang performed the method of base claim 38, using an undisclosed thermostable polymerase.[303] Chou[304] teaches that non-specific amplification and mis-priming during PCR can be avoided or at least reduced by using a "hot start" PCR in which a reagent such as the polymerase is withheld from fluid contact with the rest of the reaction mixture by a layer of solid wax until the reaction tube temperature has reached 60- 80° C.[305] Thus, Chou teaches a step of "*amplifying [which] employs a polymerase which is activated only after heating.*" This is confirmed by the '706 patent, which cites to Chou as teaching a polymerase which is activated only after heating.[306]

Obviousness: Reasons to Combine

It would have been obvious to use Chou's hot-start polymerase in Zhang's amplification reactions.

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[303] Zhang at page 5847, right column. Zhang cites to unpublished data for the PCR amplification conditions for the APOC2 locus. However, no mention of any heat-activated polymerase appears in Zhang, and conditions cited for PCR amplification of other loci (*e.g.*, ref. no. 18 in Zhang only mention Taq polymerase.

[304] Chou *et al.*, Nucleic Acids Res., 20(7): 1717–1723 (April 11, 1992). Chou forms prior art to the '706 patent under 35 U.S.C. § 102(b). (Exhibit PA-5).

[305] Chou, abstract.

[306] '706 patent, col. 10, lines 13-17.

Although a reason to combine Chou's and Zhang's teachings is not required, an apparent reason to combine the known elements as claimed may be evidenced by the teachings of the references themselves, issues in the technical area, or the skill in the art. *KSR*, 550 U.S. at 418. Here, reasons to combine are directly provided by the references themselves. Chou explicitly teaches the many advantages of a hot-start polymerase in PCR amplification reactions. Chou teaches that mispriming and spurious amplification can be reduced by his hot-start polymerase, and such problems are especially prominent in low-copy-number amplifications such as Zhang's (in particular, "PCR amplification of low-copy-number targets is vulnerable to interference by the amplified extension of primer pairs annealed to non-target nucleic acid sequences in the test sample ('mispriming') and by the amplified extension of two primers across one another's sequence without significant intervening sequence ('primer dimerization')."[307] Thus, one of ordinary skill would have had ample reason to use Chou's hot-start polymerase in Zhang's amplifications.

Under the broadest reasonable interpretation, Zhang therefore renders claim 52 obvious in view of Chou.

R. Proposed Rejection No. 18: Zhang renders claims 53-55 obvious in view of Burg under 35 U.S.C. § 103(a)

Dependent claims 53-55 recite the method of claim 38 "*wherein the step of amplifying employs at least*" 40 cycles (claim 53), 50 cycles (claim 54) or 60 cycles

[307] Chou, page 1717, left col., first paragraph.

(claim 55) *"of heating and cooling."* Zhang renders these claims obvious in light of Burg[308] in addition to anticipating base claim 38.

In particular, Zhang anticipates base claim 38, whereas Burg discloses a PCR amplification procedure generates detectable amplification product from a single cell sample, which employs at least 60 cycles of heating and cooling between three temperatures (93°C, 55°C and 72°C).[309]

For at least the reasons below, claims 53-55 would have been rendered obvious over Zhang in view of Burg.

Obviousness: Reasons to Combine

Although a reason is not required to use 60 cycles of heating and cooling as taught by Burg in Zhang's amplifying step, an apparent reason to combine may be evidenced by the teachings of the references themselves, issues in the technical area, or the skill in the art. KSR, 550 U.S. at 418. Here, strong reasons to combine are directly provided by the references themselves.

Similarly, Burg describes the amplification of a target sequence (the B1 gene of *T. gondii*),[310] and observed that increasing the number of PCR cycles from 25 to 60 gave a corresponding increase in sensitivity without any sacrifice in specificity. In particular, 25 cycles of PCR gave a faint band from assay samples containing 100 templates (Fig. 3),

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[308] Burg, *et al.*, "Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction." *J. Clin. Microbiol.* 27, 1787-1792 (1989). Burg is prior art to the '706 patent under 35 U.S.C. § 102(b). (Exhibit PA-6).

[309] Burg, abstract ("We applied the polymerase chain reaction to detection of the pathogenic protozoan Toxoplasma gondii … Using this procedure, we were able to amplify and detect the DNA of a single organism directly from a crude cell lysate"). *See also* page 1788, left col., "Amplification Procedures" ("Samples were … amplified for 25 to 60 cycles in an automated PCR machine (Perkin-Elmer- Cetus, Ericomp, or a machine built in our laboratory). Each cycle consisted of 1 min of denaturation at 93°C, 1 to 2 min at the annealing temperature of 55°C, and 1.5 to 3.0 min of extension at 72°C").

[310] Burg, page 1790, paragraph bridging left and right cols., and Fig. 4, legend.

whereas 55 cycles barely gave detectable amounts of amplification product from assay samples containing 10 template molecules (Fig. 5), but 60 cycles in contrast gave sufficient amount of amplification product from single template molecules (Fig. 4) that was "easily detected" in a "highly reproducible" manner.[311]

It would have been obvious to perform the amplifying step of base claim 38 as taught by Zhang using 60 cycles of amplification as taught by Burg, for at least the following reasons.

Obviousness: Known Technique to Improve Known Method

KSR and the MPEP provides that where a known technique has been used to improve base methods ready for improvement, a POSITA would be capable of applying the known improvement to the base method.[312] Zhang and Burg are both directed to the use of nucleic acid amplification for molecular analysis of target nucleic acids. The base methods of Zhang used PCR amplification followed by analysis of the amplified product.[313] Burg taught that the total amount of amplification product is related to the number of amplification cycles. Therefore, it would have been obvious to increase the number of amplification cycles in Zhang's amplifying step in order to ensure that amplification was reliable and to generate a larger amount of amplification product for subsequent analysis.

Obviousness: Reasons to Combine

Although a reason to use 60 cycles of amplification as taught by Burg in the Zhang's amplifying step is not required, an apparent reason to combine the known

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311 Burg, page 1790, paragraph bridging left and right cols., and Fig. 4, legend.
312 *See* MPEP at §2143(C).
313 Zhang at page 5847, paragraph bridging left and right columns.

elements as claimed may be evidenced by the teachings of the references themselves, issues in the technical area, or the skill in the art. *KSR*, 550 U.S. at 418. Here, strong reasons to combine are directly provided by the references themselves.

Burg teaches that increasing the number of cycles to sixty (60) cycles tends to increase yield of amplification product, such that the amplification product is "easily detected" and "highly reproducible."[314] Thus a skilled person would have been motivated to use 60 cycles of amplification in Zhang's methods to ensure maximal yield. Moreover, increasing the number of amplification cycles to sixty cycles as taught by Burg would have been readily performed by a POSITA, and the expected effects of such an increase, *i.e.* an increase in reliability of the amplification and the amount of amplified product, would have been well-known to a skilled person.

Therefore, Zhang renders claims 53-55 obvious in view of Burg.

S. Proposed Rejection No. 19: Zhang renders claim 56 obvious in view of Trumper under 35 U.S.C. § 103(a)

Dependent claim 56 recites the method of claim 38, "*wherein the biological sample is selected from the group consisting of stool, blood, and lymph nodes.*"

Under the broadest reasonable interpretation, Zhang renders claim 56 obvious in view of Trumper.[315] Zhang anticipates base claim 38, whereas Trumper used single-cell amplification and analysis on cells from a lymph node sample as specified by dependent claim 56.

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[314] Burg, page 1790, paragraph bridging left and right cols., and Fig. 4, legend.

[315] Trumper *et al.*, *Single-Cell Analysis of Hodgkin and Reed-Sternberg Cells: Molecular Heterogeneity of Gene Expression and p53 Mutations*, Blood, 81: 3097-3115 (1993), forming prior art to the '706 patent under 35 U.S.C. § 102(b). (Exhibit PA-7).

In particular, Zhang anticipates claim 38 by performing single-cell amplification on individual cells ("assay samples") and determining a ratio by comparing a first number of assay samples containing a selected sequence and a second number of assay samples containing a reference sequence.

Like Zhang, Trumper also performed amplification of template molecules derived from a single cell using steps corresponding to those found in claim 38. Specifically, Trumper isolated single Hodgkin and Reed-Sternberg (H&RS) cells[316] and subjected a plurality of individual cells from a single patient to single-cell RT-PCR,[317] thereby performing the "*amplifying*" step of claim 38. Trumper also determined a first "*number of assay samples which contain the selected genetic sequence*" in the form of any one of 22 different genes of interest, and a second "*number of assay samples which contain a reference genetic sequence*" in the form of a housekeeping gene[318] thereby performing the "*analyzing*" step of claim 38. Trumper also presented a detailed numerical analysis of the results, although not in Zhang's exact format.[319]

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[316] Trumper, page 3098, Section titled "Preparation of HD Lymph Nodes," paragraph bridging pages 3098-3099, indicating that cells were first diluted in "phosphate-buffered saline" and then "single cells were drawn into a glass micropipette."

[317] Trumper, page 3099, right col., last paragraph, describing the generation of cDNA by reverse transciption, and page 3100, second paragraph, indicating that "the tailed cDNA" was used as template for PCR amplification.

[318] Trumper, Tables 4-7, each summarizing the RT-PCR results from different patients, where single cells were taken from each patient and subjects to RT-PCR, and the amplified molecules were analyzed to determine both presence and amount of a reference housekeeping gene (*e.g.*, actin) and any one of 22 genes of interest, including c-myc. Any single one of Trumper's genes of interest constitutes a "selected sequence" while any single one of Trumper's housekeeping genes constitutes a "reference" sequence.

[319] Trumper, page 3104, right col. and page 418, left col.

Finally, Trumper meets the added requirements of claim 56 by isolating single cells from a biological sample in the form of "*lymph node*," as recited in claim 56.[320]

Obviousness: Reasons to Combine

Although a reason to perform Zhang's single-cell genotyping analysis on Trumper's lymph node samples is not necessarily required, strong reasons to combine are would have been known in the art. In addition to haploid sperm cells, Zhang stated the importance of genotyping single diploid cells (*e.g.*, "single cells from early embryos or polar bodies"),[321] such as those found in lymph node samples. Trumper did precisely as Zhang suggested by studying cell-to-cell variations in expression of cancer cells from lymph node samples. It would have been *prima facie* obvious to one of ordinary skill to use Zhang's single-cell amplification and analysis methods on clinically relevant biological samples such as lymph nodes in blood-cancer patients as taught by Trumper.

Thus, claim 56 would have been *prima facie* obvious at the time of invention.

T. Proposed Rejection No. 20: Zhang renders claims 57 obvious in view of Kanzler under 35 U.S.C. § 103(a)

Dependent claim 57 recites the method of claim 38 "*wherein the biological sample is blood or bone marrow of a leukemia or lymphoma patient who has received anti-cancer therapy.*"

[320] Trumper, page 3098, indicating that a "single-cell suspensions were prepared from fresh, HD-implicated lymph nodes" and "[i]ndividual H&RS cells were identified" and samples "containing a single cell" was transferred to a tube.

[321] Zhang at page 5847, left column.

Under the broadest reasonable interpretation, Zhang renders claim 57 obvious in view of Kanzler.[322] Zhang anticipates base claim 38, whereas Kanzler used single-cell amplification and analysis on the types of biological samples that are specified by dependent claim 57.

In particular, Zhang anticipates claim 38 by performing single-cell amplification on individual cells ("assay samples") and determining a ratio by comparing a first number of assay samples containing a selected sequence and a second number of assay samples containing a reference sequence.

Like Zhang, Kanzler also performed amplification of template molecules derived from a single cell using steps corresponding to those found in claim 38. Specifically, Kanzler isolated single malignant B cells designated Hodgkin/Reed-Sternberg cells from a Hodgkin's lymphoma patient[323] and subjected the individual cells to single-cell PCR,[324] thereby performing the "*amplifying*" step of claim 38. Kanzler also determined a first and second "*number of assay samples which contain the selected genetic sequence and ... a selected genetic sequence*" in the form of rearranged V_H3 and $V_\kappa3$ sequences already found in a related cell line L1236,[325] thereby performing the "*analyzing*" step of claim 38.

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[322] Kanzler *et al.*, *Molecular Single Cell Analysis Demonstrates the Derivation of Peripheral Blood-Derived Cell Line (L1236) From the Hodgkin/Reed-Sternberg Cells of a Hodgkin's Lymphoma Patient*, Blood, 87: 3429-3436 (1996), forming prior art to the '706 patent under 35 U.S.C. § 102(b).

[323] Kanzler, page 3429, right col., third paragraph, indicating that "Single cells were isolated from frozen sections of a bone marrow specimen of an HD patient by micromanipulation as previously described" by Kuppers *et al.* (Exhibit 6). Kuppers in turn explains that cell "sections were incubated with 5 mg/ml collagenase H (Boehringer, Mannheim) in PBS" buffer before micromanipulation, and "aspirated" with a micropipette and transferred to a tube with buffer, thereby indicating that the biological sample was diluted in the process of making single-cell assay samples.

[324] Kanzler, page 3429, right col., third & fourth paragraphs, referring to " single cell PCR" of 10 H-RS cells.

[325] Kanzler, abstract.

Kanzler also analyzed the numbers of cells carrying the selected sequence of interest to determine information about the composition of the biological sample.[326]

In addition, Kanzler meets the added requirements of claim 57. Kanzler used a biological sample in the form of "*bone marrow... of a ... lymphoma patient*"[327] as recited in claim 57. The patient in question had earlier "*received anti-cancer therapy*"[328] as also recited in claim 57.

Obviousness: Reasons to Combine

Although a reason to use Zhang's single-cell genotyping analysis on Kanzler's lymphoma blood samples is not necessarily required, strong reasons to combine would have been known in the art. In addition to haploid sperm cells, Zhang stated the importance of genotyping single diploid cells (*e.g.*, "single cells from early embryos or polar bodies"),[329] such as those found in bone marrow samples. Kanzler did precisely as Zhang suggested by studying DNA rearrangements in diploid cells, where prevalence of DNA rearrangements and other genetic alterations is well recognized. It would have been *prima facie* obvious to one of ordinary skill to use Zhang's single-cell amplification

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[326] Kanzler, page 3432, left col., second paragraph, noting that "amplificates obtained from the H-RS and B cells showed sequences identical to the V_H3 and $V_\kappa3$ rearrangements of cell line L1236," and page 3434, right col., third paragraph, noting that the "amplification of at least one of three V gene rearrangements carried by the cell line from 11 of 20 H-RS cells … demonstrate that H-RS cells in this patient represent a clonal population."

[327] Kanzler, page 3429, right col., third paragraph, indicating that "Single cells were isolated from frozen sections of a bone marrow specimen of an HD patient."

[328] Kanzler, page 3429, bottom paragraph, stating that the bone marrow sample used in the study was obtained in April 1994, and citing to an "accompanying article by Wolf *et al.*" for further details on the history of the same patient. The Wolf article (Exhibit 7) indicates that the same patient was treated with radiotherapy in 1991 and with chemotherapy in 1993, before Kanzler obtained his blood marrow sample in 1994. Wolf, Exhibit 7, paragraph bridging pages 3418-3419.

[329] Zhang at page 5847, left column.

and analysis methods on clinically relevant biological samples such as lymph nodes from a treated lymphoma patient as taught by Kanzler.

Thus, claim 57 would have been *prima facie* obvious over Zhang in view of Kanzler.

U. Proposed Rejection No. 21: Zhang renders claim 58 obvious in view of Gravel under 35 U.S.C. § 103(a)

Dependent claim 58 recites the method of claim 38, "*wherein the selected genetic sequence is a translocated allele.*"

Under the broadest reasonable interpretation, Zhang renders claim 58 obvious in view of Gravel.[330] Zhang anticipates base claim 38, whereas Gravel used single-cell amplification and analysis using a translocated allele[331] as his selected sequence.

In particular, Zhang anticipates claim 38 by performing single-cell amplification on individual cells ("assay samples") and determining a ratio by comparing a first number of assay samples containing a selected sequence and a second number of assay samples containing a reference sequence.

Like Zhang, Gravel also performed amplification of template molecules derived from a single cell using steps corresponding to those found in claim 38. Specifically, Gravel isolated single malignant B cells designated Hodgkin/Reed-Sternberg cells from a

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[330] Gravel *et al.*, *Single-cell analysis of the t(14;18)(q32;q21) chromosomal translocation in Hodgkin's disease demonstrates the absence of this translocation in neoplastic Hodgkin and Reed-Sternberg cells.* Blood. 91(8):2866-74 (Apr 15, 1998), forming prior art to the '706 patent under 35 U.S.C. § 102(b).

[331] In particular, Gravel used a translocated allele in the form of a t(14;18)(q32;q21) translocation, which Gravel also referred to as the" bcl-2/JH rearrangement" since the translocation placed "the bcl-2 gene of the 18q21 chromosomal region under the transcriptional control of the Ig heavy chain gene (IgH) region." Gravel, abstract and page 2866, left col.

Hodgkin's lymphoma patient[332] and subjected the individual cells to single-cell PCR,[333] thereby performing the "amplifying" step of claim 38. Gravel also determined a "*first number of assay samples*" containing a selected sequence in the form of a t(14;18) translocation sequence, and a "*second number of assay samples*" containing a reference sequence in the form of the c-raf-1 gene,[334] thereby performing the "analyzing" step of claim 38. Gravel also explicitly compared the first and second numbers of assay samples to determine information about the composition of the biological sample.[335]

In addition, Gravel meets the added requirements of claim 58 by using "*a translocated allele*" in the form of a t(14;18)(q32;q21) translocation[336] as his "*selected genetic sequence.*"

Obviousness: Reasons to Combine

Although a reason to use a translocated allele as a selected sequence in Zhang's genotyping analysis is not necessarily required for obviousness, strong reasons to do so would have been known in the art. In addition to the demonstrated haploid sperm cells, Zhang stated the importance of genotyping single diploid cells (*e.g.*, "single cells from early embryos or polar bodies"),[337] such as those used by Gravel. Gravel did precisely as Zhang suggested by studying cell-to-cell variations in expression of cancer cells. Thus, Gravel did precisely as Zhang suggested by studying DNA translocation rearrangements

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[332] Gravel, page 2867, right col., second paragraph, indicating that "stained sections were overlaid with phosphate buffered saline (PBS)" and "[s]ingle cells were picked up … and then transferred by aspiration," thereby diluting the biological sample to form a set comprising a plurality of assay samples.

[333] Gravel, page 2869, right col., bottom paragraph.

[334] Gravel, page 2869, right col., bottom paragraph.

[335] Gravel, page 2869, right col., bottom paragraph

[336] In particular, Gravel used a translocated allele in the form of a t(14;18)(q32;q21) translocation. Gravel, abstract and page 2866, left col.

[337] Zhang at page 5847, left column.

in cancer cells, where prevalence of DNA rearrangements and other genetic alterations is well recognized. It would have been *prima facie* obvious to one of ordinary skill to use a translocated allele as taught by Gravel as the selected sequence in Zhang's single-cell amplification and analysis methods.

Thus, claim 58 would have been *prima facie* obvious at the time of invention over Zhang in view of Gravel.

V. Proposed Rejection No. 22: Zhang renders claims 60 & 61 obvious in view of Marcucci under 35 U.S.C. § 103(a)

1. Detailed explanation of the pertinency and manner of applying Zhang and Marcucci to claim 60

Dependent claim 60 recites the method of claim 38, "*wherein the selected genetic sequence is within an amplicon which is amplified during neoplastic development.*"

Under the broadest reasonable interpretation, Zhang renders claim 60 obvious in view of Marcucci.[338] Zhang anticipates base claim 38, whereas Marcucci analyzed biological cancer samples for the presence of "*an amplicon which is amplified during neoplastic development*" as recited in claim 60.

In particular, Zhang anticipates claim 38 by performing single-cell amplification on individual cells in separate wells ("assay samples") and determining a ratio by comparing a first number of assay samples containing a selected sequence and a second number of assay samples containing a reference sequence.

In addition, Marcucci analyzed biological samples by PCR to determine the presence of a "*selected genetic sequence*" in the form of an "*ALL1* [gene] rearrangement"

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[338] Marcucci *et al.*, *Detection of Unique ALLÃ•(MLL) Fusion Transcripts in Normal Human Bone Marrow and Blood: Distinct Origin of Normal versus Leukemic ALL1 Fusion Transcripts.* Cancer Res, 58:790-793. (February 15, 1998), forming prior art under 35 U.S.C. § 102(b).

which is expressed as an aberrant mRNA in the form of an "*ALL1* fusion transcript."[339] It had been previously discovered that this rearrangement involves a "partial tandem duplication (PTD)" of a portion of the *ALL1* gene, and is associated with leukemia.[340] As explained in detail below, such a duplication is "*an amplicon which is amplified during neoplastic development*" as recited by claim 60, under the broadest reasonable interpretation.

More specifically, Marcucci's goal was to "determine if the *ALL1* fusion transcript is specific for leukemic blasts or instead can be found with any frequency in normal cells."[341] Upon finding by RT-PCR analysis on RNA samples that 10 of 60 apparently normal individuals appeared to express a fusion mRNA transcript, Marcucci followed up with a genomic PCR analysis on the genomic DNA of eight such individuals to check for the presence of an actual rearrangement of the *ALL1* gene as a PTD amplicon.[342] In his genomic PCR assay, Marcucci used "primers specific for the exons involved in the fusion"[343] to check for the PTD amplicon as his "*selected genetic sequence*." In the same assay, Marcucci also amplified a β-actin gene[344] as a "*reference genetic sequence*." Marcucci found that in none of the eight normal samples could he amplify the PTD

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[339] Marcucci, Abstract.

[340] Marcucci, Abstract, noting that the "partial tandem duplication (PTD) of ALLÃ• (MLL) is one of the more common molecular abnormalities in adult *de novo* acute myeloid leukemia (AML) and carries a poor prognosis."

[341] Marcucci, Abstract.

[342] Marcucci, Abstract ("we analyzed … [RNA] samples from 60 normal donors by nested RT-PCR. Ten of 60 samples … contained a unique transcript showing a fusion of two *ALL1* exons that was consistent with the PTD of *ALL1*. However, a corresponding genomic rearrangement or a unique genomic fusion of *ALL1* could not be demonstrated by Southern analysis or DNA PCR, respectively.") *See also* page 791, left col., bottom paragraph ("[RNA] samples from 60 healthy normal donors were analyzed for the PTD of *ALL1* by nested RT-PCR. Ten of 60 samples … amplified a transcript showing a unique fusion of two *ALL1* exons.")

[343] Marcucci, page 791, left col., "DNA Analysis" Section, first paragraph.

[344] Marcucci, page 791, left col., "DNA Analysis" Section, first paragraph ("DNA integrity was demonstrated by successful β-actin amplification in each sample.")

amplicon at the genomic level, despite the apparent expression of a fusion transcript at the mRNA level.[345] In particular, "[a]ll eight normal donor samples that were positive by RT-PCR failed to show a genomic fusion" by genomic PCR, although "[i]ntegrity of the DNA for all the samples was verified by the [successful] amplification of the β-actin gene."[346] Thus, Marcucci compared the number of samples containing his "*selected genetic sequence*" and his "*reference genetic sequence*."

In addition, Marcucci meets all additional limitations recited in the body of claim 60 itself. First, Marcucci's partial tandem duplication (PTD) of the *ALL1* gene sequence is an "*amplicon*" as recited in claim 60 under the broadest reasonable interpretation, at least because (1) the art recognized duplication of any particular portion of a chromosome as a type of genetic amplification,[347] (2) claim 60 does not limit the scope of "amplicon" in any way, and (3) the '706 specification does not give "amplicon" a special definition contrary to the art.[348]

Second, Marcucci's PTD amplicon is "*amplified during neoplastic development,*" as required by claim 60. In particular, Marcucci explains that his PTD amplicon "is one

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[345] Marcucci, Abstract ("Ten of 60 [RNA] samples … contained a unique [*ALL1* fusion] transcript … that was consistent with the PTD of *ALL1*. However, a corresponding … unique genomic fusion of *ALL1* could not be demonstrated by … DNA PCR"). *See also* page 791, left col., "DNA Analysis" Section, first paragraph ("no evidence of fusion at the genomic level could be found following DNA PCR amplification across the putative introns involved in the unique fusion").

[346] Marcucci, Fig. 4, legend.

[347] Mandahl *et al.*, Int. J. Cancer: 67,632-635 (1996), (Exhibit 8), at Abstract (explaining that genetic "duplication of 12q sequences may be a sufficient level of amplification" to cause cancerous change in cells), and at page 633, right col., second paragraph, last sentence, describing " low-level amplification, resulting in 1.5 to 2.5 times the normal copy number").

[348] The '706 patent only uses amplicon once in the specification, and that is in Table 1, where "Gene amplifications" is listed as a potential application of digital PCR, of which one non-limiting example is to "Determine presence or extent of amplification" using a first probe to a "sequence within [an] amplicon" and a second probe to a "sequence from another part of [the] same chromosome arm." As mentioned, the '706 patent indicates that this is a non-limiting example of analyzing gene amplifications using the claimed methods.

of the more common molecular abnormalities in adult de novo acute myeloid leukemia (AML) and carries a poor prognosis."[349]

Obviousness: Reasons to Combine

It would have been *prima facie* obvious to one of ordinary skill to use a "*selected genetic sequence ... within an amplicon which is amplified during neoplastic development*" in the form of a PTD sequence in the ALL1 oncogene, as taught by Marcucci, in Zhang's single-cell amplification and analysis methods. Marcucci amplified a "*selected genetic sequence*" (*i.e.*, a PTD amplicon) and a "*reference genetic sequence*" (*i.e.*, β-actin gene) directly on his biological cell sample instead of dividing his biological sample into a plurality of single-cell assay samples as Zhang did. However, Zhang explicitly taught the benefits of using his single-cell format to study differences between individual cells due to "cell-to-cell variation in developmental processes involving DNA rearrangements,"[350] which bulk amplification is incapable of assessing. Marcucci teaches that his PTD amplicon sequence is precisely the kind of "rearrangement" that Zhang recognized as a suitable target – specifically, Marcucci noted that the "PTD of ALL1 is identified in leukemic blasts at the genomic level by ALL1 rearrangement upon Southern analysis."[351] Marcucci's specific goal was to "determine if the ALL1 fusion transcript is specific for leukemic blasts or instead can be found with any frequency in normal cells."[352] Marcucci designed his PCR assays to be sensitive enough to detect the PTD amplicon "when it is present in 1% of the cells in the processed sample,"[353] because his

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[349] Marcucci, Abstract, first sentence.
[350] Li, page 417, right col., third paragraph.
[351] Marcucci, Abstract.
[352] Marcucci, Abstract.
[353] Marcucci, page 791, left col., second paragraph.

biological samples (*i.e.*, lymph node biopsies) would normally contain at least some normal cells as well as leukemic cells, potentially obfuscating his data. This problem would have been resolved byZhang's single-cell amplification format.

It would thus have been *prima facie* obvious to one of ordinary skill to used Marcucci's "*selected genetic sequence*" (*i.e.*, a PTD amplicon) and his "*reference genetic sequence*" (*i.e.*, β-actin gene), in Zhang's single-cell amplification and analysis methods.

2. Detailed explanation of the pertinency and manner of applying Zhang and Marcucci to claim 61

Dependent claim 61 recites the method of claim 38, "*wherein the selected genetic sequence is a rare exon sequence.*"

Under the broadest reasonable interpretation, Zhang renders claim 61 obvious in view of Marcucci.[354] Zhang anticipates base claim 38, whereas Marcucci analyzed biological cancer samples for the presence of a "*selected genetic sequence [that] is a rare exon sequence*" as recited by claim 61.

In particular, Zhang anticipates claim 38 by performing single-cell amplification on individual cells in separate wells ("assay samples") and determining a ratio by comparing a first number of assay samples containing a selected sequence and a second number of assay samples containing a reference sequence.

In addition, Marcucci analyzed single cells from B-lymphoma patients containing an *ALL1* gene rearrangement by RT-PCR, in order to study cell-to-cell expression of the

[354] Marcucci *et al.*, *Detection of Unique ALLÃ•(MLL) Fusion Transcripts in Normal Human Bone Marrow and Blood: Distinct Origin of Normal versus Leukemic ALL1 Fusion Transcripts.* Cancer Res, 58:790-793. (February 15, 1998), forming prior art under 35 U.S.C. § 102(b).

unique *ALL1* mRNA "fusion transcript"[355] encoded by the rearranged gene, which as discussed below is a "*rare exon sequence*." Marcucci's goal was to "determine if the *ALL1* fusion transcript is specific for leukemic blasts or instead can be found with any frequency in normal cells."[356] Marcucci detected an *ALL1* fusion transcript in the RNA of 10 out of 60 apparently normal individuals by RT-PCR.[357] Marcucci ensured that his RT-PCR assay would selectively amplify only *ALL1* fusion transcripts instead of normal *ALL1* mRNA by using a primer pair specific for the fusion.[358] Marcucci also ensured that each RNA sample was amplifiable by amplifying the β-actin gene as a reference control ("*reference genetic sequence*") in each sample.[359] Despite demonstrating amplification of the actin reference in each sample, Marcucci found that only "[t]en of 60 [RNA] samples … amplified a transcript showing a unique fusion of two *ALL1* exons,"[360] Thus, Marcucci compared the number of samples containing the *ALL1* fusion transcript (a "*selected genetic sequence*") with the number of samples containing the actin transcript (a "*reference genetic sequence*") although the comparison was not explicitly presented as a ratio.

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[355] Marcucci, Abstract.

[356] Marcucci, Abstract.

[357] Marcucci, Abstract ("we analyzed … [RNA] samples from 60 normal donors by nested RT-PCR. Ten of 60 samples … contained a unique transcript showing a fusion of two *ALL1* exons that was consistent with the PTD of *ALL1*.") *See also* page 791, left col., bottom paragraph ("[RNA] samples from 60 healthy normal donors were analyzed for the PTD of *ALL1* by nested RT-PCR. Ten of 60 samples … amplified a transcript showing a unique fusion of two *ALL1* exons.")

[358] Marcucci, Fig. 2, providing a "schematic illustration of … unique exon fusion transcripts detected by nested RT-PCR" and also showing the positions of the "primers that amplify the PTD of ALL1," so that "[e] ach transcript is consistent with a PTD of the *ALL1* gene."

[359] Marcucci, page 790, right col., Section on "RT-PCR Analysis," explaining that "each RNA sample was also amplified for β-actin transcript. *See also* page 792, left col., "Poly(A)+ RNA Analysis" section (Each poly(A)+ RNA sample was successfully amplified for the β-actin transcript.)" *See also* page 792, right col., top paragraph ("Integrity of the RNA was verified by successful amplification of the β-actin transcript.")

[360] Marcucci, page 791, left col., last sentence.

In addition, Marcucci meets the added requirements of claim 61 in that his "*selected genetic sequence is a rare exon sequence*." Marcucci's "*selected genetic sequence*" in the form of an *ALL1* fusion transcript is "a unique transcript showing a fusion of two *ALL1* exons."[361] In Fig. 3B, Marcucci also provided an exemplary sequence one of his observed "fusion transcripts in which the 3' exon involved in the fusion is spliced, not at the consensus spliced site of the 5' exon but rather in the middle of the sequence, resulting in a frameshift of the ORF."[362] Because Marcucci's *ALL1* fusion transcript contains an aberrant fusion exon that is not found in most normal individuals, it is a "*rare exon sequence*" as recited in claim 61 under the broadest reasonable interpretation. Claim 61 does not limit the scope of "*rare exon*" in any way, and the '706 specification does not give "*rare exon*" a special definition contrary to the art.[363] Thus, Marcucci's fusion transcript is a "*selected genetic sequence [which] is a rare exon sequence*" as required by claim 61.

Obviousness: Reasons to Combine

It would have been *prima facie* obvious to one of ordinary skill to use a "*selected genetic sequence ... within an amplicon which is amplified during neoplastic development"* in the form of a PTD sequence in the ALL1 oncogene, as taught by Marcucci, in Zhang's single-cell amplification and analysis methods. Marcucci amplified a "*selected genetic sequence*" (*i.e.*, a PTD amplicon) and a "*reference genetic sequence*"

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[361] Marcucci, Abstract.

[362] Marcucci, Fig 3B, legend.

[363] The '706 patent only uses amplicon once in the specification, and that is in Table 1, where "Gene amplifications" is listed as a potential application of digital PCR, of which one non-limiting example is to "Determine presence or extent of amplification" using a first probe to a "sequence within [an] amplicon" and a second probe to a "sequence from another part of [the] same chromosome arm." As mentioned, the '706 patent indicates that this is a non-limiting example of analyzing gene amplifications using the claimed methods.

(*i.e.*, β-actin gene) directly on his biological cell sample instead of dividing his biological sample into a plurality of single-cell assay samples as Zhang did. However, Zhang explicitly taught the benefits of using his single-cell format to study differences between individual cells due to "cell-to-cell variation in developmental processes involving DNA rearrangements,"[364] which a bulk amplification procedure like Marcucci's is incapable of detecting. Marcucci teaches that his PTD amplicon sequence is precisely the kind of "rearrangement" that Zhang recognized as a suitable target – specifically, Marcucci noted that the "PTD of ALL1 is identified in leukemic blasts at the genomic level by ALL1 *rearrangement*."[365] Marcucci's specific goal was to "determine if the ALL1 fusion transcript is specific for leukemic blasts or instead can be found with any frequency in normal cells."[366] Marcucci designed his PCR assays to be sensitive enough to detect the PTD amplicon "when it is present in 1% of the cells in the processed sample,"[367] because his biological samples (*i.e.*, lymph node and blood samples) would normally contain both normal and leukemic cells, potentially obfuscating his data. This problem would have been addressed by Zhang's single-cell PCR format.

It would thus have been *prima facie* obvious to one of ordinary skill to used Marcucci's "*selected genetic sequence*" (*i.e.*, a PTD amplicon) and his "*reference genetic sequence*" (*i.e.*, β-actin gene), in Zhang's single-cell amplification and analysis methods.

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[364] Li, page 417, right col., third paragraph.
[365] Marcucci, Abstract.
[366] Marcucci, Abstract.
[367] Marcucci, page 791, left col., second paragraph.

W. Proposed Rejection No. 23: Zhang renders claims 62 obvious in view of Flint under 35 U.S.C. § 103(a)

Dependent claim 62 recites the method of claim 38, "*wherein the nucleic acid template molecules comprise cDNA of RNA transcripts and the selected genetic sequence is present on a cDNA of a first transcript and the reference genetic sequence is present on a cDNA of a second transcript.*"

Under the broadest reasonable interpretation, Zhang renders claim 62 obvious in view of Flint.[368] Zhang anticipates base claim 38, whereas Flint used single-cell RT-PCR in order to amplify a selected and reference transcript as recited by claim 62.

In particular, Zhang anticipates claim 38 by performing single-cell amplification on individual cells in separate wells ("assay samples") and determining a ratio by comparing a first number of assay samples containing a selected sequence and a second number of assay samples containing a reference sequence.

In addition, Flint performed all steps of claims 1 and 38. Flint started by "*diluting nucleic acid template molecules in a biological sample to form a set comprising a plurality of assay samples*," as recited in claim 1. Flint took a slice of "rat somatosensory cortex at postnatal ages P3/4 or P8/9" (*i.e.*, a "*biological sample*")[369] and extracted the cytoplasmic contents (including mRNA) of individual neurons and transferred the contents of each individual neuron to a PCR reaction tube containing PCR reaction

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[368] Flint *et al. NR2A Subunit Expression Shortens NMDA Receptor Synaptic Currents in Developing Neocortex.* J. Neurosci., 17(7):2469–2476 (April 1, 1997), forming prior art to the '706 patent under 35 U.S.C. § 102(b).

[369] Flint, page 2470, right col., last paragraph ("Whole-cell recordings were made in slices of rat somatosensory cortex at postnatal ages P3/4 or P8/9… After characterization of intrinsic firing pattern and NMDAR EPSCs (Fig. 1E,F), cytoplasmic harvest was performed").

mixture,[370] thereby "*diluting nucleic acid template molecules ... to form a set comprising a plurality of assay samples*" from the biological sample.

Flint then "*amplified the template molecules*" in each single-cell mRNA assay sample, where the template molecules were in the form of NR2 molecules by RT-PCR, in which "[r]everse-transcription … was followed by a PCR designed to coamplify all four NR2 subunits."[371]

Flint then "*analyzed the amplified molecules*"[372] to determine a "*number of assay samples*" which originally contained mRNA of each different NR2 subunit, as well as the levels of each subunit. In particular, Flint dotted his PCR products onto a membrane by a dot-blot apparatus and hybridized the blotted product with "radiolabeled NR2 subunit-specific oligonucleotide probes."[373]

From this analysis, Flint determined a "*first number of assay samples*" containing a "*selected genetic sequence*" in the form of NR2A mRNA, and a "*second number of assay samples*" containing a "*reference genetic sequence*" in the form of NR2B mRNA,

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[370] Flint, page 2470, right col., last paragraph ("After characterization of [neuron cell behavior] … cytoplasmic harvest was performed" followed by RT-PCR on "single-cell mRNA"). *See also* page 2471, Fig. 1 legend, showing amplification "products obtained by RT-PCR for NR2A–D subunits on cytoplasmic material harvested from physiologically characterized neurons.")

[371] Flint, page 2470, right col., last paragraph. *See also* same page, left col., last paragraph ("Coamplification of NR2A–D subunits was performed by nested hot-start PCR" using a single pair of primers in a primary PCR reaction).

[372] Flint, page 2470, paragraph bridging left and right cols., (samples were analyzed by first re-amplifying one "microliter of the first-round PCR product … in a second PCR" with a single pair of hemi-nested primers, and performing "dot-blot hybridization" in which "PCR products of expected size … were … extracted… [and a] serial dilution of each PCR product was dotted onto four different nitrocellulose membranes, each containing … one of the four "NR2 standards" … obtained by cloning PCR fragments (244 bp) of each NR2 subunit ").

[373] Flint, page 2470, right col., "Ratiometric analysis" section ("PCR products and standards were denatured with NaOH, neutralized with NH4Ac, and dotted in triplicate for each concentration point by a dot-blot apparatus … The membranes were hybridized with radiolabeled NR2 subunit-specific oligonucleotide probes").

subsequently "*comparing*" these two numbers to "*ascertain a ratio which reflects the composition of the biological sample*." As discussed earlier, a direct explicit numerical comparison between the first and second number constitutes "*comparing the first number to the second number to ascertain a ratio*" under the broadest reasonable interpretation, even without any explicit mention of a "ratio" between the two numbers. Flint made such direct and explicit numerical comparisons, thereby "*ascertain[ing] a ratio*." In particular, Flint provided bar graphs of the number of assay samples containing each NR2 subunit in the P8/9 age group in Fig. 4, and remarked that "Two groups were apparent at P8/9 on the basis of NR2A expression. B1, A majority of cells (n = 12) expressed NR2A along with NR2B. B2, Eight of 20 cells expressed no significant NR2A and expressed high relative levels of NR2B."[374] Flint concluded that "one group [of assay samples] at P8/9 [postnatal age] expressed NR2A along with NR2B (n = 12 of 20, Fig. 4B1), and another had background levels of NR2A expression with high NR2B expression (n = 8 of 20, Fig. 4B2)."[375] In short, Flint determined that all 20 single-cell mRNA assay samples contained NR2B mRNA while "12 of [those] 20" samples also contained NR2A mRNA, and "8 of 20" did not.[376]

Finally, the ratio of the two numbers "*reflect[ed] the composition of the biological sample*," at least because the two groups of cells differed in their NR2 mRNA compositional makeup. Accordingly, Flint performed all steps of claim 1.

Flint also meets the added requirements of claim 38, as follows:

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[374] Flint, Fig. 4, legend.
[375] Flint, page 2472, right col.,, last paragraph.
[376] *Id.*

- Flint "*form[ed] a population of amplified molecules in each of the assay samples of the set*," as required by claim 38. As mentioned, Flint detected amplification products of NR2B in all 20 assay samples
- Flint also found that "*at least one-fiftieth of the assay samples in the set comprise a number (N) of molecules such that 1/N is larger than the ratio of selected genetic sequences to total genetic sequences required to determine the presence of the selected genetic sequence.*" Flint detected his "*selected sequence*" in the form of NR2A in 12 of 20 assay sample, which is more than one-fiftieth of his assay samples.

In addition, Flint meets the added requirements of claim 62:

- Because Flint performed "reverse-transcription of single-cell mRNA … followed by PCR,"[377] Flint used "*nucleic acid template molecules compris[ing] cDNA of RNA transcripts.*"
- In addition, Flint's "*selected genetic sequence is present on a cDNA of a first transcript*" whereas the "*reference genetic sequence is present on a cDNA of a second transcript.*" As discussed, Flint assayed for cDNA of NR2A as his "*selected genetic sequence,*" and for cDNA of NR2B as his "*reference genetic sequence.*"[378]

Obviousness: Reasons to Combine

It would have been obvious to use Flint's single-cell RT-PCR analysis in which two different cDNAs are used as the "*selected genetic sequence*" and "*reference genetic sequence*," in Zhang's own single-cell methods of genotyping. Zhang's single-sperm PCR

[377] Flint, page 2470, right col., last paragraph.
[378] Flint, page 2472, right col., section on "Single-cell expression of NR2 subunit mRNA."

needed to be extremely sensitive in order to detect single template molecules, thus being correspondingly prone to contamination and artifacts. In contrast, single-cell samples generally have multiple mRNA transcripts of each gene, making it easier to genotype cells by presence or absence of mRNA transcripts of a gene of interest (in Zhang's case, APOC2). Moreover, Flint teaches the usefulness of single-cell RT-PCR, which has "the advantage of directly linking expression of … mRNA in normal cells with the function of receptors constructed from … these mRNAs."[379]. It would have been *prima facie* obvious to one of ordinary skill to use Zhang's methods in an RT-PCR format in order to study such correlations, using a first cDNA as a "*selected genetic sequence*" of interest, and a second cDNA as a "*reference genetic sequence.*"

Thus, claim 62 would have been *prima facie* obvious at the time of invention over Zhang in view of Flint.

X. Proposed Rejection No. 24: Zhang renders claims 63 obvious in view of Ponten under 35 U.S.C. § 103(a)

Dependent claim 63 recites the method of claim 38, "*wherein the selected genetic sequence comprises a first mutation and the reference genetic sequence comprises a second mutation.*"

Under the broadest reasonable interpretation, Zhang renders claim 63 obvious in view of Ponten.[380] Zhang anticipates base claim 38, whereas Ponten used single-cell RT-PCR in order to amplify a selected and reference sequence each comprising a different mutation as recited by claim 63.

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[379] Flint, page 2470, left col., first paragraph.

[380] Ponten *et al.*, *Genomic analysis of single cells from human basal cell cancer using laser-assisted capture microscopy*. Mutation Research Genomics 382, 45–55 (1997).

In particular, Zhang anticipates claim 38 by performing single-cell amplification on individual cells in separate wells ("assay samples") and determining a ratio by comparing a first number of assay samples containing a selected sequence and a second number of assay samples containing a reference sequence.

In addition, Ponten performed single-cell amplification, a method explicitly suggested by Zhang, in order to perform "mutational analysis of genomic DNA … on single somatic cells."[381] Specifically, Ponten isolated "[e]ighty-nine single tumor cells" from a *biological sample* in the form of a "stained tissue section" of a "human basal cell cancer BCC" thereby generating a plurality of single-cell assay samples from a biological sample.[382] Ponten then "*amplified the template molecules*" by PCR, in which "[e]xons 4–9, of the human p53 gene, and the HLA-DQB1 locus were amplified in a multiplex/nested configuration."[383] Ponten then "*analyzed the amplified molecules*" in the single-cell samples by performing a secondary (nested) amplification and DNA sequencing.[384] Ponten checked each cell for a "*selected genetic sequence*" in the form of a first p53 allele mutated in exon 7 (codon 245), and a "*reference genetic sequence*" in the form of second p53 allele mutated in exon 8 (codon 266).[385] Ponten then determined a "*first number of assay samples*" containing the "*selected* " exon 7-mutated p53 allele

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[381] Ponten, Abstract. *See also* page 46, right col., second paragraph ("Single cells were picked up, with the aid of the micromanipulator, on the tip of a small glass capillary … The tip of the capillary, with the attached cell, was broken off against the bottom of a PCR tube.")

[382] Ponten, page 45, Abstract.

[383] Ponten, page 46, Section 2.3.

[384] Ponten, page 46, Section 2.3, and page 44, Section 2.3.

[385] Ponten, page 49, paragraph bridging left and right cols., explaining that 'crude' microdissection … had [already] uncovered two point mutations in the tumor (Fig. 2). One was in exon 7 codon 245. and the other in exon 8 codon 266 … Cloning of this fragment revealed that the mutations were situated on different alleles." *See also* Fig. 4, Table 1 and page 50, left col., bottom paragraph, describing the results of the single-cell PCR analysis, finding that "[t]wo mutations were dominant ... Both mutations were identical to those found in the previous crude microdissections."

and a "*second number of assay samples*" containing the "*reference*" exon 8-mutated p53 allele, and compared the two numbers. In particular, out of 44 single-cell samples which yielded an amplification product of exon 7 and/or exon 8, Ponten found that "[t]wo mutations were dominant, codon 245 (GGC to GTC), [in] exon 7, mutated in 20 cells, and codon 266 (GGA to GAA), [in] exon 8, mutated in 13 cells."[386]

In addition, Ponten meets the added requirements of claim 63. Ponten's *selected genetic sequence*" (*i.e.*, exon 7-mutated p53) "*comprises a first mutation and the reference genetic sequence*" (*i.e.*, exon 8-mutated p53) "*comprises a second mutation.*"

Obviousness: Reasons to Combine

It would have been obvious to use Ponten's single-cell RT-PCR analysis in which the number of samples containing two different mutant sequences are compared, in Zhang's own single-cell methods of analysis. Zhang explicitly recognized the usefulness of importance of genotyping single diploid cells (*e.g.*, "single cells from early embryos or polar bodies.")[387] Ponten did precisely as Zhang suggested for precisely the same reason – specifically, to "resolve important and fundamental questions determining cancer heterogeneity," *i.e.*, cell-to-cell variation.[388] Ponten explained that "[i]n crude microdissections … mutations [in single cells] would be 'diluted' and thus not detected … while microdissection of single cells would disclose such mutations.[389] Ponten also noted that two separate mutations on either allele commonly take place in cancer

[386] Ponten, page 50, paragraph bridging left and right cols.
[387] Zhang at page 5847, left column.
[388] Ponten, Abstract, and page 46, left col., last paragraph of Introduction section.
[389] Ponten, page 54, second paragraph.

progression,[390] thereby rendering it obvious that a mutant "*selected genetic sequence*" in the form of a first mutant allele and a mutant "*reference genetic sequence*" in the form of a second mutant allele could often be found in cancer samples.

In view of Ponten, it would have been *prima facie* obvious to one of ordinary skill to use Zhang's methods on cancer samples such as Ponten's in which "*the selected genetic sequence comprises a first mutation and the reference genetic sequence comprises a second mutation.*"

Thus, claim 63 would have been *prima facie* obvious at the time of invention over Zhang in view of Ponten.

VII. CONCLUSION

Claims 1-12, 14-16, 19-32, 38-44, 46-48 and 51-64 are anticipated or alternatively obvious over one or more prior-art references applied herein. Accordingly, reexamination of claims 1-12, 14-16, 19-32, 38-44, 46-48 and 51-64 of the '706 patent is respectfully requested.

VIII. CONCURRENT LITIGATION AND REEXAMINATION PROCEEDINGS

The '706 patent is presently involved in litigation in the United States District Court for the Middle District of North Carolina Greensboro Division (Esoterix Genetic Laboratories, LLC vs. Life Technologies Corporation, Applied Biosystems, LLC, and Ion Torrent Systems, Inc., Case No. 12-cv-411 (filed April 26, 2012)).

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[390] Ponten, page 46, left col., last paragraph of Introduction section ("we have microdissected different parts of individual BCC's and found that p53 mutations often affect both alleles and that progression of p53 alterations can take place within a tumor.")

IX. AUTHORITY TO ACT AND CORRESPONDENCE ADDRESS

The real party in interest is Life Technologies Corporation, a Delaware corporation, having its principle place of business at 5791 Van Allen Way, Carlsbad, CA, 92008. Undersigned counsel states that it is acting on behalf of the real party in interest either in a representative capacity pursuant to C.F.R. § 1.34(a), or under any power of attorney provided herewith.

Please send all correspondence to the address associated with customer number 52059, to the attention of: Legal – Intellectual Property Group, Life Tech Docket, Bldg. 5781, Office 8304.

X. REQUIRED FEES AND DEPOSIT ACCOUNT AUTHORIZATION

The Commissioner is authorized to charge the fee of $17,750.00 set forth in 37 C.F.R. § 1.20(c)(1) to Life Technologies Deposit Account No. 50-3994. The Commissioner is authorized to charge any additional fees or credit any overpayment to Deposit Account No. 50-3994, as well as any and all other fees that have been or may be required from Requester, referencing Docket No. LT00831 REX.

Dated: June 17, 2013

Respectfully submitted,

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APPLICANTS

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**** CONTINUING DATA ***************************

This application is a REX of 09/613,826 07/11/2000 PAT 6440706
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**** FOREIGN APPLICATIONS *********************

Foreign Priority claimed	STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS
☐ yes ☐ no			64	5
35 USC 119 (a-d) conditions met ☐ yes ☐ no ☐ Met after Allowance				
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ADDRESS
11332

TITLE
Digital Amplification

FILING FEE RECEIVED	FEES: Authority has been given in Paper	
12000	No. _____ to charge/credit DEPOSIT ACCOUNT No. _____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

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NOTICE OF ASSIGNMENT OF REEXAMINATION REQUEST

The above-identified request for reexamination has been assigned to Art Unit 3991. All future correspondence to the proceeding should be identified by the control number listed above and directed to the assigned Art Unit.

A copy of this Notice is being sent to the latest attorney or agent of record in the patent file or to all owners of record. (See 37 CFR 1.33(c)). If the addressee is not, or does not represent, the current owner, he or she is required to forward all communications regarding this proceeding to the current owner(s). An attorney or agent receiving this communication who does not represent the current owner(s) may wish to seek to withdraw pursuant to 37 CFR 1.36 in order to avoid receiving future communications. If the address of the current owner(s) is unknown, this communication should be returned within the request to withdraw pursuant to Section 1.36.

NOTICE OF USPTO EX PARTE REEXAMINATION PATENT OWNER STATEMENT WAIVER PROGRAM

The USPTO has implemented a pilot program where, after a reexamination proceeding has been granted a filing date and before the examiner begins his or her review, the patent owner may orally waive the right to file a patent owner's statement. See *"Pilot Program for Waiver of Patent Owner's Statement in Ex Parte Reexamination Proceedings,"* 75 FR 47269 (August 5, 2010). One goal of the pilot program is to reduce the pendency of reexamination proceedings and improve the efficiency of the reexamination process.

Ordinarily when ex parte reexamination is ordered, the USPTO must wait until after the receipt of the patent owner's statement and the third party requester's reply, or after the expiration of the time period for filing the statement and reply (a period that can be as long as 5 to 6 months), before mailing a first determination of patentability. The USPTO's first determination of patentability is usually a first Office action on the merits or a Notice of Intent to Issue Reexamination Certificate (NIRC).

Under the pilot program, the patent owner's oral waiver allows the USPTO to act on the first determination of patentability immediately after determining that reexamination will be ordered, and in a suitable case issue the reexamination order and the first determination of patentability (which could be a NIRC if the claims under reexamination are confirmed) at the same time.

Benefits to the Patent Owner for participating in this pilot program include reduction in pendency.

To participate in this pilot program, Patent Owners may contact the USPTO's Central Reexamination Unit (CRU) at 571-272-7705. The USPTO will make the oral waiver of record in the reexamination file in an interview summary and a copy will be mailed to the patent owner and any third party requester.

cc: Third Party Requester(if any)
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/jawhitfield/

Legal Instruments Examiner
Central Reexamination Unit 571-272-7705; FAX No. 571-273-9900

REEXAM CONTROL NUMBER	FILING OR 371 (c) DATE	PATENT NUMBER
90/012,894	06/17/2013	6440706

CONFIRMATION NO. 8442

LIFE TECHNOLOGIES CORPORATION
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REEXAMINATION REQUEST NOTICE

OC000000062249285

Date Mailed: 06/27/2013

NOTICE OF REEXAMINATION REQUEST FILING DATE

(Third Party Requester)

Requester is hereby notified that the filing date of the request for reexamination is 06/17/2013, the date that the filing requirements of 37 CFR § 1.510 were received.

A decision on the request for reexamination will be mailed within three months from the filing date of the request for reexamination. (See 37 CFR 1.515(a)).

A copy of the Notice is being sent to the person identified by the requester as the patent owner. Further patent owner correspondence will be the latest attorney or agent of record in the patent file. (See 37 CFR 1.33). Any paper filed should include a reference to the present request for reexamination (by Reexamination Control Number).

cc: Patent Owner
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Litigation Search Report CRU 3999

Reexam Control No. 90/012,894

To: Examiner **Art Unit: 3991** **Date: 06/27/13** **Case Serial Number: 90/012,894**	**From: Karen L. Ward** **Location: CRU 3999** **MDW 7C76** **Phone: (571) 272-7932** **Karen.Ward@uspto.gov**

Search Notes

Litigation was found involving U.S. Patent No. 6,440,706.

1:12CV1173 – OPEN

1) I performed a KeyCite Search in Westlaw, which retrieves all history on the patent including any litigation.

2) I performed a search on the patent in Lexis CourtLink for any open dockets or closed cases.

3) I performed a search in Lexis in the Federal Courts and Administrative Materials databases for any cases found.

4) I performed a search in Lexis in the IP Journal and Periodicals database for any articles on the patent.

5) I performed a search in Lexis in the news databases for any articles about the patent or any articles about litigation on this patent.

Westlaw.

Date of Printing: Jun 27, 2013

KEYCITE

C US PAT 6440706 DIGITAL AMPLIFICATION, Assignee: Johns Hopkins University (Aug 27, 2002)

History

Direct History

=> 1 **DIGITAL AMPLIFICATION,** US PAT 6440706, 2002 WL 1977277 (U.S. PTO Utility Aug 27, 2002)

Patent Family

2 DETECTING MUTANT NUCLEIC ACIDS IN A MIXED POPULATION, USEFUL E.G. FOR DETECTING TUMOR-ASSOCIATED MUTATIONS, BY AMPLIFICATION OF DILUTED SAMPLES TO GENERATE A LINEAR DIGITAL SIGNAL, Derwent World Patents Legal 2001-182981+

Assignments

3 ACTION: ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS). NUMBER OF PAGES: 002, (DATE RECORDED: Dec 15, 2000)

Docket Summaries

4 ESOTERIX GENETIC LABORATORIES, LLC ET AL v. LIFE TECHNOLOGIES CORPORATION, (M.D.N.C. Oct 31, 2012) (NO. 1:12CV01173), (28 USC 1338 PATENT INFRINGEMENT)

Prior Art (Coverage Begins 1976)

C 5 DETECTABLY LABELED DUAL CONFORMATION OLIGONUCLEOTIDE PROBES, ASSAYS AND KITS, US PAT 5925517Assignee: The Public Health Research Institute of, (U.S. PTO Utility 1999)

C 6 METHOD AND ASSAY FOR DETECTION OF THE EXPRESSION OF ALLELE-SPECIFIC MUTATIONS BY ALLELE-SPECIFIC IN SITU REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION, US PAT 5804383Assignee: The Regents of the University of, (U.S. PTO Utility 1998)

C 7 METHOD FOR THE DETECTION OF CLONAL POPULATIONS OF TRANSFORMED CELLS IN A GENOMICALLY HETEROGENEOUS CELLULAR SAMPLE, US PAT 5670325Assignee: Exact Laboratories, Inc., (U.S. PTO Utility 1997)

C 8 METHOD FOR THE RAPID AND ULTRA-SENSITIVE DETECTION OF LEUKEMIC CELLS, US PAT 5858663Assignee: Life Technologies, Inc., (U.S. PTO Utility 1999)

C 9 METHOD OF SAMPLING, AMPLIFYING AND QUANTIFYING SEGMENT OF NUCLEIC

ACID, POLYMERASE CHAIN REACTION ASSEMBLY HAVING NANOLITER-SIZED SAMPLE CHAMBERS, AND METHOD OF FILLING ASSEMBLY, US PAT 6143496Assignee: Cytonix Corporation; The United States of America as, (U.S. PTO Utility 2000)

C 10 METHODS FOR THE DETECTION OF LOSS OF HETEROZYGOSITY, US PAT 6020137Assignee: Exact Laboratories, Inc., (U.S. PTO Utility 2000)

C 11 METHODS FOR THE DETECTION OF LOSS OF HETEROZYGOSITY, US PAT 5928870Assignee: Exact Laboratories, Inc., (U.S. PTO Utility 1999)

C 12 WAVELENGTH-SHIFTING PROBES AND PRIMERS AND THEIR USE IN ASSAYS AND KITS, US PAT 6037130Assignee: The Public Health Institute of the City of, (U.S. PTO Utility 2000)

US District Court Civil Docket

U.S. District - North Carolina Middle
(Ncmd)

1:12cv1173

Esoterix Genetic Laboratories, Llc et al v. Life Technologies Corporation, et al

This case was retrieved from the court on Wednesday, June 26, 2013

Date Filed:	**10/31/2012**	**Class Code:**	**OPEN**
Assigned To:	**Judge CATHERINE C. EAGLES**	**Closed:**	**No**
Referred To:	**Magistrate Judge Joi Elizabeth Peake**	**Statute:**	**28:1338**
Nature of suit:	**Patent (830)**	**Jury Demand:**	**Both**
Cause:	**Patent Infringement**	**Demand Amount:**	**$0**
Lead Docket:	**None**	**NOS Description:**	**Patent**
Other Docket:	**1:12cv00411**		
Jurisdiction:	**Federal Question**		

Litigants

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Date	#	Proceeding Text	Source
10/31/2012	1	COMPLAINT for Patent Infringement against LIFE TECHNOLOGIES CORPORATION, APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., (Filing fee $350 receipt number 0418-1203651), filed by ESOTERIX GENETIC LABORATORIES, LLC, THE JOHNS HOPKINS UNIVERSITY. (Attachments: # 1 Exhibit 1, # 2 Exhibit 2, # 3 Exhibit 3) (GARDNER, JOHN) (Entered: 10/31/2012)	
10/31/2012	2	Corporate Disclosure Statement by ESOTERIX GENETIC LABORATORIES, LLC identifying Corporate Parent LABORATORY CORPORATION OF AMERICA HOLDINGS for ESOTERIX GENETIC LABORATORIES, LLC. (GARDNER, JOHN) (Main Document 2 replaced on 11/1/2012 with correct PDF form) (Garland, Leah) (Entered: 10/31/2012)	
10/31/2012	3	Corporate Disclosure Statement by THE JOHNS HOPKINS UNIVERSITY. (GARDNER, JOHN) (Main Document 3 replaced on 11/1/2012 with corrected PDF image) (Garland, Leah) (Entered: 10/31/2012)	
11/01/2012	5	Summons Issued as to LIFE TECHNOLOGIES CORPORATION, APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC. (Attachments: # 1 Summons for Applied Biosystems, LLC, # 2 Summons for Ion Torrent Systems, INC.) (Garland, Leah) (Entered: 11/01/2012)	
11/01/2012	6	Notice of Right to Consent. Counsel shall serve the attached form on all parties. (Garland, Leah) (Entered: 11/01/2012)	
11/01/2012		CASE REFERRED to Mediation pursuant to Local Rule 83.9b of the Rules of Practice and Procedure of this Court. Please go to our website under Attorney Information for a list of mediators which must be served on all parties. (Garland, Leah) (Entered: 11/01/2012)	
11/01/2012		CASE REFERRED to Standing Order 30. (Garland, Leah) (Entered: 11/01/2012)	
11/01/2012	7	NOTICE of Appearance by attorney MATIAS FERRARIO on behalf of Plaintiff ESOTERIX GENETIC LABORATORIES, LLC (FERRARIO, MATIAS) (Entered: 11/01/2012)	
11/01/2012	8	NOTICE of Appearance by attorney LESLIE THOMAS GRAB on behalf of Plaintiff ESOTERIX GENETIC LABORATORIES, LLC (GRAB, LESLIE) (Entered: 11/01/2012)	
11/05/2012	9	NOTICE of Appearance by attorney PAUL K. SUN, JR on behalf of Plaintiff THE JOHNS HOPKINS UNIVERSITY (SUN, PAUL) (Entered: 11/05/2012)	
11/08/2012	10	SUMMONS Returned Executed by ESOTERIX GENETIC LABORATORIES, LLC as to LIFE TECHNOLOGIES CORPORATION served on 11/2/2012, answer due 11/23/2012. (GARDNER, JOHN) (Entered: 11/08/2012)	
11/08/2012	11	SUMMONS Returned Executed by ESOTERIX GENETIC LABORATORIES, LLC as to APPLIED BIOSYSTEMS, LLC served on 11/2/2012, answer due 11/23/2012. (GARDNER, JOHN) (Entered: 11/08/2012)	
11/08/2012	12	SUMMONS Returned Executed by ESOTERIX GENETIC LABORATORIES, LLC as to ION TORRENT SYSTEMS, INC. served on 11/2/2012, answer due 11/23/2012. (GARDNER, JOHN) (Entered: 11/08/2012)	
11/12/2012	13	Consent MOTION for Extension of Time to File Answer by APPLIED BIOSYSTEMS, LLC, ESOTERIX GENETIC LABORATORIES, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION, THE JOHNS HOPKINS UNIVERSITY. (Attachments: # 1 Text of Proposed Order)(FERRARIO, MATIAS) (Entered: 11/12/2012)	
11/14/2012		Motions Referred: RE: 13 Consent MOTION for Extension of Time to File Answer , to MAG/JUDGE JOI ELIZABETH PEAKE (Garrett, Kim) (Entered: 11/14/2012)	
11/20/2012	14	ORDER signed by MAG/JUDGE JOI ELIZABETH PEAKE on 11/20/2012; that Plaintiffs' Agreed Motion Extending Time of Defendants Life Technologies Corporation, Applied	

		Biosystems, LLC and ION Torrent Systems Inc. to Respond to Complaint [Doc. # 13] is GRANTED, and Defendants have to and including January 10, 2013, within which to file an Answer or other responsive pleading to Plaintiffs' Complaint. Answer due by 1/10/2013. (Sheets, Jamie) (Entered: 11/20/2012)
01/10/2013	15	NOTICE of Appearance by attorney ALLISON O. VAN LANINGHAM on behalf of Defendants APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION (VAN LANINGHAM, ALLISON) (Entered: 01/10/2013)
01/10/2013	16	NOTICE of Appearance by attorney STEPHEN MCDANIEL RUSSELL, JR on behalf of Defendants APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION (RUSSELL, STEPHEN) (Entered: 01/10/2013)
01/10/2013	17	NOTICE by APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION OF SPECIAL APPEARANCE OF KATHERINE NOLAN-STEVAUX (VAN LANINGHAM, ALLISON) (Entered: 01/10/2013)
01/10/2013	18	MOTION TO DISMISS FOR FAILURE TO STATE A CLAIM by APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION. Responses due by 2/4/2013 (VAN LANINGHAM, ALLISON) (Entered: 01/10/2013)
01/10/2013	19	BRIEF re 18 MOTION TO DISMISS FOR FAILURE TO STATE A CLAIM by Defendants APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION filed by APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION. (VAN LANINGHAM, ALLISON) (Entered: 01/10/2013)
01/10/2013	20	Corporate Disclosure Statement by LIFE TECHNOLOGIES CORPORATION. (VAN LANINGHAM, ALLISON) (Entered: 01/10/2013)
01/10/2013	21	Corporate Disclosure Statement by APPLIED BIOSYSTEMS, LLC. (VAN LANINGHAM, ALLISON) (Entered: 01/10/2013)
01/10/2013	22	Corporate Disclosure Statement by ION TORRENT SYSTEMS, INC. (VAN LANINGHAM, ALLISON) (Entered: 01/10/2013)
01/11/2013	23	NOTICE of Appearance by attorney KATRINA M. QUICKER on behalf of Plaintiff THE JOHNS HOPKINS UNIVERSITY (QUICKER, KATRINA) (Entered: 01/11/2013)
01/29/2013	24	Consent MOTION for Extension of Time to File Response/Reply by ESOTERIX GENETIC LABORATORIES, LLC, THE JOHNS HOPKINS UNIVERSITY. (Attachments: # 1 Text of Proposed Order)(FERRARIO, MATIAS) (Entered: 01/29/2013)
01/30/2013		Motions Referred: RE: 24 Consent MOTION for Extension of Time to File Response/Reply , to MAG/JUDGE JOI ELIZABETH PEAKE (Garrett, Kim) (Entered: 01/30/2013)
01/31/2013	25	ORDER signed by MAG/JUDGE JOI ELIZABETH PEAKE on 1/31/2013; that Plaintiffs' Agreed Motion Extending Time of Plaintiffs Esoterix Genetic Laboratories LLC and the Johns Hopkins University to Respond to Defendants' Motion to Dismiss [Doc. # 24] is GRANTED, and Plaintiffs have to and including February 18, 2013, within which to respond to Defendants' Motion to Dismiss for Failure to State a Plausible Claim. Responses due by 2/18/2013. (Sheets, Jamie) (Entered: 01/31/2013)
02/11/2013	26	Notice to Parties RE: SO30. Responses due by 3/11/2013 (Winchester, Robin) (Entered: 02/11/2013)
02/19/2013	27	RESPONSE filed by Plaintiffs ESOTERIX GENETIC LABORATORIES, LLC, THE JOHNS HOPKINS UNIVERSITY re 18 MOTION TO DISMISS FOR FAILURE TO STATE A CLAIM filed by LIFE TECHNOLOGIES CORPORATION, ION TORRENT SYSTEMS, INC., APPLIED BIOSYSTEMS, LLC. Replies due by 3/8/2013. (FERRARIO, MATIAS) Modified on 2/20/2013 to remove duplicate text. (Sheets, Jamie) (Entered: 02/19/2013)
03/08/2013	28	REPLY, filed by Defendants ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION, to Response to 18 MOTION TO DISMISS FOR FAILURE TO STATE A CLAIM by all Defendants filed by ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION. (VAN LANINGHAM, ALLISON) (Entered: 03/08/2013)
03/11/2013		Motions Referred: RE: 18 MOTION TO DISMISS FOR FAILURE TO STATE A CLAIM , to MAG/JUDGE JOI ELIZABETH PEAKE (Garrett, Kim) (Entered: 03/11/2013)
03/12/2013		Motions Submitted: 18 MOTION TO DISMISS FOR FAILURE TO STATE A CLAIM to JUDGE CATHERINE C. EAGLES. (Sanders, Marlene) (Entered: 03/12/2013)
03/15/2013		Case Reassigned to JUDGE CATHERINE C. EAGLES. UNASSIGNED no longer assigned to the case. (Powell, Gloria) (Entered: 03/15/2013)

03/19/2013	29	NOTICE of Hearing: Motion Hearing set for 4/23/2013 02:00 PM in Greensboro Courtroom #1 before JUDGE CATHERINE C. EAGLES. (Sanders, Marlene) (Entered: 03/19/2013)
03/19/2013	30	NOTICE by APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION OF SPECIAL APPEARANCE OF ANNE S. TOKER (RUSSELL, STEPHEN) (Entered: 03/19/2013)
03/19/2013	31	NOTICE by APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION OF SPECIAL APPEARANCE OF PETER J. ARMENIO (RUSSELL, STEPHEN) (Entered: 03/19/2013)
03/21/2013	32	NOTICE of Appearance by attorney ALLISON O. VAN LANINGHAM on behalf of Defendants APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION (VAN LANINGHAM, ALLISON) (Entered: 03/21/2013)
04/10/2013	33	NOTICE OF CANCELLATION of Motion Hearing set for 4/23/2013 at 2:00 PM in Greensboro Courtroom #1 before JUDGE CATHERINE C. EAGLES. (Sanders, Marlene) (Entered: 04/10/2013)
04/19/2013	34	Suggestion of Subsequently Decided Authority re 18 MOTION TO DISMISS FOR FAILURE TO STATE A CLAIM by Defendants APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION. (Attachments: # 1 Exhibit A)(VAN LANINGHAM, ALLISON) (Entered: 04/19/2013)
04/19/2013	35	WITHDRAWAL of Motion by Defendants APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION re 18 MOTION TO DISMISS FOR FAILURE TO STATE A CLAIM filed by LIFE TECHNOLOGIES CORPORATION, ION TORRENT SYSTEMS, INC., APPLIED BIOSYSTEMS, LLC (VAN LANINGHAM, ALLISON) (Entered: 04/19/2013)
05/01/2013	36	NOTICE of Initial Pretrial Conference Hearing set for 6/7/2013 at 11:00 AM in Greensboro Courtroom #3 before JUDGE CATHERINE C. EAGLES. (Sanders, Marlene) (Entered: 05/01/2013)
05/03/2013	37	ANSWER to 1 Complaint, with Jury Demand, Counterclaim against THE JOHNS HOPKINS UNIVERISTY, ESOTERIX GENETIC LABORATORIES, LLC, by APPLIED BIOSYSTEMS, LLC, LIFE TECHNOLOGIES CORPORATION, ION TORRENT SYSTEMS, INC. (VAN LANINGHAM, ALLISON) Modified on 5/6/2013 to add countercliam parties. (Sheets, Jamie) (Entered: 05/03/2013)
05/22/2013	38	NOTICE by ESOTERIX GENETIC LABORATORIES, LLC of Special Appearance of Susan A. Cahoon (FERRARIO, MATIAS) (Entered: 05/22/2013)
05/28/2013	39	RESPONSE re 37 Answer to Complaint, Counterclaim,, Esoterix Genetic Laboratories, LLC's Answer to Defendants Counterclaims by ESOTERIX GENETIC LABORATORIES, LLC. (FERRARIO, MATIAS) Modified on 5/29/2013 to remove reply deadline. (Sheets, Jamie) (Entered: 05/28/2013)
05/28/2013	40	RESPONSE re 37 Answer to Complaint, Counterclaim, The Johns Hopkins University's Answer to Defendants' Counterclaims filed by THE JOHNS HOPKINS UNIVERSITY. (SUN, PAUL) Modified on 5/29/2013 to remove reply deadline. (Sheets, Jamie) (Entered: 05/28/2013)
05/28/2013	41	Rule 26(f) Report (Individual). Responses due by 6/21/2013 by ESOTERIX GENETIC LABORATORIES, LLC, THE JOHNS HOPKINS UNIVERSITY. (Attachments: # 1 Exhibit 1 - Proposed Rule 26(f) Schedule of Pre-Markman Hearing Dates, # 2 Exhibit 2 - Competing Proposed Rule 26(f) Schedules of Post-Markman Hearing Dates)(FERRARIO, MATIAS) (Entered: 05/28/2013)
05/28/2013	42	Rule 26(f) Report (Individual). Responses due by 6/21/2013 by APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION. (Attachments: # 1 Exhibit 1 - Proposed Case Management Schedule Through The Markman Claim Construction Hearing, # 2 Exhibit 2 - Competing Proposed Case Management Schedules For Post-Markman Claim Construction Hearing Dates)(VAN LANINGHAM, ALLISON) (Entered: 05/28/2013)
05/29/2013		Motions Submitted: 41 Rule 26(f) Report (Individual), 42 Rule 26(f) Report (Individual). to JUDGE CATHERINE C. EAGLES. (Sanders, Marlene) (Entered: 05/29/2013)
06/07/2013		Minute Entry for proceedings held before JUDGE CATHERINE C. EAGLES: Initial Pretrial Conference held on 6/7/2013. Attorneys Susan Cahoon, Matias Ferrario and Paul Sun present for plaintiffs and Attorneys Allison Van Laningham and Peter Armenio present for defendants. Written Scheduling Order forthcoming, the parties may proceed as to the matters agreed upon in the Rule 26(f) reports. (Court Reporter Lori Russell.) (Sanders,

		Marlene) (Entered: 06/07/2013)
06/11/2013	43	SCHEDULING ORDER signed by JUDGE CATHERINE C. EAGLES on 06/11/2013, the Court adopts the Rule 26(f) Reports as to subjects on which the parties agree, as reflected in Documents 41 and 42 . The Court agrees with the plaintiff that fact discovery should not be barred until after the Markman claim construction hearing and may instead proceed upon filing of this order. The Court will also grant the defendants request that discovery be held open for 90 days following the Courts entry of a claim construction order. This will allow each party flexibility in deciding whether to conduct none, some, or all of their fact discovery before or after the claim construction hearing.The Court enters the following Scheduling Order, and includes recently elapsed deadlines as set out herein. Parties agree that mediation should be conducted late in the discovery period, after the Claim Construction briefing and order. Parties agrree to select a mediator 60 days before the close of all discovery. Parties agree that Plaintiff should be allowed to join additional parties or amend pleadings without leave up until the Plaintiff's final contentions are due. Parties agree that Life should be be allowed to join additional parties or amend pleadings without leave up until the date Defendnat's final contentions are due. Parties do not consent to a magistrate judge. A jury trial has been demanded. (Taylor, Abby) (Entered: 06/11/2013)
06/17/2013	44	MOTION to Stay Pending Reexamination of Patents-In-Suit by APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION. Responses due by 7/11/2013 (VAN LANINGHAM, ALLISON) (Entered: 06/17/2013)
06/17/2013	45	BRIEF re 44 MOTION to Stay Pending Reexamination of Patents-In-Suit by Defendants APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION filed by APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION. (Attachments: # 1 Exhibit 1 - USPTO Acknowledgement Receipts, # 2 Exhibit 2 - USPTO Ex Parte Reexamination Filing Data, # 3 Exhibit 3 - Sealy Tech, LLC v. Simmons Bedding Co.)(VAN LANINGHAM, ALLISON) (Entered: 06/17/2013)
06/17/2013	46	DECLARATION filed by Defendants APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION re 44 MOTION to Stay Pending Reexamination of Patents-In-Suit (Declaration of Rosy Lee) filed by APPLIED BIOSYSTEMS, LLC, ION TORRENT SYSTEMS, INC., LIFE TECHNOLOGIES CORPORATION. (VAN LANINGHAM, ALLISON) (Entered: 06/17/2013)
06/24/2013		MEDIATION SCHEDULING ORDER ; Mediation to be conducted late in the discovery period, after the Claim Construction briefing and order. The parties agree to discuss a specific date for mediation within 10 days after the Court's issuance of a decision on claim construction. Mediator to be selected 60 days before the close of all discovery. (Gammon, Cheryl) (Entered: 06/24/2013)
06/24/2013	47	ORDER signed by JUDGE CATHERINE C. EAGLES on 06/24/2013; that the Motion to Stay Pending Reexamination of Patents-in-Suit, (Doc. 44), is DENIED without prejudice. (Garland, Leah) (Entered: 06/24/2013)

Source: **Command Searching > Utility, Design and Plant Patents**
Terms: **6440706** (Suggest Terms for My Search)

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1. 20080241830 (Note: This is a Patent Application only.), October 2, 2008, Digital amplification, Vogelstein, Bert, Baltimore, Maryland, United States of America(US), United States of America(); Kinzler, Kenneth W., BelAir, Maryland, United States of America(US), United States of America(); 709742, The Johns Hopkins University, Baltimore, Maryland, United States of America(US), United States company or corporation

   CORE TERMS: sequence, sample, amplification, mutation, probe, template, mutant, ratio, prime, molecule, digital, genetic, assay, fluorescence, cell, allele, primer, amplified, analyzed, diluted, detection, gene, detect, loop, sequencing, experiment, transcript, molecular, beacon, stool

   ... 6753147 , which is a Continuation of Ser. No. 09613826, July 11, 2000, GRANTED **6440706** Provisional Application Ser. No. 60146792, August 2, 1999, PENDING Prior ...

2. RE 43365, May 8, 2012, Apparatus for chemical amplification based on fluid partitioning in an immiscible liquid, Anderson, Brian L., Lodi, California, United States of America(US), United States of America(); Colston, Bill W., San Ramon, California, United States of America(US), United States of America(); Elkin, Christopher J., San Ramon, California, United States of America(US), United States of America(); 891733, Lawrence Livermore National Security, LLC, Livermore, California, United States of America(US), United States company or corporation

   CORE TERMS: sample, fluid, partitioned, detection, microdroplet, nucleic acid, amplification, partitioning, cycle, target, template, immiscible, molecule, sequence, performing, primer, optical, reduction, reagent, polymerase, partition, optically, dilution, reactant, carrier, ligand, fluorescence, absorption, indicator, mixture

   ... 6429025, August 6, 2002, Parce et al., United States of America (US) **6440706**, August 27, 2002, Vogelstein et al., United States of America (US) 6466713, ...

3. RE 41780, September 28, 2010, Chemical amplification based on fluid partitioning in an immiscible liquid, Anderson, Brian L., Lodi, California, United States of America(US), United States of America(); Colston, Bill W., San Ramon, California, United States of America(US), United States of America(); Elkin, Christopher J., San Ramon, California, United States of America(US), United States of America(); 118418, June 26, 2008, CONFIRMATORY LICENSE (SEE DOCUMENT FOR DETAILS)., ENERGY, U.S. DEPARTMENT OF, GC-62 6F-067, 1000 INDEPENDENCE

AVENUE S.W., WASHINGTON, DISTRICT OF COLUMBIA, UNITED STATES OF AMERICA(US), 20585-0162, reel-frame:021154/0499; January 14, 2010, ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS)., THE REGENTS OF THE UNIVERSITY OF CALIFORNIA, 1111 FRANKLIN STREET, OAKLAND, CALIFORNIA, UNITED STATES OF AMERICA(US), 94612-3550, reel-frame:023790/0855; January 14, 2010, ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS)., LAWRENCE LIVERMORE NATIONAL SECURITY LLC, 7000 EAST AVENUE, L-703, LAWRENCE LIVERMORE NATIONAL LABORATORY, LIVERMORE, CALIFORNIA, UNITED STATES OF AMERICA(US), 94551-9234, reel-frame:023790/0864, Lawrence Livermore National Security, LLC, Livermore, California, United States of America(US), United States company or corporation

CORE TERMS: sample, fluid, partitioned, detection, microdroplet, nucleic acid, amplification, partitioning, cycle, target, template, immiscible, molecule, sequence, performing, primer, optical, reduction, reagent, polymerase, partition, optically, dilution, reactant, carrier, ligand, colorimetric, fluorescence, absorption, indicator

... 6429025, August 6, 2002, Parce et al., United States of America (US) **6440706**, August 27, 2002, Vogelstein et al., United States of America (US) 6466713, ...

☐ 4. 8460872, June 11, 2013, Quantification of a minority nucleic acid species, Nygren, Anders, San Diego, California, United States of America(US), United States of America(); 458341, May 11, 2012, ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS)., SEQUENOM, INC., 3595 JOHN HOPKINS COURT, SAN DIEGO, CALIFORNIA, UNITED STATES OF AMERICA(US), 92121, reel-frame:028193/0870, Sequenom, Inc., San Diego, California, United States of America(US), United States company or corporation

CORE TERMS: nucleic acid, amplification, sequence, primer, oligonucleotide, competitor, region, sample, species, target, genomic, nucleotide, minus, inhibitory, prime, chr21, fetal, loci, base pairs, chromosome, hypermethylation, hybridization, assay, sequencing, amplified, cell, amplicon, methylated, independently, quantification

... 6368834, April 9, 2002, Senapathy et al., United States of America (US) **6440706**, August 27, 2002, Vogelstein et al., United States of America (US) 6664056, ...

☐ 5. 8455221, June 4, 2013, Quantification of a minority nucleic acid species, Nygren, Anders, San Diego, California, United States of America(US), United States of America(); 458036, May 11, 2012, ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS)., SEQUENOM, INC., 3595 JOHN HOPKINS COURT, SAN DIEGO, CALIFORNIA, UNITED STATES OF AMERICA(US), 92121, reel-frame:028193/0870, Sequenom, Inc., San Diego, California, United States of America(US), United States company or corporation

CORE TERMS: nucleic acid, amplification, sequence, primer, oligonucleotide, competitor, region, sample, species, target, genomic, nucleotide, minus, inhibitory, prime, chr21, fetal, loci, base pairs, chromosome, hypermethylation, hybridization, assay, sequencing, amplified, cell, amplicon, methylated, independently, quantification

... 6368834, April 9, 2002, Senapathy et al., United States of America (US) **6440706**, August 27, 2002, Vogelstein et al., United States of America (US)

6664056, ...

6. 8450061, May 28, 2013, Quantification of a minority nucleic acid species, Nygren, Anders, San Diego, California, United States of America(US), United States of America(); 457978, May 11, 2012, ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS)., SEQUENOM, INC., 3595 JOHN HOPKINS COURT, SAN DIEGO, CALIFORNIA, UNITED STATES OF AMERICA(US), 92121, reel-frame:028193/0870, Sequenom, Inc., San Diego, California, United States of America(US), United States company or corporation

CORE TERMS: nucleic acid, amplification, sequence, primer, oligonucleotide, competitor, region, sample, species, target, genomic, nucleotide, minus, inhibitory, prime, chr21, fetal, loci, base pairs, chromosome, hypermethylation, hybridization, assay, sequencing, amplified, cell, amplicon, methylated, independently, quantification

... 6368834, April 9, 2002, Senapathy et al., United States of America (US) **6440706**, August 27, 2002, Vogelstein et al., United States of America (US) 6664056, ...

7. 8442774, May 14, 2013, Diagnosing fetal chromosomal aneuploidy using paired end, Lo, Yuk-Ming Dennis, Kowloon, Hong Kong(HK), Hong Kong(); Chiu, Rossa Wai Kwun, New Territories, Hong Kong(HK), Hong Kong(); Chan, Kwan Chee, Kowloon, Hong Kong(HK), Hong Kong(); 433110, The Chinese University of Hong Kong, Hong Kong SAR, People's Republic of China(CN), Foreign company or corporation

CORE TERMS: chromosome, sequence, sequencing, sample, sequenced, nucleic acid, plasma, fetal, maternal, fragment, molecule, genome, chromosomal, percentage, trisomy, fetus, euploid, tag, nucleotide, aneuploidy, specimen, biological, pregnant, aligned, cutoff, proportion, region, subset, concentration, pregnancy

... 6391559, May 21, 2002, Brown et al., United States of America (US) **6440706**, August 27, 2002, Vogelstein et al., United States of America (US) 6566101, ...

8. 8372584, February 12, 2013, Rare cell analysis using sample splitting and DNA tags, Shoemaker, Daniel, San Diego, California, United States of America(US), United States of America(); Toner, Mehmet, Wellesley, Massachusetts, United States of America(US), United States of America(); Kapur, Ravi, Sharon, Massachusetts, United States of America(US), United States of America(); Stoughton, Roland, The Sea Ranch, California, United States of America(US), United States of America(); 763421, January 18, 2008, ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS)., CELLPOINT DIAGNOSTICS, INC., 265 NORTH WHISMAN ROAD, MOUNTAIN VIEW, CALIFORNIA, UNITED STATES OF AMERICA(US), 94043, reel-frame:020386/0032; January 18, 2008, ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS)., LIVING MICROSYSTEMS INC., 480 ARSENAL STREET, SUITE 130, WATERTOWN, MASSACHUSETTS, UNITED STATES OF AMERICA(US), 02472, reel-frame:020387/0228; January 18, 2008, CHANGE OF NAME (SEE DOCUMENT FOR DETAILS)., ARTEMIS HEALTH, INC., 480 ARSENAL STREET, SUITE 130, WATERTOWN, MASSACHUSETTS, UNITED STATES OF AMERICA(US), 02472, reel-frame:020388/0062; September 30, 2008, CHANGE OF NAME (SEE DOCUMENT FOR DETAILS)., CELLECTIVE DX CORPORATION, 265 NORTH WHISMAN ROAD, MOUNTAIN VIEW, CALIFORNIA, UNITED STATES OF AMERICA(US), 94043, reel-

frame:021611/0738; January 28, 2010, ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS)., GENERAL HOSPITAL CORPORATION, THE, 55 FRUIT STREET, BOSTON, MASSACHUSETTS, UNITED STATES OF AMERICA(US), 02114, reel-frame:023865/0919; August 5, 2011, CHANGE OF NAME (SEE DOCUMENT FOR DETAILS)., VERINATA HEALTH, INC., 1531 INDUSTRIAL ROAD, SAN CARLOS, CALIFORNIA, UNITED STATES OF AMERICA(US), 94070, reel-frame:026711/0626; September 29, 2011, ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS)., VERINATA HEALTH, INC., 1531 INDUSTRIAL ROAD, SAN CARLOS, CALIFORNIA, UNITED STATES OF AMERICA(US), 94070, reel-frame:026992/0687; September 29, 2011, ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS)., VERINATA HEALTH, INC., 1531 INDUSTRIAL ROAD, SAN CARLOS, CALIFORNIA, UNITED STATES OF AMERICA (US), 94070, reel-frame:026992/0868; October 11, 2011, ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS)., THE GENERAL HOSPITAL CORPORATION, 55 FRUIT STREET, BOSTON, MASSACHUSETTS, UNITED STATES OF AMERICA(US), 02114, reel-frame:027044/0055; October 11, 2011, ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS)., GPB SCIENTIFIC, LLC, 800 EAST LEIGH STREET, RICHMOND, VIRGINIA, UNITED STATES OF AMERICA (US), 23219, reel-frame:027044/0055; October 11, 2011, QUIT CLAIM ASSIGNMENT, TONER, MEHMET, 106 BRISTOL ROAD, WELLESLEY, MASSACHUSETTS, UNITED STATES OF AMERICA(US), 02481, reel-frame:027046/0173, The General Hospital Corporation, Boston, Massachusetts, United States of America(US), United States company or corporation; GPB Scientific, LLC, Richmond, Virginia, United States of America(US), United States company or corporation; Verinata Health, Inc., Redwood City, California, United States of America(US), United States company or corporation

CORE TERMS: cell, fetal, sample, allele, primer, emsp, maternal, enriched, sequence, chromosome, cancer, bin, obstacle, array, sequencing, region, minus, amplification, module, blood cells, enrichment, tag, probe, epithelial, genomic, magnetic, ratio, blood sample, mutation, capture

... States of America (US) 6432630, August 13, 2002, Blankenstein, United States of America (US) **6440706**, August 27, 2002, Vogelstein et al., United States of America (US) 6444461, ...

☐ 9. 8343442, January 1, 2013, Microfluidic device and methods of using same, McBride, Lincoln, Belmont, California, United States of America(US), United States of America(); Lucero, Michael, South San Francisco, California, United States of America(US), United States of America(); Unger, Marc, San Mateo, California, United States of America(US), United States of America(); Nassef, Hany Ramez, San Mateo, California, United States of America(US), United States of America(); Facer, Geoffrey, San Francisco, California, United States of America(US), United States of America(); 859176, Fluidigm Corporation, South San Francisco, California, United States of America(US), United States company or corporation

CORE TERMS: channel, site, sample, reagent, microfluidic, probe, nucleic acid, primer, amplification, blind, utilized, layer, elastomeric, nucleotide, detection, guard, target, substrate, analyses, horizontal, fluid, valve, gene, concentration, cell, deposited, sequence, region, polymerase, allele

... 6409832, June 25, 2002, Weigl et al., United States of America (US) **6440706**, August 27, 2002, Vogelstein et al., United States of America (US) 6488832, ...

☐ 10. 8296076, October 23, 2012, Noninvasive diagnosis of fetal aneuoploidy by

sequencing, Fan, Hei-Mun Christina, Fremont, California, United States of America (US), United States of America(); Quake, Stephen R., Stanford, California, United States of America(US), United States of America(); 452083, April 20, 2012, ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS)., THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY, 1705 EL CAMINO REAL, PALO ALTO, CALIFORNIA, UNITED STATES OF AMERICA(US), 94306-1106, reel-frame:028083/0262; June 1, 2012, CONFIRMATORY LICENSE (SEE DOCUMENT FOR DETAILS)., NATIONAL INSTITUTES OF HEALTH (NIH), U.S. DEPT. OF HEALTH AND HUMAN SERVICES (DHHS), U.S. GOVERNMENT, 6705 ROCKLEDGE DRIVE, SUITE 310, MSC 7980, NIH DIVISION OF EXTRAMURAL INVENTIONS AND TECHNOLOGY RESOURCES (DEITR), BETHESDA, MARYLAND, UNITED STATES OF AMERICA(US), 20892-7980, reel-frame:028309/0777, The Board of Trustees of the Leland Stanford Junior University, Palo Alto, California, United States of America(US), United States company or corporation

CORE TERMS: sequence, chromosome, plasma, fetal, tag, sample, sequencing, maternal, window, genome, density, pregnancy, male, cell-free, fragment, blood, genomic, aneuploidy, cell, trisomy, mapped, median, sequenced, fraction, digital, minus, bias, fetus, estimated, region

... States of America (US) 6432630, August 13, 2002, Blankenstein, United States of America (US) **6440706**, August 27, 2002, Vogelstein et al., United States of America (US) 6444461, ...

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and	and	w/p	in same paragraph
or	or	w/seg	in same segment
w/N	within N words	w/s	in same sentence
pre/N	precedes by N words	and not	and not

More Connectors & Commands...

How Do I...?
- Combine sources?
- Restrict by date?
- Restrict by document segment?
- Use wildcards as placeholders for one or more characters in a search term?

View Tutorials

In

No Documents Found

No documents were found for your search terms
"6440706 or 6,440,706"

Click "Save this search as an Alert" to schedule your search to run in the future.

- OR -
Click "Search Using Natural Language" to run your search as Natural Language search.

- OR -

Click "Edit Search" to return to the search form and modify your search.

Suggestions:

- Check for spelling errors.
- Remove some search terms.
- Use more common search terms, such as those listed in "Suggested Words and Concepts."
- Use a less restrictive date range.
- Use "OR" in between terms to search for one term or the other.

| **Save this Search as an Alert** || **Search Using Natural Language** || **Edit Search** |

In

Search | View Tutorial | Help

Select Search Type and Enter Search Terms

Terms & Connectors | Natural Language | Easy Search™

6440706 or 6,440,706

Suggest terms for my search

Check spelling

Search

Restrict by Document Segment

Select a document segment, enter search terms for the segment, then click Add.

Select a Segment | Add

Note: Segment availability differs between sources. Segments may not be applied consistently across sources.

Restrict by Date

No Date Restrictions | From | To | Date formats...

Search Connectors

and	and	w/p	in same paragraph
or	or	w/seg	in same segment
w/N	within N words	w/s	in same sentence
pre/N	precedes by N words	and not	and not

More Connectors & Commands...

How Do I...?

Combine sources?
Restrict by date?
Restrict by document segment?
Use wildcards as placeholders for one or more characters in a search term?

View Tutorials

In

Source: **Command Searching > News, All (English, Full Text)**
Terms: **6440706 or 6,440,706** (Suggest Terms for My Search)

Select for FOCUS™ or Delivery

1. Instrument Business Outlook, October 31, 2012, Pg. 1(3), 323857056, 1733 words, Litigation round up: more settlements.

   CORE TERMS: patent, IBO, Illumina, prejudice, infringement, dismissed, District Court, Esoterix, stipulated, judgment, defendant, Biosciences, plaintiff's, genetic, patent infringement, Genomics, Helicos, dismissal, motion, probes, Affymetrix, Panagene, Ion, non-infringement, counterclaims, complaint, summary, Nucleic Acid, Bio-Synthesis, eBioscience

   ... Esoterix Genetic Life **6,440,706** ...

2. RIA Novosti, February 22, 2007, 6440706, 212 words, Bird flu now confirmed in eight districts near Moscow

   CORE TERMS: virus, flu, avian, Odintsovo, veterinary, confirmed, deadly, bird

Source: **Command Searching > News, All (English, Full Text)**
Terms: **6440706 or 6,440,706** (Suggest Terms for My Search)
View: Cite
Date/Time: Thursday, June 27, 2013 - 8:19 AM EDT

In