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(54) **RECOMBINASE POLYMERASE AMPLIFICATION**

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(57) **ABSTRACT**

This disclosure describe three related novel methods for Recombinase-Polymerase Amplification (RPA) of a target DNA that exploit the properties of recombinase and related proteins, to invade double-stranded DNA with single stranded homologous DNA permitting sequence specific priming of DNA polymerase reactions. The disclosed methods have the advantage of not requiring thermocycling or thermophilic enzymes. Further, the improved processivity of the disclosed methods may allow amplification of DNA up to hundreds of megabases in length.
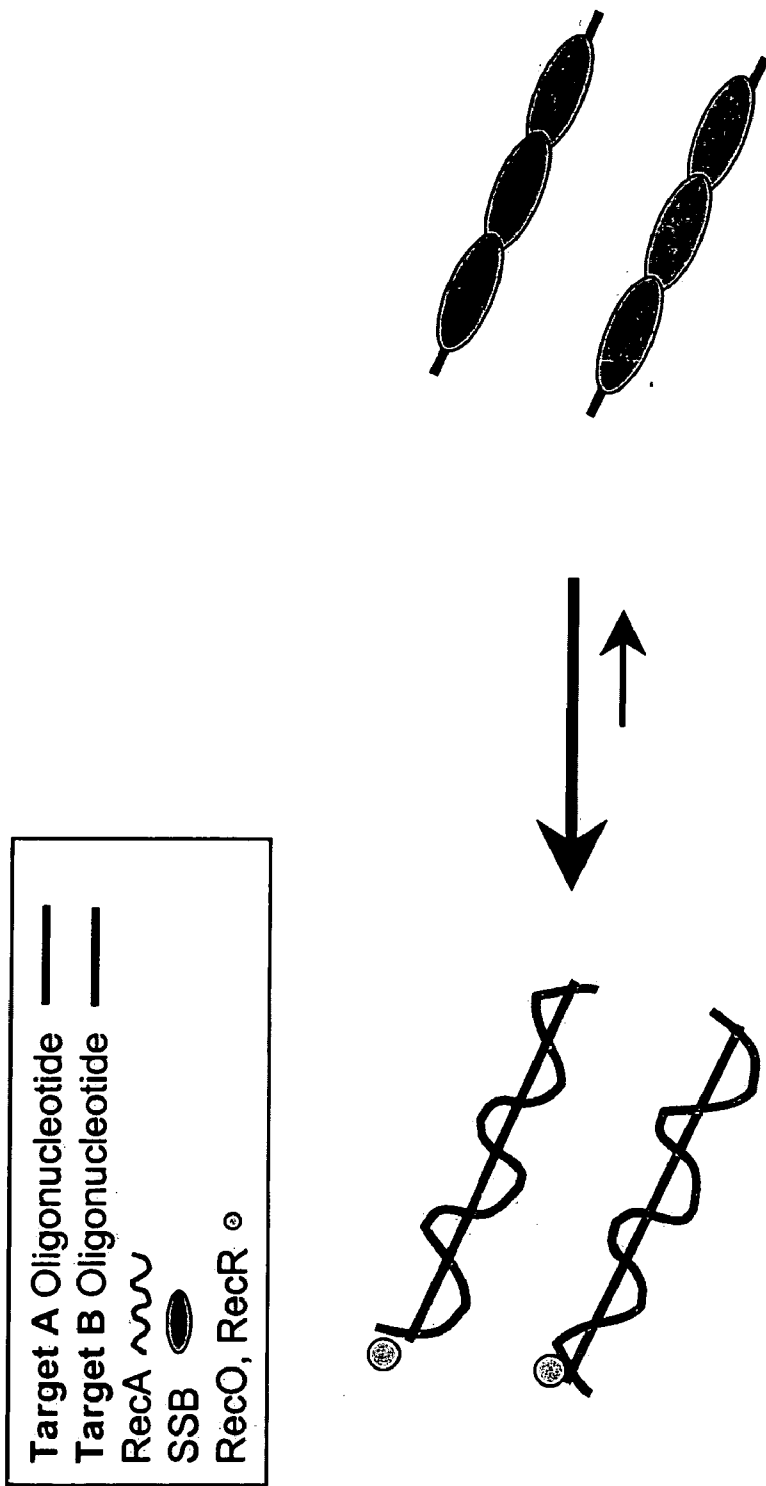
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Figure 1

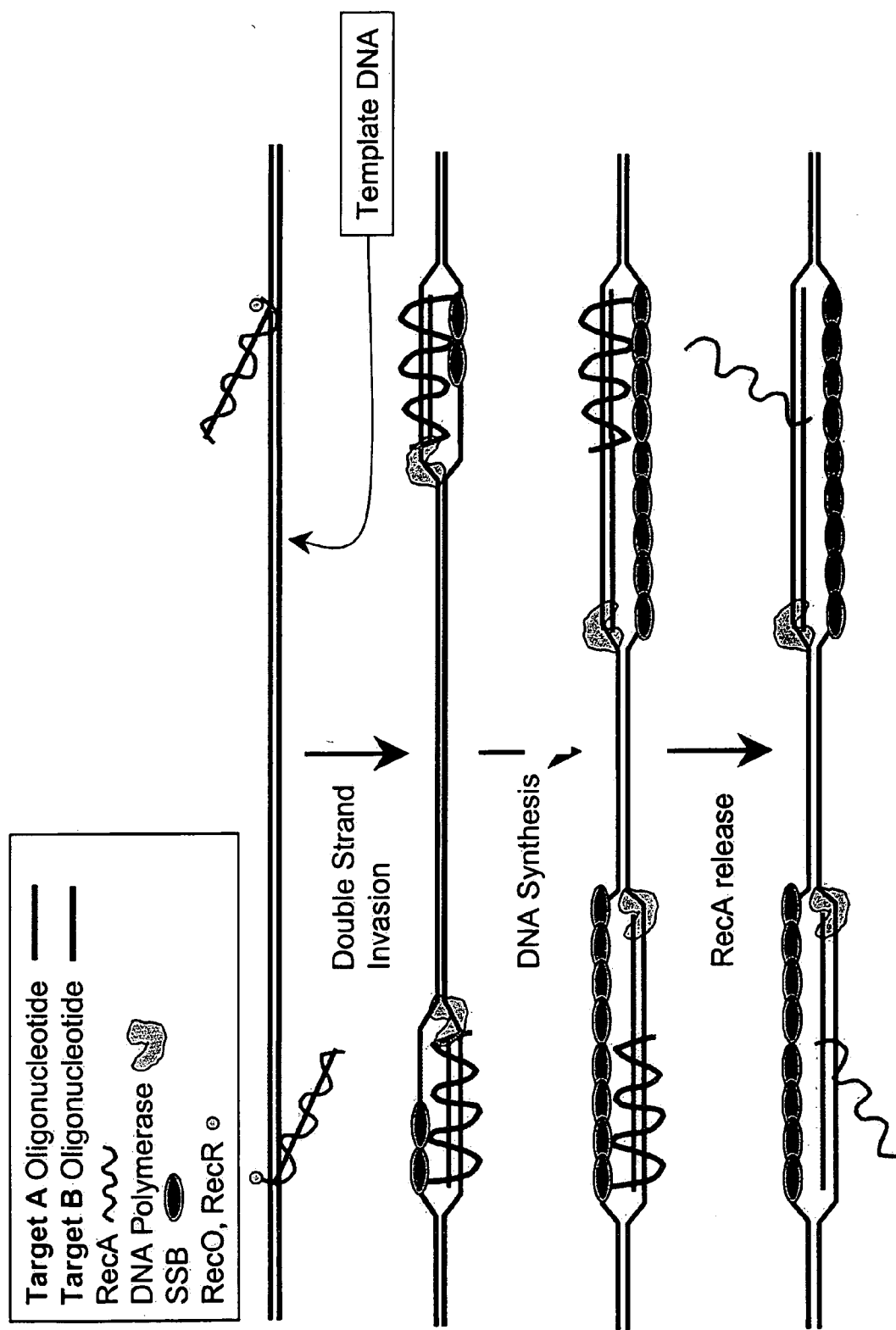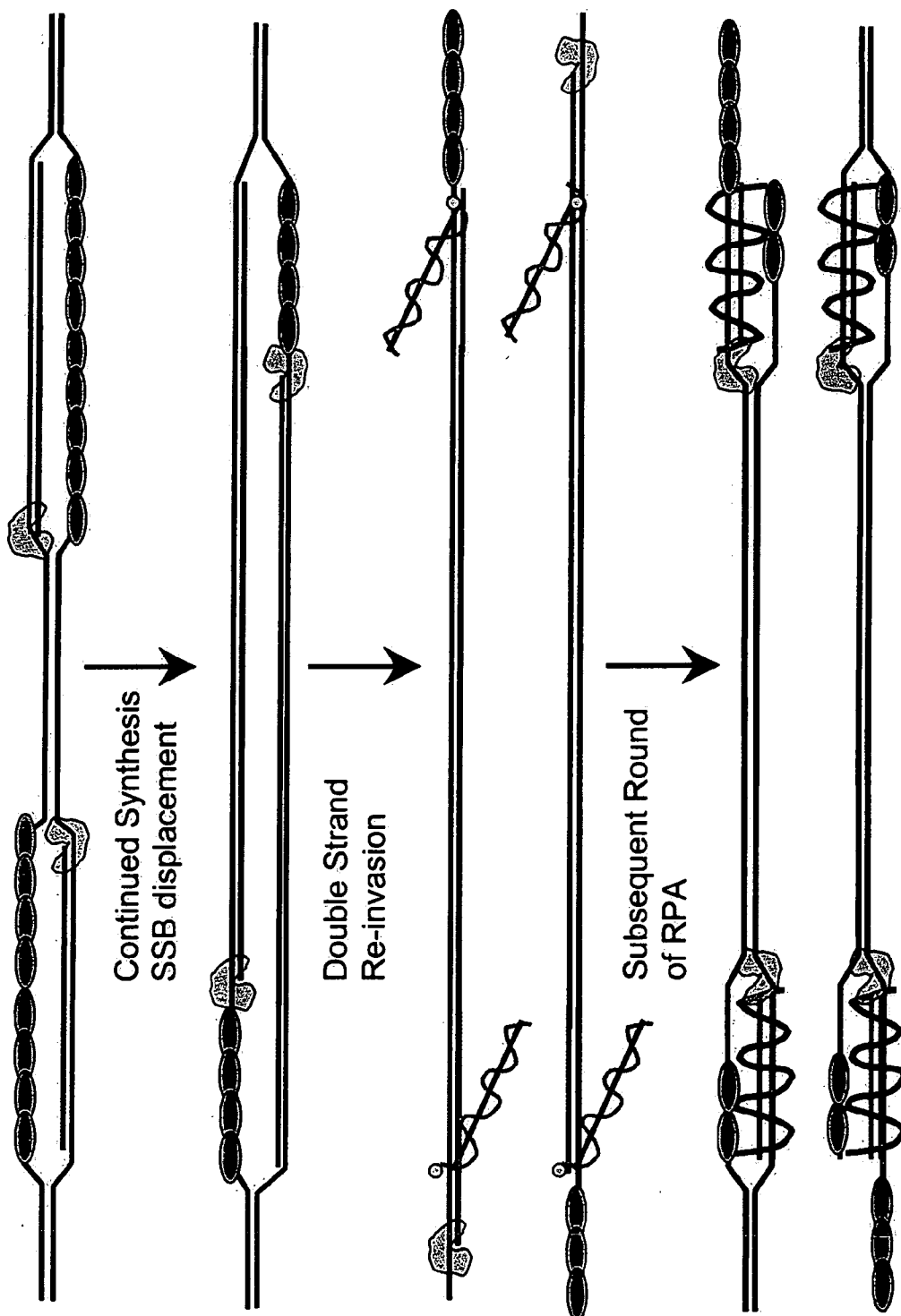Figure 2A

Continued Synthesis
SSB displacement

Double Strand
Re-invasion

Subsequent Round
of RPA

Figure 2B

Figure 3A

- Leading and Lagging Strand synthesis
- SSB Binding

- Lagging Strand Clamp Unloading
- New RNA Primer Synthesis

Figure 3B

•Leading Strand
 Synthesis Continues
•New Lagging Strand
 Clamp Loading

•Leading Strand
 Synthesis Continues
•New Lagging Strand
 Synthesis Back to
 Previous Okazaki Fragment

Figure 3C

•DNA Pol I removes
The upstream RNA primer

•DNA Pol I synthesis fills
in the gap
•DNA Ligase connects the
two strands

DNA Pol I

DNA Ligase

Figure 3D

Figure 4

Primer set 1
Primer set 2
Primer set 3
Primer set 4
Primer set 5

FIGURE 5.   EXAMPLES OF SUITABLE DOUBLE STRANDED TEMPLATE
NUCLEIC ACIDS.

Blunt ended
5' –-–-–-–-–-–-–-–-–-–-–-–-–-–-–-–-–- 3'
3' –-–-–-–-–-–-–-–-–-–-–-–-–-–-–-–-–- 5'

5' overhang and 3' overhang
5' –-–-–-–-–-–-–-–-–-–-–-–-–-–-–-–-–- 3'
      3' –-–-–-–-–-–-–-–-–-–-–- 5'

Blunt and 5' overhang
5' –-–-–-–-–-–-–-–-–-–-–-–-–-–-–-–-–- 3'
3' –-–-–-–-–-–-–-–-–-–-–-–-–-– 5'

Blunt and 3' overhang
5' –-–-–-–-–-–-–-–-–-–-–-–-–-–-–-–-–- 3'
3' –-–-–-–-–-–-–-–-–-–-–-–-–- 5'

Two 5' overhang
5' –-–-–-–-–-–-–-–-–-–-–-–-–-–- 3'
      3' –-–-–-–-–-–-–-–-–-–-– 5'

Two 3' overhang
3' –-–-–-–-–-–-–-–-–-–-–-–-–-–-–-–-–- 5'

Figure 6A   Orientation of nucleic acid primer pairs.

Figure 6B

Figure 7A   R.P.A. Reaction in Progress

Figure 7B

Figure 7C

Figure 8A

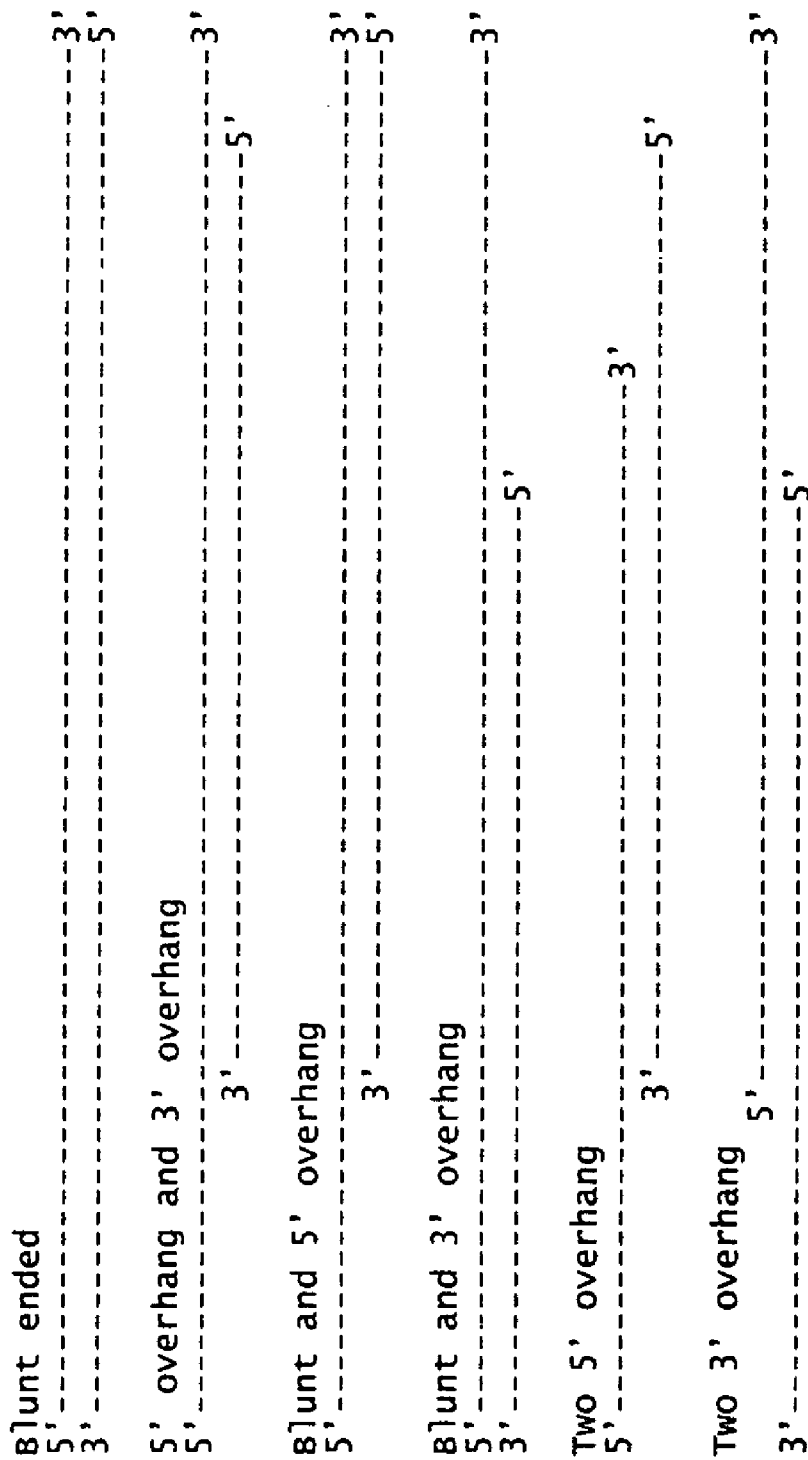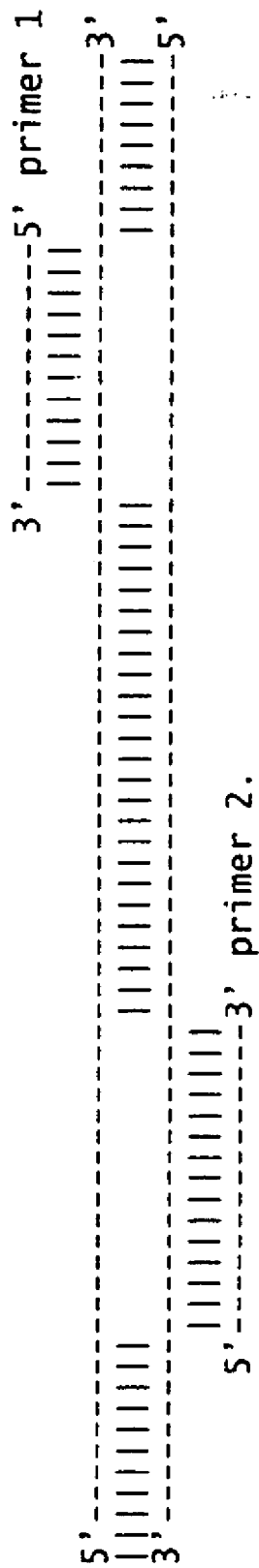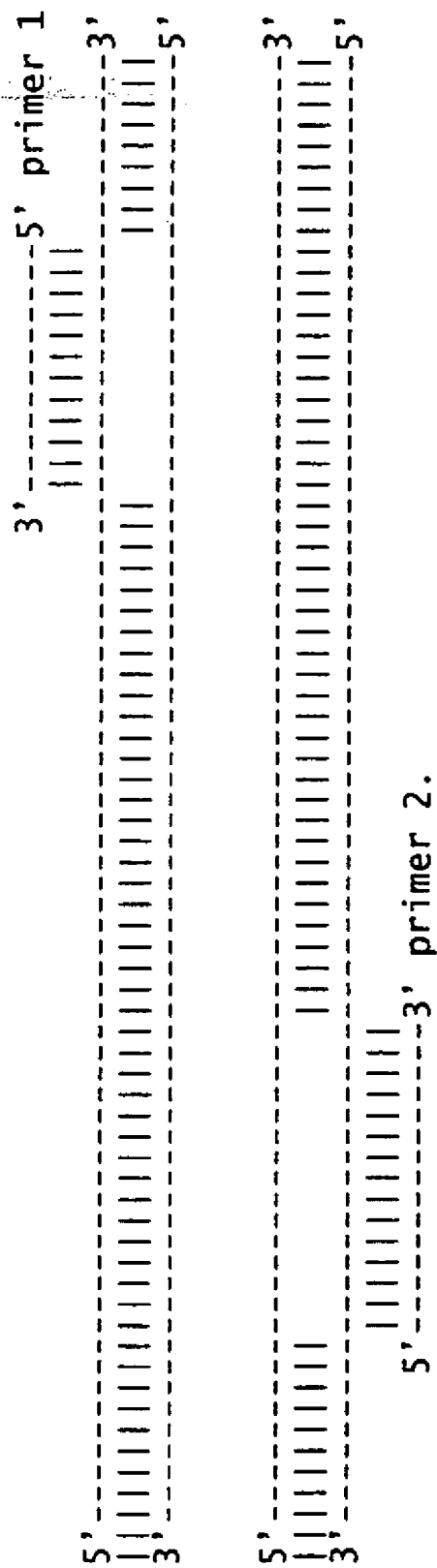Invading Strand
Non-invading strand

Invading Strand
Non-invading strand

Invading Strand
Non-invading strand

Figure 8B

Invading Strand

Second primer

Non-invading strand

Elongated Invading

Figure 8C

Invading Strand

Elongated second primer

Non-invading strand

Elongated Invading

Figure 9

Figure 10

Figure 11

E.coli SSB and T4 phage gp32(N) both support recombination/extension reactions

Conditions:

10mM MgAcetate
6 μg recA
8.8μg gp32 or 1μg SSB
27mM Phosphocreatine
1 unit creatine kinase
0.3mM Tester3bio
3mM ATP
200μM dNTPs
50 units Klenow
0.5 pmoles template
(120 ng recO)
(120 ng recR)

Reaction volume 30 μl

37°C 1 hr

Figure 12

End-directed target (RV)

Gen2bio

RV

Tester3bio

RV

10mM MgAcetate
27mM Phosphocreatine
1 unit creatine kinase
0.2 μM Tester3bio, or Gen2bio
10mM dATP
3mM ATP
200μM dNTP mix
50 units Klenow, or Phi29 polymerase
13 μg recA(C)
1 μg E.coli SSB
0.5 pmoles template

Final volume 30μl

37°C 2 hours
2ul of reaction loaded

CON

recA
Gen2bio
Tester3bio
Klenow
Phi29

Figure 13

Targeting ends vs targeting embedded sequences

'Paranemic joint' formation

'Plectonemic joint' formation

○ Recombinase

● Single-stranded DNA binding protein

Interaction of recombinase filament with DNA stimulates unwinding

'Unwound' region moves with homology search

Homology found

Recombinase dissociates and new duplex attempts to rewind

'Outgoing' strand is forced to rewind around new duplex as it is topologically constrained. This highly unfavourable state is unstable and even SSB's cannot always cope

Interaction of recombinase filament with DNA stimulates unwinding

If strand exchange overlaps a DNA end the 'outgoing' strand is freed and can relax as the incoming oligo and its complement rewind as the recombinase dissociates

A deconstrained product forms and a single-strand DNA binding protein inhibits branch migration

Figure 14

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Conditions:
10mM MgAcetate
10.6 µg recA
8.8µg gp32
27mM Phosphocreatine
1 unit creatine kinase
0.3mM Tester3bio, or Tester1bio
3mM ATP
200µM dNTPs
50 units Klenow
0.5 pmoles RV template
PEG species as indicated

Conditions:
10mM MgAcetate
10.6 µg recA
8.8µg gp32
27mM Phosphocreatine
1 unit creatine kinase
0.3mM Tester3bio, or Tester1bio
3mM ATP
200µM dNTPs
50 units Klenow
0.5 pmoles template,
Species as indicated
(120 ng recO)
(120 ng recR)
PEG8000 as indicated
Final Vol 30µl
1 hour 37°C

High X

Figure 15

Figure 16

Figure 17

Figure 18

Figure 19

Figure 20

Schematic structure of gp32 proteins used in this study

Name

gp32

gp32(N)

gp32(C)

gp32(C)K3A

gp32(C)R4Q

gp32(C)R4T

gp32 K3A

gp32 R4Q

gp32 R4T

B domain

6x His

Figure 21

Full length run-on

0.5 pmoles PCR product

N-tag C-tag K3-A R4-Q R4-T Control
C-tag C-tag C-tag

Reaction conditions
10mM Mgacetate
3mM ATP
200 μM dNTPs
400nM Tester3bio
27mM Phosphocreatine
Creatine kinase 100ngμl-1
10 units chicken myokinase
C-tag uvsX 8μg
7.5 or 15 μg gp32 each species
50 units Klenow fragment
0.5 pmoles template
Volume=30 μl

37°C 2 hours
NO PEG included

RV

~300bp

Tester3bio

16

15 7.5 7.5 15 7.5 7.5 15 15 7.5 7.5 15 15 7.5 7.5

2μl of 30μl loaded onto gel

Figure 22

27

21 residues

C uvsX(C)

uvsXΔ21(C)

C

N

N

End-directed target (RV)

RV

RV

10mM MgAcetate
27mM Phosphocreatine
1 unit creatine kinase
0.4 μM Tester3bio
3mM ATP
200μM dNTPs
50 units Klenow
1 unit Chicken myokinase
uvsX(C) or uvsXΔ21(C)
8.8 μg gp32(N)
0.5 pmoles RV template

Final volume 30μl

37°C 2 hours
2ul of reaction loaded

Figure 23

μg protein

uvsX(C) uvsXΔ(C)

11 5.5 2.7   11 5.5 2.7

Full-length product

Partially Elongated products

Partially Elongated products

primer

Figure 24

C-terminally tagged gp32 is qualitatively different to untagged gp32

Reaction conditions
10mM Mgacetate
3mM ATP
200 μM dNTPs
300nM Sizer2
300nM Tester3bio
27mM Phosphocreatine
Creatine kinase 100ngul-1
10 units chicken myokinase
uvsX(C) 300ng/μl
gp32 (untagged) 300 ng/ml
or gp32(C) 100, 200, 300, 400,
500, or 600 ng/ml
50 units Klenow fragment
PEG as indicated
2 hours 37°C

Figure 25

Titration of untagged gp32, and requirement for uvs Y

Figure 26

Figure 27

Figure 28

8.12.03

First round invasion/synthesis using 'end-targeting' and oligonucleotide overhang

Additional
~10 to 15

~30 residues i.e. roughly minimal requirement for strand exchange

Invasion........release of deconstrained outgoing strand........'backfire' synthesis

New duplex generated
by 'backfire'

Most nucleoprotein filaments that are coated enough to complete strand exchange will catalyse complete
and deconstrained release of the outgoing strand

Subsquent rounds of invasion/synthesis

Few, or no, nucleoprotein filaments can exchange to end of target

Providing only 1, or no, gp32 molecules

Recombinase loading

Branch migration

Branch migration

Scenario 1

Scenario 2

Scenario 3

Figure 29

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RPA reaction from human genomic DNA:
compare uncut vs cut template; compare
primer pairs

Reaction conditions
10mM Mg-acetate
3mM ATP
200 µM dNTPs
300nM Up3 primer
300nM Down 1, 2, or 3 primer
27mM Phosphocreatine
Creatine kinase 100ngul-1
10 units chicken myokinase
uvsX(C) 300ng/µl
gp32(C)K3A 300 ng/µl
uvsY(N) 50 ng/µl
50 units Klenow fragment
10% PEG1450

Final reaction volume 30 µl
37°C 5 hours
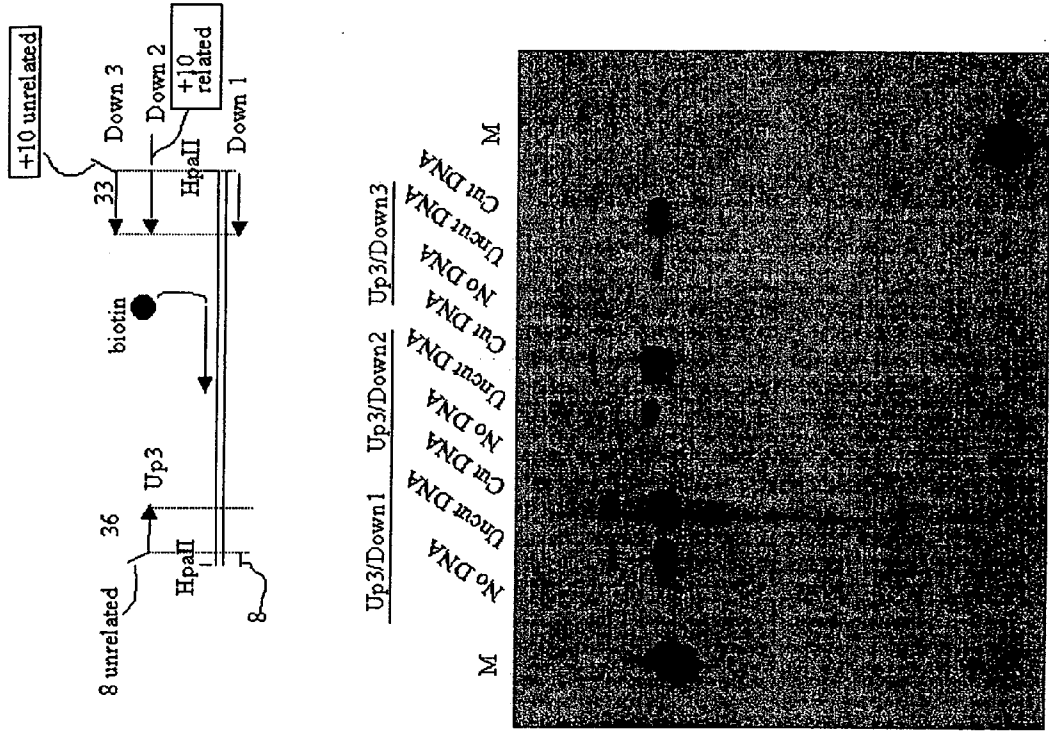
Figure 30
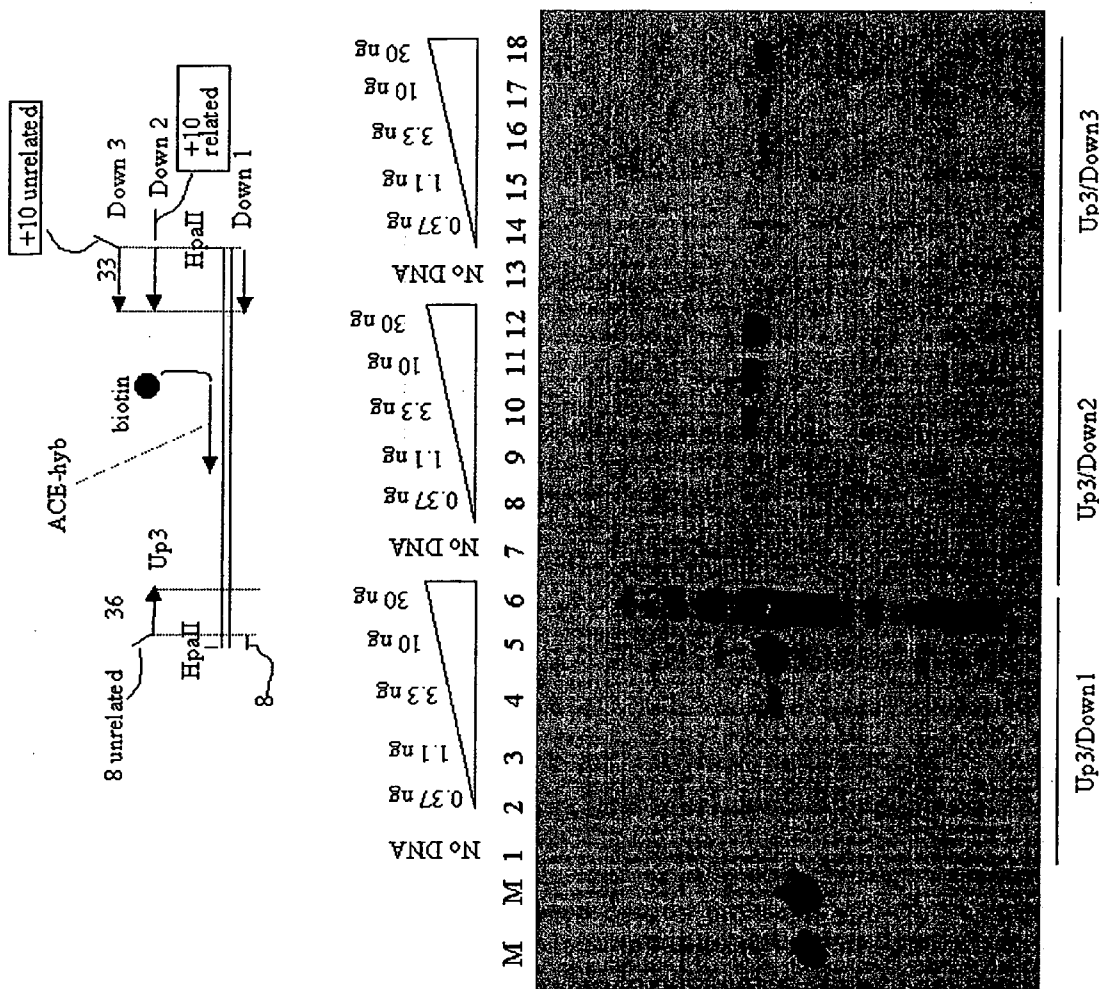
Figure 31

Reaction conditions
10mM Mg-acetate
3mM ATP
200 μM dNTPs
300nM Up3 primer
300nM Down 1, 2, or 3 primer
27mM Phosphocreatine
Creatine kinase 100ngul⁻1
10 units chicken myokinase
uvsX(C) 300ng/μl
gp32(C)K3A 300 ng/μl
uvsY(N) 50 ng/μl
50 units Klenow fragment
10% PEG1450

Final reaction volume 30 μl
37°C 5 hours

Probe ACE-hyb
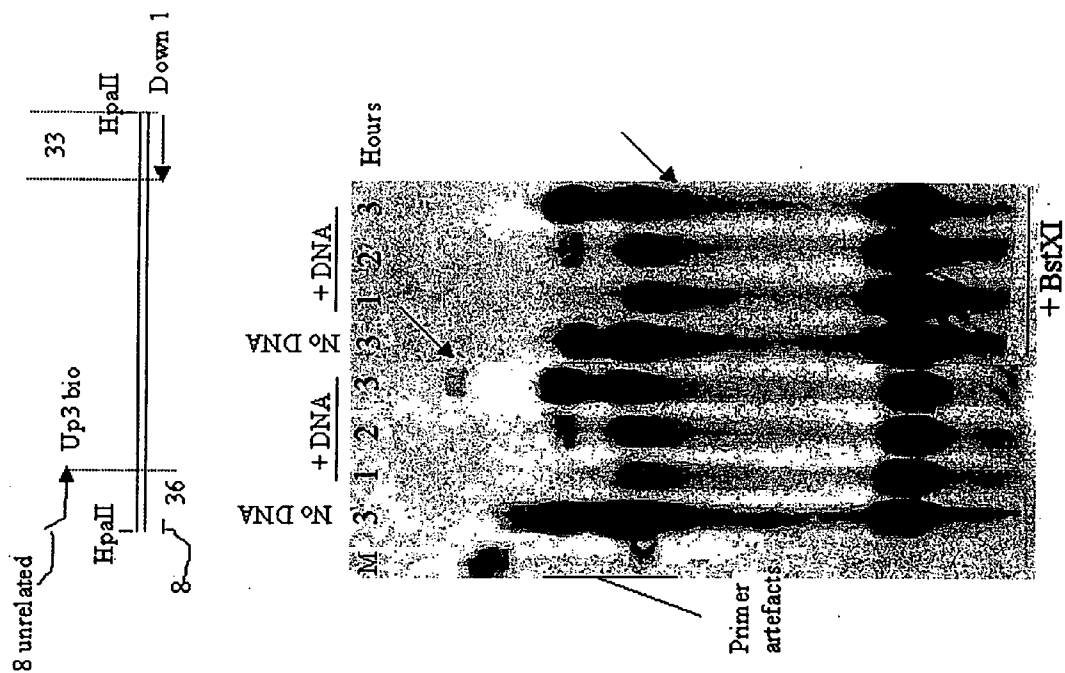
Figure 32

Reaction conditions
10mM Mg-acetate
3mM ATP
200 μM dNTPs
300nM Up3 bio primer
300nM Down 1 primer
27mM Phosphocreatine
Creatine kinase 100ngul-1
10 units chicken myokinase
uvsX(C) 300ng/μl
gp32(C) 300 ng/μl
uvsY(N) 50 ng/μl
50 units Klenow fragment
10% PEG1450

Final reaction volume 30 μl
37°C 5 hours

Mode by which primer artefacts may be generated under low temperature conditions

Hairpin formation

Primer-primer annealing

DNA polymerase, dNTPs

Duplex hairpin

Primer dimer

A

B

Figure 33

Methods to limit primer noise

Description

A. A short 3'-blocked oligonucleotide complementary to the 3' region of the targeting oligonucleotide competes efficiently with secondary structure formation

B. A short 3'-blocked oligonucleotide complementary to the 3' region of the targeting oligo competes efficiently with secondary structure formation. It is tethered at its 3' end to the 5' end of the targeting oligo

C. A short 3'-blocked oligonucleotide complementary to the 3' region of the targeting oligo competes efficiently with secondary structure formation. It is tethered at its 5' end to the 5' end of the targeting oligo

D. A short complementary region to the 3' region of the targeting oligo is added to the 5' region of the targeting oligo. It competes efficiently with secondary structure formation.

Figure 34

Deliberate use of 'hairpin' oligonucleotides can lead to self-priming of displaced strands

Figure 35

Establishment of conditions for sensitive noise-free amplification
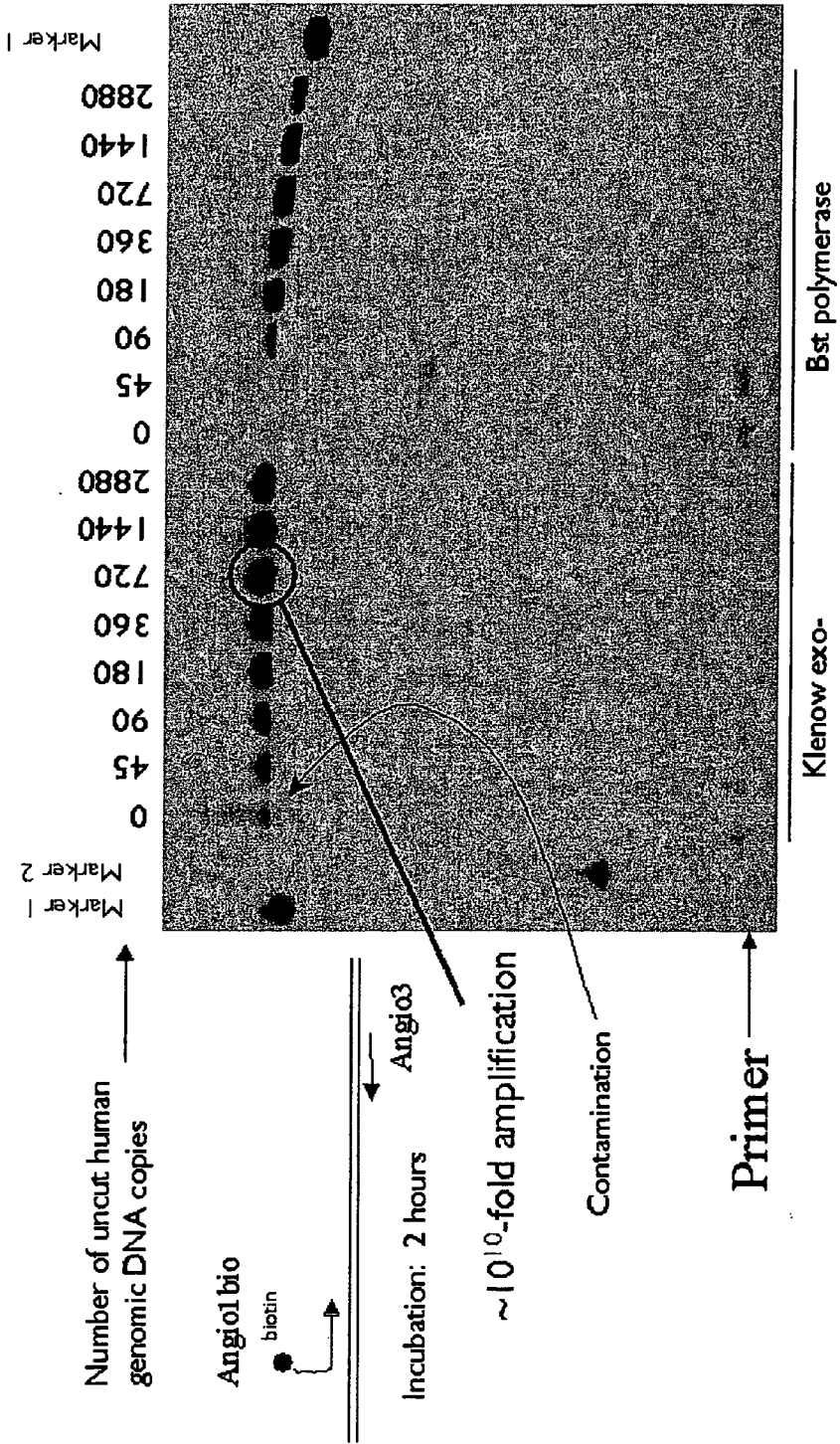
Figure 36
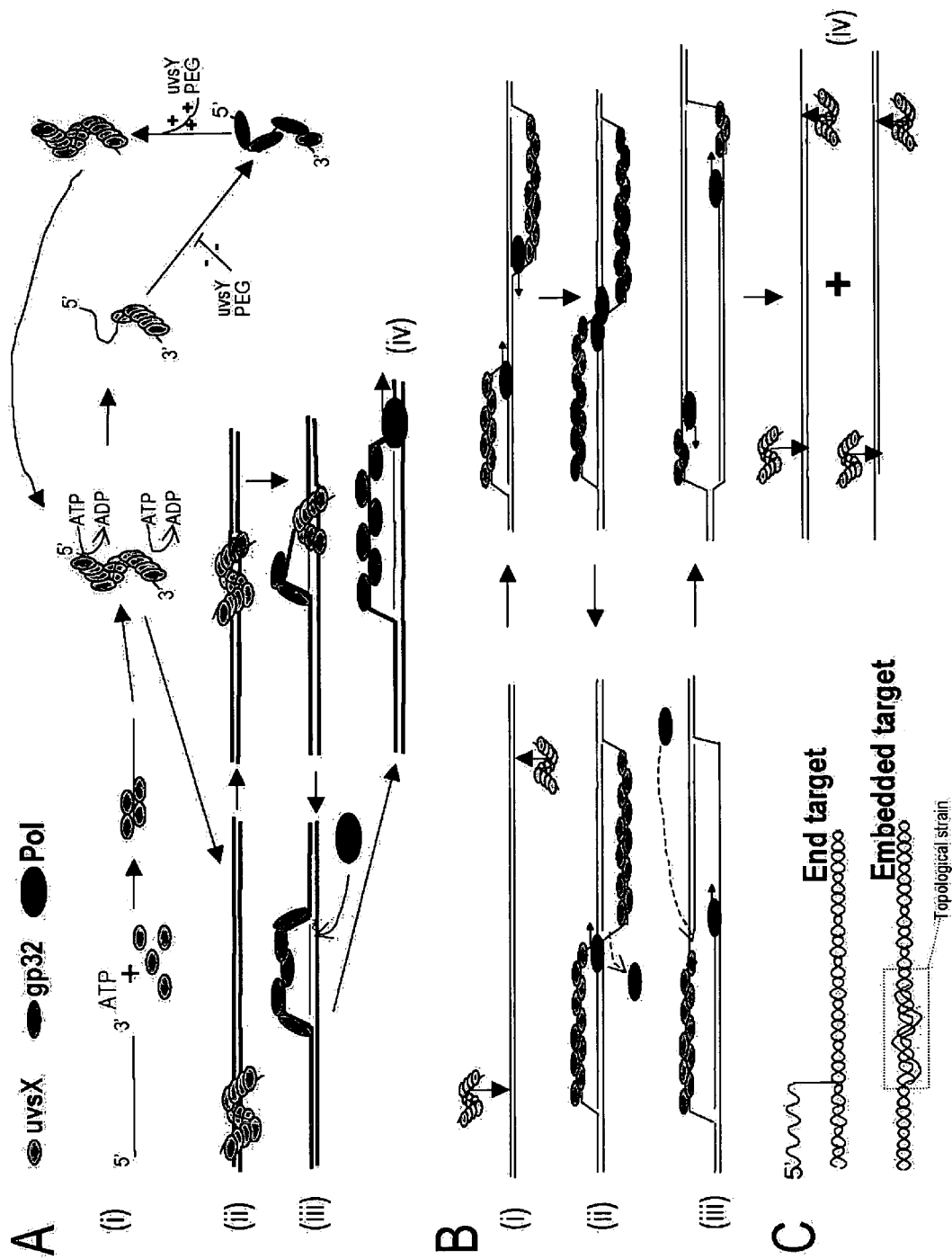
Figure 37
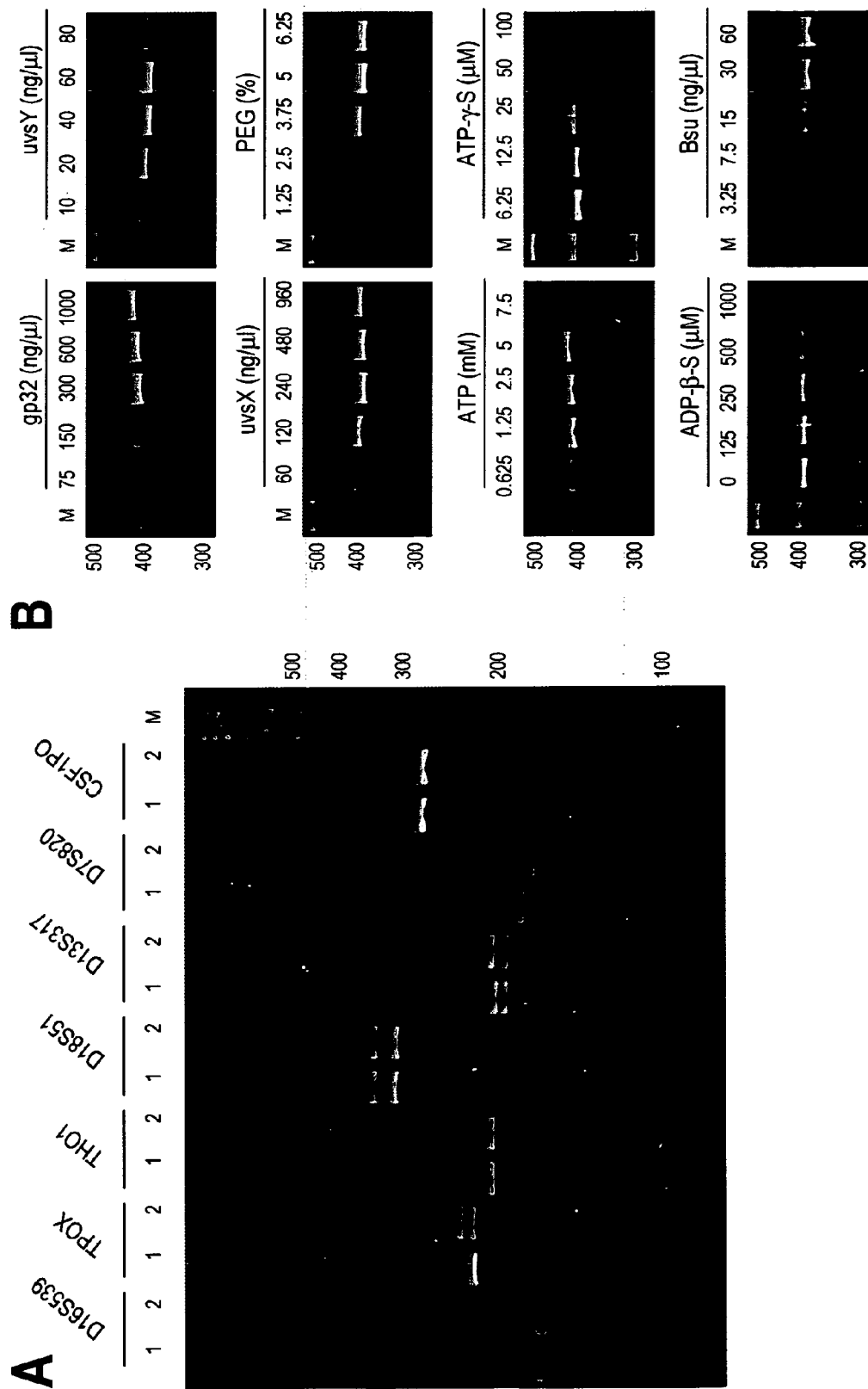
Figure 38
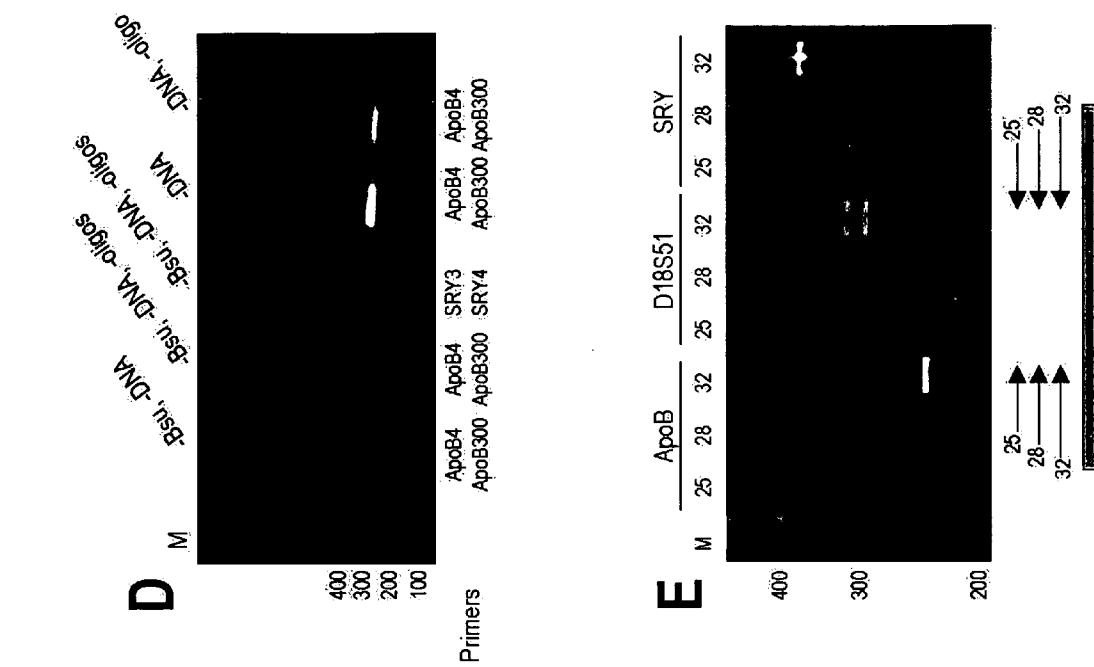
Figure 39

Figure 40

RECOMBINASE POLYMERASE AMPLIFICATION

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. application 60/553,999 filed Mar. 16, 2004, and is a continuation-in-part of U.S. application Ser. No. 10/371,641 filed Feb. 21, 2003, which claims the benefit of U.S. Application 60/358,563 filed Feb. 21, 2002. All patents and patent applications cited in this specification are hereby incorporated by reference herein in their entirety.

BACKGROUND OF THE INVENTION

[0002] The ability to amplify DNA lies at the heart of modem biological and medical research. This is because most molecular biology techniques rely on samples containing many identical molecules to increase the sensitivi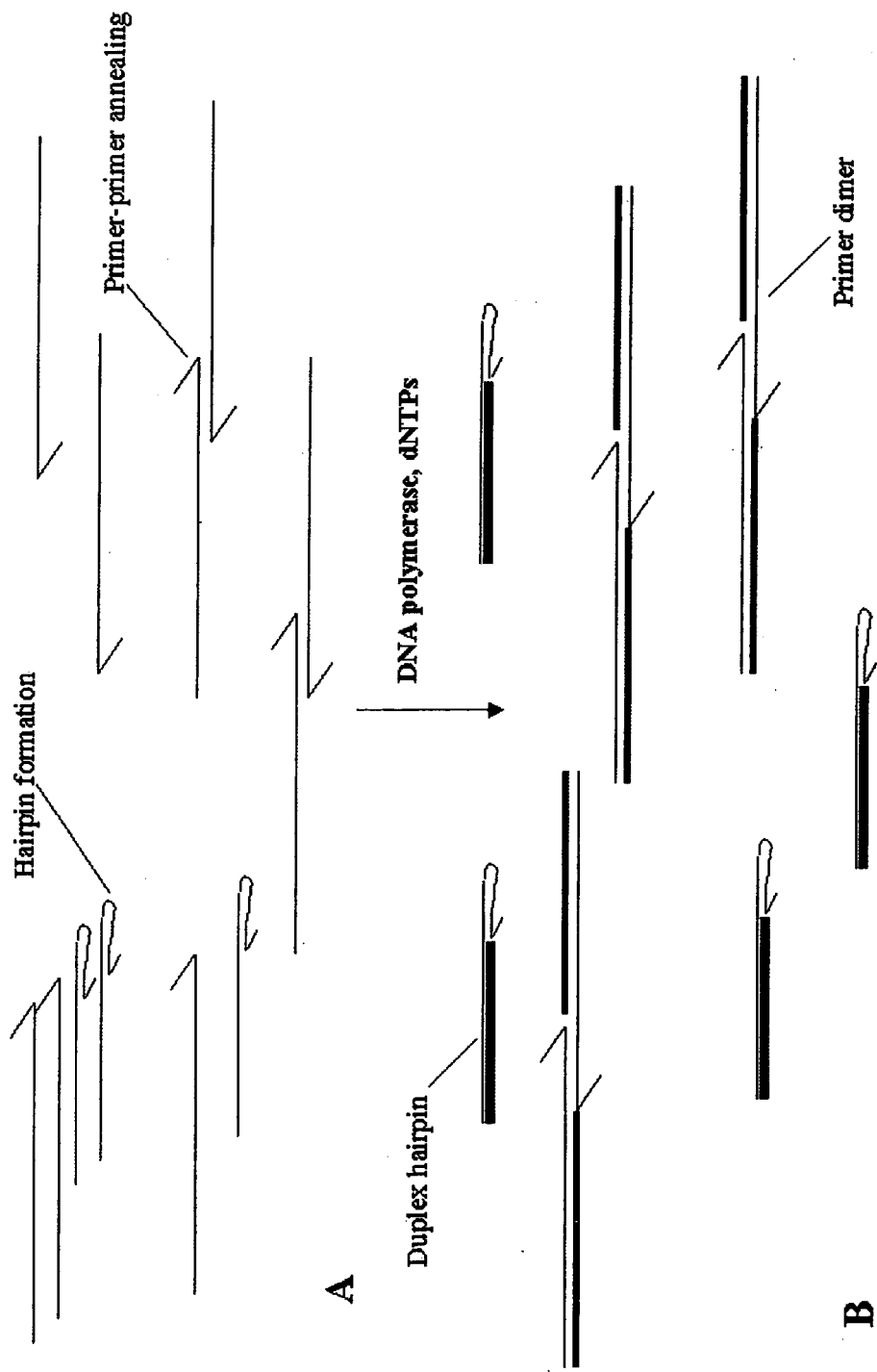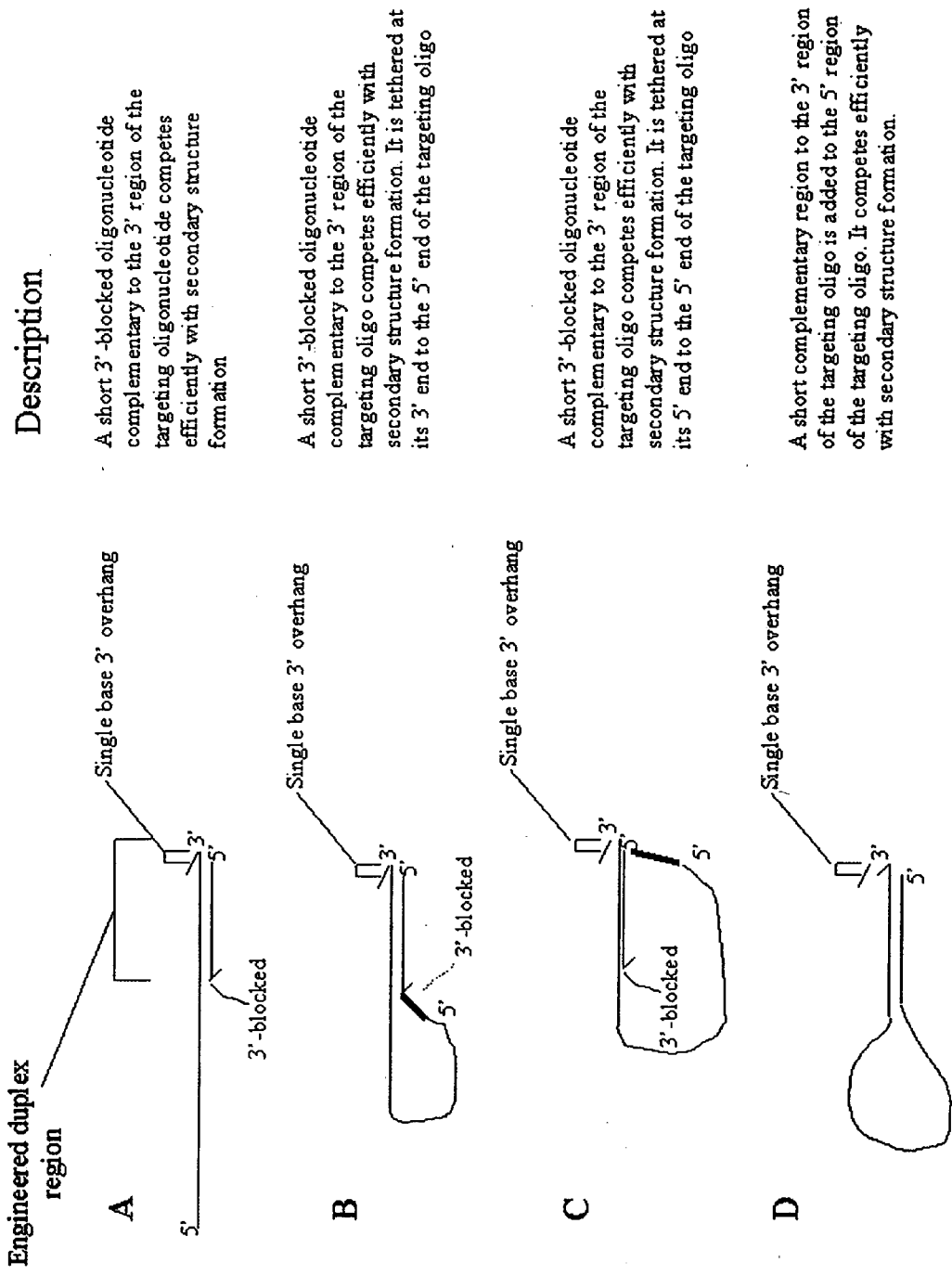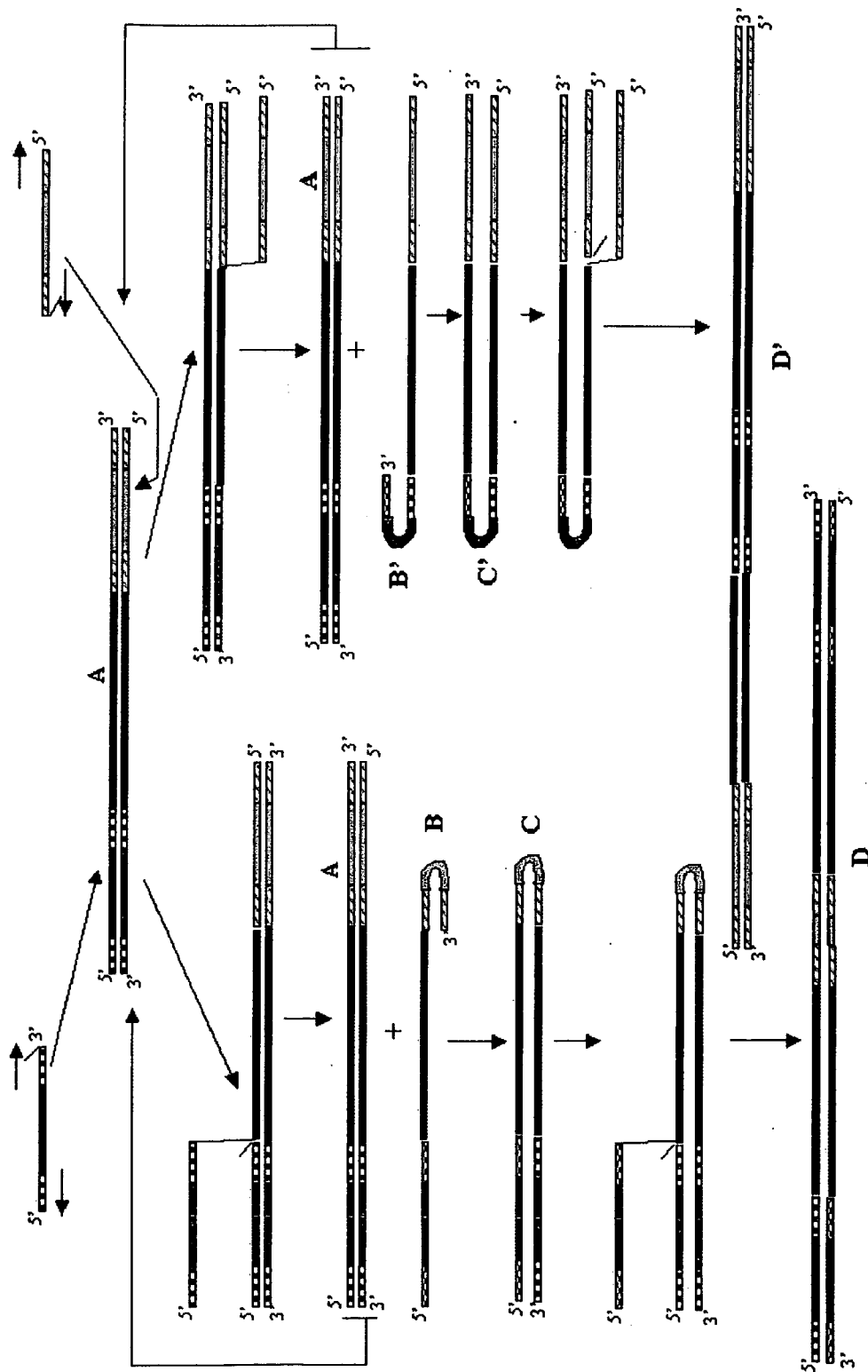ty of an assay or to prepare enough material for further processing. Among the various nucleic acid amplification techniques, polymerase chain reaction (PCR) is the most common because of its sensitivity and efficiency at amplifying short nucleic acid sequences.

[0003] While PCR is of great utility, it is also limited in a number of ways. The first limitation of PCR is that it relies on multiple cycles of thermal melting (denaturing) at high temperatures followed by hybridization and elongation at a reduced temperature. To maximize efficiency and to minimize noise, complex temperature control of multiple reactions is required. This necessitates the use of a thermocycler controllable rapid heating/cooling block made with exotic material (e.g., gold plated silver blocks), or a robotic mechanism to move samples between temperature-controlled zones. Because of the high-temperature required to melt DNA in physiological salt conditions, PCR technology requires either the addition of fresh polymerase per cycle or the use of thermostable polymerases. The approach of adding fresh polymerase has not been automated and is thus labor intensive and prone to errors (e.g., contamination, dropped tubes, labeling errors). Furthermore, the need to add enzymes and to mix each reaction individually presents serious drawbacks that have limited adaptation of enzyme-addition PCR methods to the small scale.

[0004] Compared to methods involving the addition of fresh polymerase, the use of thermostable polymerases in PCR is the most widely practiced. This approach suffers from the fact that thermostable polymerases are found in a limited number of organisms, and the replication mechanisms used by thermophilic organisms are poorly understood. The available repertoire of thermostable polymerases is limited to single polypeptide polymerase enzymes involved in DNA repair, and/or lagging strand synthesis. DNA repair and/or lagging strand polymerases are poor choices for DNA amplification because they exhibit poor processivity (distributive synthesis). In part as a consequence of using repair and/or lagging strand polymerases (e.g. Taq, Pfu, Vent polymerases), and due to the formation of inhibitory secondary or tertiary nucleic acid structures following thermal melting, current PCR protocols do not readily amplify sequences longer than several thousands of base pairs. Reliable synthesis (and amplification) of longer templates will rely on polymerases and auxiliary enzymatic complexes collectively exhibiting much higher levels of processivity, strand displacement, and secondary structure resolution, as well as limiting the formation of inhibitory higher order nucleic acid structures that may form on cooling heat-denatured DNA.

[0005] A second limitation of PCR is that it relies on solution hybridization between oligonucleotides (PCR primers) and denatured template DNA (i.e., the DNA to be amplified) in an aqueous environment. To be effective, PCR reactions are performed in a short time because the thermostable polymerases have a rapidly declining activity at PCR temperatures. Further, for effective hybridization in a short time, a feature critical to rapid turnaround, it is necessary to perform PCR in an environment with high concentrations of oligonucleotides. The high oligonucleotide concentration also ensures rapid interaction of target sequences with the oligonucleotides in competition with the heat-denatured complementary strand still present in solution. High oligonucleotide primer concentrations can cause problems, particularly when the copy number of the target sequence is low and present in a complex mixture of DNA molecules. This would be the case, for example, in a PCR of a genome to determine the genetic polymorphism in one locus.

[0006] One problem with using high oligonucleotide concentrations is that it enhances the degree of false priming at only partly matched sequences in the complex DNA mixture. False priming refers to the hybridization of a primer to a template DNA in PCR even when the primer sequence is not completely complementary to the template nucleic acid, which can lead to non-specific amplification of nucleic acids. Noise, due to false priming, increases with the oligonucleotide concentration and the complexity of total starting DNA. In addition, the possibility of false priming increases as the copy number of target sequences decreases. Where the conditions for false priming are favorable (i.e., high oligonucleotide concentration, high complexity, low copy number), errant amplified sequences can become a dominant reaction product. Consequently it can be difficult to identify conditions, and oligonucleotides, for clean amplification of target sequences from a sample DNA without an excess of false priming background. Thus a further disadvantage of using PCR is the limited success at cleanly amplifying rare target DNAs from complex sequences mixtures.

[0007] One solution to the problems of specificity and template-melting problem incurred by PCR is to employ methods that rely on the biological properties of the bacterial RecA recombinase protein, or its prokaryotic and eukaryotic relatives. These proteins coat single-stranded DNA (ssDNA) to form filaments, which then scan double-stranded DNA (dsDNA) for regions of sequence homology. When homologous sequences are located, the nucleoprotein filament strand invades the dsDNA creating a short hybrid and a displaced strand bubble known as a D-loop. The free 3'-end of the filament strand in the D-loop can be extended by DNA polymerases to synthesize a new complementary strand. The complementary strand displaces the originally paired strand as it elongates. By utilizing pairs of oligonucleotides in a manner similar to that used in PCR it should be possible to amplify target DNA sequences in an analogous fashion but without any requirement for thermal melting (thermocycling). This has the advantage both of allowing the use of heat labile polymerases previously unusable in PCR, and increasing the fidelity and sensitivity by template scanning and strand invasion instead of hybridization.

[0008] Although the use of RecA and its homologues for in vitro amplification of nucleic acids has been previously described (U.S. Pat. No. 5,223,414 to Zarling et al., referred to herein as "Zarling"), the method and results are limited. Zarling's method has critical failings that limit its ability to achieve exponential amplification of double-stranded DNA. The failure of the Zarling method to achieve exponential amplification may be due to its specification for the use of ATPγS rather than ATP. The Zarling method urges the use of ATPγS, instead of ATP, in the assembly of RecA nucleoprotein filaments because it results in a more stable RecA/ssDNA filament structure. Normally, filaments are assembled in a 5' to 3' direction and will spontaneously disassemble in the same 5' to 3' direction as RecA hydrolyzes ATP. This process is dynamic in that assembly and disassembly occurs at the same time and the amount of assembled filaments is at equilibrium. If the non-hydolyzable ATP analog, ATPγS, is used, hydrolysis of the ATPγS and the 5' to 3' disassembly of the filaments are inhibited. The great stability of RecA/ATPγS filaments, both before and after strand exchange, while helpful in the method of targeting (i.e., the Zarling method) is detrimental and unpractical for DNA amplification.

[0009] In the Zarling method, RecA protein involved in strand invasion will remain associated with the double-stranded portion of the exchanged material after strand exchange. This interaction occurs because the newly formed duplex is bound in the high-affinity site of RecA. The displaced strand occupies a different low-affinity site, unless it is bound to another single-stranded DNA binding protein (SSB), such as E. coli SSB. If ATP had been utilized to generate the exchange structure, spontaneous 5' to 3' disassembly might occur, although the exchange complex can be quite stable and may require additional factors to stimulate ATP-dependent disassembly. Regardless of whether spontaneous or stimulated, in the presence of ATPγS, 5' to 3' disassembly of the RecA filament is inhibited (Paulus, B. F. and Bryant, F. R. (1997). Biochemistry 36, 7832-8; Rosselli, W. and Stasiak, A. (1990). J Mol Biol 216, 335-52; Shan, Q. et al., (1997). J Mol Biol 265, 519-40).

[0010] These RecA/dsDNA complexes are precisely the sites targeted by the RecA/ssDNA primer complexes used to initiate subsequent rounds of invasion and synthesis. Indeed, with the RecA bound, the intermediate may not be accessible to polymerase, and certainly the dsDNAs can no longer be invaded by RecA/ssDNA primer complexes and are therefore not amplifiable from this point. Further synthesis from these templates might occur if initiated at the other end of the template, which is free of RecA, and this might eventually lead to physical displacement of the bound RecA. It is not clear, however, whether many polymerases can displace RecA in this manner. Moreover, the initiation site for that synthetic round will now be 'blocked' instead. In such a situation, amplification is only linear with time, and will predominately generate single-stranded DNA amplification products.

[0011] Thus, the described Zarling method, at best, is likely to generate little more than small quantities of ssDNA copies from each template. The linear amplification potentially given by the Zarling method will only occur in the presence of SSB, since the displaced strand will continue to bind to the second interaction site on RecA, and single-stranded DNA will not be released (Mazin, A. V. and Kowalczykowski, S. C. (1998). EMBO J. 17, 1161-8). This probably explains why the Zarling method observed additional faster-migrating fragments when they included SSB. These additional fragments were most likely displaced single-stranded fragments. Hence, in the Zarling method only linear amplification of single-stranded DNA will occur at best. There is, therefore, a need in the art for an improved recombinase-dependent DNA amplification method.

[0012] This invention utilizes two new amplification strategies that avoid any requirement for thermal melting of DNA or thermostable components. These strategies also overcome the inefficiencies of the Zarling method. As with the Zarling strategy, these methods rely on the biological properties of the bacterial RecA protein, or its prokaryotic and eukaryotic relatives, in particular, the phage T4 uvsX protein. However, in contrast to the Zarling method, these methods are devised to achieve exponential amplification of dsDNA. They achieve this by permitting rapid regeneration of targetable sequences in the target nucleic acid in the presence of dynamic recombinase/DNA filaments. Furthermore one of the methods obviates any requirement for phased replication initiation from both ends of the target nucleic acid by coupling leading and lagging strand synthesis to simultaneously generate 2 double-stranded products.

SUMMARY OF THE INVENTION

[0013] The invention provides a method of DNA amplification, termed RPA, which comprises the following steps. First, a recombinase agent is contacted with a first and a second nucleic acid primer to form a first and a second nucleoprotein primer. Second, the first and second nucleoprotein primers are contacted to a double stranded target sequence to form a first double stranded structure at a first portion of said first strand and form a double stranded structure at a second portion of said second strand so the 3' ends of said first nucleic acid primer and said second nucleic acid primer are oriented towards each other on a given template DNA molecule. Third, the 3' end of said first and second nucleoprotein primers are extended by DNA polymerases to generate first and second double stranded nucleic acids, and first and second displaced strands of nucleic acid. Finally, the second and third steps are repeated until a desired degree of amplification is reached.

[0014] The invention also provides for a method of nested RPAs. In a nested RPA, a first region of nucleic acid is amplified by RPA to form a first amplified region. Then a second region of nucleic acid that is completely within the first amplified region is amplified using RPA to form a second amplified region. This process may be repeated as often as necessary. For example, a third region of nucleic acid, which is completely within the second region, may be amplified from the second amplified region by RPA. In addition to the one, two and three rounds of RPA discussed above, the invention contemplates at least 4, and preferably at least 5 rounds of nested RPAs also.

[0015] The invention also provides for methods of detecting a genotype using RPA. This method is useful for genotyping, for detecting a normal or diseased condition, a predisposition, or a lack of a disposition for a diseased condition. Further, RPA can be used for detecting the presence of a genome, such as for example, a genome of a pathogen. In this use, the method is useful for diagnosis and detection.

[0016] The invention also details the nature and concentrations of recombinases, single-stranded binding proteins, polymerases, and nucleotides necessary to establish an effective amplification reaction. The invention further provides detailed enablement on the nature of the target DNA, the length, and composition of targeting oligonucleotides, and the inter-oligonucleotide length optimal for amplification under various conditions. The invention provides for the inclusion of additional components, or the use of modified components, that contribute to establishing a recombination-polymerase amplification system that is sensitive, robust, and with optimal signal-to-noise properties. In particular the use of more than one species of recombinase is demonstrated, and the utility of engineered and modified analogues of the recombinases *E. coli* recA and T4 bacteriophage uvsX, of polymerases including the *E. coli* DNA polymerase I Klenow fragment, Bst polymerase, Phi-29 polymerase, *Bacillus subtilis* Pol I (Bsu), as well as single-stranded DNA binding proteins from *E. coli* and T4 (the gp32 protein) is detailed.

[0017] The utility of forms of gp32 with altered cooperativity and/or strand assimilation properties is demonstrated. Also shown is the use of T4 uvsY protein, and of molecular crowding agents in particular PEG compound, to aid in establishing an optimal reaction environment. Further the present invention details effects and the possible use of other enzymes involved in DNA metabolism including toposiomerases, helicases and nucleases, in order to improve the amplification behaviour. The present invention also includes the use of optimised conditions for repeated invasion/extension of a primer targeted to a supercoiled or linear template to generate a linear amplification, and the use of this method for DNA sequencing. The present invention also describes the use of a recombinase in detection of a specific amplified product of a reaction by directing oligonucleotides labeled in some manner to the specific product species and measuring a change in the appearance or property of the reactants as a consequence.

[0018] Other embodiments, objects, aspects, features, and advantages of the invention will be apparent from the accompanying description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] FIG. 1 depicts a schematic representation of RecA/Primer Loading.

[0020] FIGS. 2A-2B depict a schematic of succeeding steps, shown in panels (A) and (B), of Leading Strand Recombinase Polymerase Amplification (lsRPA).

[0021] FIGS. 3A-3D depict a schematic of succeeding steps, shown in panels (A), (B), (C), and (D), of Leading and Lagging Strand Recombinase Polymerase Amplification.

[0022] FIG. 4 depicts an example of nested primers chosen for nested RPA.

[0023] FIG. 5 depicts examples of suitable double stranded template nucleic acids.

[0024] FIGS. 6A-6B depict the various orientations of the RPA primer pairs in hybridization with the target nucleic acid.

[0025] FIGS. 7A-7C depict a schematic representation of an RPA reaction in progress.

[0026] FIGS. 8A-8C depict (A) examples of double stranded primers; (B) double stranded primers after elongation and after annealing of the second member of a primer pair; (C) after the elongation of the second member of a primer pair with the non-invading strand displaced.

[0027] FIG. 9 depicts investigation into the nature of double-stranded DNA targets and targeting oligonucleotides. Experiments using either supercoiled templates or linearized DNAs suggest that recA catalyses the formation of intermediates capable of supporting polymerase elongation most readily on supercoiled DNA or at the ends of linearized DNA. Tester3bio oligonucleotide (SEQ ID NO:66) is shown.

[0028] FIGS. 10A-10B depict backfire synthesis. Backfire synthesis occurs when a recA-coated targeting oligonucleotide possessing a 5' overhang invades a duplex DNA end in the presence of a suitable polymerase and dNTPs. This new duplex region is stable to subsequent branch migration and can be utilised as a platform for other activities. Forward fire, that is the elongation from the invading oligonucleotide, can also occur.

[0029] FIGS. 11A-11C depict uses of backfire synthesis. Backfire synthesis can be useful because it generates a branch migration resistant structure that can be used for application other than normal oligonucleotide priming. Some examples are shown here including introduction of a nicking enzyme target site, introduction of an RNA polymerase promoter, and the linear generation of short dsDNA fragments through successive invasion/synthesis/cleavage events.

[0030] FIG. 12 depicts single stranded binding proteins facilitate recombinase invasion and primer extension. Both *E. coli* SSB and T4 gp32 with an N-terminal His tag (gp32(N)) stimulate recA-mediated invasion/elongation on a linear DNA template.

[0031] FIG. 13 depicts the requirement for a minimal oligonucleotide length or overhang for invasion and elongation during end targeting of linear templates. When the invading oligonucleotide is targeted at ends of a linearized template a minimal oligonucleotide length, or an overhang is needed for invasion/elongation to occur.

[0032] FIGS. 14A-14H depict paranemic and pletonemic joints. Schematic description of the formation of paranemic and plectonemic joints by recombination events involving DNA ends or embedded sequences.

[0033] FIGS. 15A-15B depict the effect of crowding agents. Crowding agents can alter the reaction behaviour. In the presence of polyethylene glycols, gp32(N) and recA recombinase, multiple invasion events can be stimulated on single templates without the requirement for 5' overhang in the targeting oligonucleotide.

[0034] FIG. 16 depicts end targeted amplification using leading strand RPA. Amplification of a target DNA using end-directed oligonucleotides, recA(C) protein, and the Klenow fragment of *E. coli* DNA polymerase I.

[0035] FIG. 17 depicts leading strand RPA and limits of Klenow processivity. Limited amplification of an approximately 300 base pair fragment when only 0.5 fmoles of starting template is utilised with the recA protein, gp32(N), and the Klenow fragment of *E. coli*. Strong accumulation of

shorter products suggests that the poor processivity of Klenow (10-50 nucleotides) may underlie the template concentration dependence of the reaction.

[0036] FIG. 18 depicts spacing dependence of RPA primers. There is an optimal inter-oligonucleotide length for RPA when using the Klenow fragment of *E. coli* DNA polymerase I. The template (SEQ ID NO:67) and EcoRI overhang (SEQ ID NO:68) sequences are shown.

[0037] FIGS. 19A-19C depict RPA products that are largely double stranded. RPA reaction can generate double-stranded DNA products as evidenced by agarose gel electrophoresis and restriction enzyme cleavage.

[0038] FIG. 20 depicts activity of a recA C-terminal truncation mutant. Mutant recA protein with a deletion of the C-terminal acidic peptide (recA(C)delta) can promote strand exchange and extension in a linear template run-on assay.

[0039] FIG. 21 depicts modified gp32 proteins. Shown is a schematic representation of the bacteriophage T4 gp32 proteins used in this study and the position of various modification and mutations.

[0040] FIG. 22 depicts activity of gp32 protein. Modified gp32 proteins show a variety of activities in linear invasion/run-on assays.

[0041] FIG. 23 depicts invasion and extension using uvsX. Modified uvsX protein with a C terminal His tag (uvsX(C)), or an additional deletion of the C-terminal acidic peptide, stimulates invasion/extension in a linear template run-on assay.

[0042] FIG. 24 depicts RPA using uvsX(C). The modified recombinase uvsX(C) can support DNA amplification in the presence of gp32(N), the Klenow fragment of *E. coli* DNA polymerase I and polyethylene glycol (PEG).

[0043] FIG. 25 depicts wild-type versus modified gp32. The modified version of gp32, gp32(C) is qualitatively different to wild-type untagged gp32.

[0044] FIG. 26A-26B depicts titration of gp32 and effect of uvsY. Titration of gp32 reveals a requirement for a minimal quantity of gp32, and a requirement for uvsY(N) protein when untagged gp32 is employed.

[0045] FIGS. 27A-27F depict factors affecting reaction rate and noise. Shown is a schematic representation of the factors affecting reaction behaviour, particularly reaction rate and noise. The predicted effects and interactions of gp32, uvsX, UvsY and PEG are suggested, with the conclusion that an optimal balance between reaction rate and noise must be struck.

[0046] FIG. 28 depicts effects of PEG. PEG can reduce the average length of linear invasion/run-on products in an uvsX-mediated linear run-on experiment in the presence of gp32(C).

[0047] FIGS. 29A-29I depict DNA end directed invasion. Shown is a schematic depicting possible outcomes of end-directed invasion events.

[0048] FIG. 30 depicts RPA in a complex sample. The amplification of specific DNA targets from human genomic DNA.

[0049] FIG. 31 depicts RPA sensitivity. The sensitivity of amplification of specific DNA targets from human genomic DNA.

[0050] FIG. 32 depicts RPA sensitivity and template independent artifacts. The sensitivity of amplification of specific DNA targets from human genomic DNA, and the existence of competing template-independent primer artifacts.

[0051] FIG. 33 depicts how primer artifacts may arise. Shown is a schematic representation of the possible mechanism by which primer artifacts may arise.

[0052] FIG. 34A-34D depicts primer artifact suppression. Shown in schematic are methods to suppress primer artifacts.

[0053] FIG. 35 depicts the use of hairpin oligonucleotides to stimulate self-priming of displaced strands. Shown is a schematic diagram of an amplification using self-complementary hairpin oligonucleotides deliberately to stimulate self-priming of displaced strands.

[0054] FIG. 36 depicts conditions that enable highly efficient noise-free amplification from complex DNA sources. The sensitivity of amplification of specific DNA targets from human genomic DNA under optimised conditions that reduce or eliminate primer artifacts.

[0055] FIGS. 37A-37C depict a schematic representation of RPA method as shown in (A)(i-iv), (B)(i-iv), and (C).

[0056] FIGS. 38A-38B depict: (A) STR markers from two individuals (1 and 2, father and son) amplified with primer pairs for seven independent markers using RPA conditions C4; (B) Titration of reaction components to determine concentrations that support in vitro amplification.

[0057] FIGS. 39A-39F depict: (A) Size limits of RPA reactions; (B) Elongation efficiencies from embedded or end sequences; (C) Sensitivity of RPA reactions; (D) Human DNA of the indicated copy number amplified with primers ApoB4 and Apo300 generating a 300 bp fragment using conditions C2; (E,F) Human DNA from single individuals amplified with primers D18S51 5' and 3'. Conditions employed were C2 in (E), and C4 in (F).

[0058] FIGS. 40A-40E depict specificity of RPA reactions for: (A) Primers BsA3 and BsB3, which amplify a 380 bp fragment using conditions C3. An asterisk indicates the position of the expected reaction product, and an arrow indicates the position of the genomic DNA; (B,C) Oligonucleotides Apo600bio and Apo300rev which generate a 345 bp fragment using conditions C4; (D) Mixtures of reaction components assembled in the absence of the indicated components, PEG and buffer. Primers used are indicated. Target DNA was human male genomic DNA at 150 copies/μl; (E) Oligonucleotides targeting three independent loci in human genomic DNA incubated with overlapping primer pairs of 25, 28, or 32 bases as indicated.

DETAILED DESCRIPTION OF THE INVENTION

[0059] The present invention provides for Recombinase-Polymerase Amplification (RPA)—a method for the amplification of target nucleic acid polymers. One benefit of RPA is that it may be performed without the need for thermal melting of double-stranded templates. Therefore, the need

for expensive thermocyclers is also eliminated. The present invention describes two related strategies by which RPA can be configured to permit exponential amplification of target nucleic acid polymers.

[0060] Throughout this specification, various patents, published patent applications and scientific references are cited to describe the state and content of the art. Those disclosures, in their entireties, are hereby incorporated into the present specification by reference.

[0061] Leading Strand Recombinase-Polymerase Amplification (lsRPA)

[0062] In leading strand Recombinase-polymerase Amplification (lsRPA) single-stranded, or partially single-stranded, nucleic acid primers are targeted to homologous double-stranded, or partially double-stranded, sequences using recombinase agents, which would form D-loop structures. The invading single-stranded primers, which are part of the D-loops, are used to initiate polymerase synthesis reactions. A single primer species will amplify a target nucleic acid sequence through multiple rounds of double-stranded invasion followed by synthesis. If two opposing primers are used, amplification of a fragment—the target sequence—can be achieved. LsRPA is described briefly in **FIGS. 1 and 2**.

[0063] The target sequence to be amplified, in any of the methods of the invention, is preferably a double stranded DNA. However, the methods of the invention are not limited to double stranded DNA because other nucleic acid molecules, such as a single stranded DNA or RNA can be turned into double stranded DNA by one of skill in the arts using known methods. Suitable double stranded target DNA may be a genomic DNA or a cDNA. An RPA of the invention may amplify a target nucleic acid at least 10 fold, preferably at least 100 fold, more preferably at least 1,000 fold, even more preferably at least 10,000 fold, and most preferably at least 1,000,000 fold.

[0064] The target sequence is amplified with the help of recombinase agents. A recombinase agent is an enzyme that can coat single-stranded DNA (ssDNA) to form filaments, which can then scan double-stranded DNA (dsDNA) for regions of sequence homology. When homologous sequences are located, the nucleoprotein filament (comprising the recombinase agent) strand invades the dsDNA creating a short hybrid and a displaced strand bubble known as a D-loop. Suitable recombinase agents include the *E. coli* RecA protein, the T4 uvsX protein, or any homologous protein or protein complex from any phyla. Eukaryotic RecA homologues are generally named Rad51 after the first member of this group to be identified. Other non-homologous recombinase agents may be utilized in place of RecA, for example as RecT or RecO. Recombinase agents generally require the presence of ATP, ATPγS, or other nucleoside triphosphates and their analogs. It is preferred that recombinase agents are used in a reaction environment in which regeneration of targeting sites can occur shortly following a round of D-loop stimulated synthesis. Completed recombination events involving recombinase disassembly will avoid a stalling of amplification or very inefficient linear amplification of ssDNA caused by oscillating single-sided synthesis from one end to the other.

[0065] Naturally, any derivatives and functional analogs of the recombinase agent above may also function itself as a recombinase agent and these derivatives and analogs are also contemplated as embodiments of the invention. For example, a small peptide from recA, which has been shown to retain some aspects of the recombination properties of recA, may be used. This peptide comprises residues 193 to 212 of *E. coli* recA and can mediate pairing of single stranded oligos (Oleg N. Voloshin, Lijang Wang, R. Daniel Camerini-Otero, Homologous DNA pairing Promoted by a 20-amino Acid Peptide Derived from RecA. Science Vol.272 10 May 1996).

[0066] Since the use of ATPγS results in the formation of stable Recombinase-agent/dsDNA complexes that are likely incompatible with efficient amplification, it is preferable to use ATP and auxiliary enzymes to load and maintain the Recombinase-agent/ssDNA primer complexes. Alternatively, the limitations of the use of ATPγS may be overcome by the use of additional reaction components capable of stripping recA bound to ATPγS from exchange complexes. This role might be played by helicases such as the RuvA/ RuvB complex.

[0067] The terms 'nucleic acid polymer' or 'nucleic acids' as used in this description can be interpreted broadly and include DNA and RNA as well as other hybridizing nucleic-acid-like molecules such as those with substituted backbones e.g. peptide nucleic acids (PNAs), morpholino backboned nucleic acids, locked nucleic acid or other nucleic acids with modified bases and sugars.

[0068] Structurally similar to RNA, LNA monomers are bicyclic compounds that bear a methylene linker that connects the nucleotide sugar ring's 2'-oxygen to its 4'-carbon. LNA polymers obey standard base-pairing rules, but their physical properties make them suitable for mismatch discrimination applications. LNA are available from Exiqon (Denmark) or Proligo (USA, Colorado).

[0069] One embodiment of the invention is directed to a method of performing RPA. The method comprises two steps. In the first step, the following reagents are combined in a reaction: (1) at least one recombinase; (2) at least one single stranded DNA binding protein; (3) at least one DNA polymerase; (4) dNTPs or a mixture of dNTPs and ddNTPs; (5) a crowding agent; (6) a buffer; (7) a reducing agent; (8) ATP or ATP analog; (9) at least one recombinase loading protein; (10) a first primer and optionally a second primer; and (11) a target nucleic acid molecule. In the second step, the reagents are incubated until a desired degree of amplification is achieved.

[0070] The recombinase may be uvsX, recA or a combination of both. The recombinase may also comprise a C terminal deletion of acidic residues to improve its activity. While any recombinase concentration disclosed in the specification may be used, preferred recombinase concentrations may be, for example, in the range of 0.2-12 μM, 0.2-1 μM, 1-4 μM, 4-6 μM, and 6-12 μM.

[0071] The single stranded DNA binding protein may be the *E. coli* SSB or the T4 gp32 or a derivative or a combination of these proteins. gp32 derivative may include, at least, gp32(N), gp32(C), gp32(C)K3A, gp32(C)R4Q, gp32(C)R4T, gp32K3A, gp32R4Q, gp32R4T and a combination thereof (See **FIG. 13**). The DNA binding protein may be present at a concentration of between 1 μM and 30 μM.

[0072] The DNA polymerase may be a eukaryotic polymerase. Examples of eukaryotic polymerases include pol-α,

pol-β, pol-δ, pol-ε and derivatives and combinations thereof. Examples of prokaryotic polymerase include *E. coli* DNA polymerase I Klenow fragment, bacteriophage T4 gp43 DNA polymerase, *Bacillus stearothermophilus* polymerase I large fragment, Phi-29 DNA polymerase, T7 DNA polymerase, *Bacillus subtilis* Pol I, *E. coli* DNA polymerase I, *E. coli* DNA polymerase II, *E. coli* DNA polymerase III, *E. coli* DNA polymerase IV, *E. coli* DNA polymerase V and derivatives and combinations thereof. In a preferred embodiment, the DNA polymerase is at a concentration of between 10,000 units/ml to 10 units/ml, such as between 5000 units/ml to 500 units/ml. In another preferred embodiment, the DNA polymerase lacks 3'-5' exonuclease activity. In yet another preferred embodiment, the DNA polymerase contains strand displacing properties.

[0073] Any of the proteins mentioned in the methods of the invention is understood to also include its derivative. These proteins includes at least the following: recombinases, polymerase, recombinase loading protein, single stranded DNA binding protein, accessory agents, RecA/ssDNA nucleoprotein filaments stabilizing agent and the like. A derivative of these proteins include, at least, a fusion protein comprising a C terminus tag, N terminus tag, or C and N terminus tags. Non-limiting examples of suitable sequence tags include 6-histidine (6×-His; HHHHHH; SEQ ID NO:69), Glu-Glu, c-myc epitope (EQKLISEEDL; SEQ ID NO:70), FLAG® octapeptide (DYKDDDDK; SEQ ID NO:71), Protein C (EDQVDPRLIDGK; SEQ ID NO:72), Tag-100 (EETARFQPGYRS; SEQ ID NO:73), V5 epitope (GKPIPNPLLGLDST; SEQ ID NO:74), VSV-G (YTDIEM-NRLGK; SEQ ID NO:75), Xpress (DLYDDDDK; SEQ ID NO:76), and hemagglutinin (YPYDVPDYA; SEQ ID NO:77). Non-limiting examples of suitable protein tags include α-galactosidase, thioredoxin, His-patch thioredoxin, IgG-binding domain, intein-chitin binding domain, T7 gene 10, glutathione-S-transferase (GST), green fluorescent protein (GFP), and maltose binding protein (MBP). It will be understood by those in the art that sequence tags and protein tags can be used interchangeably, e.g., for purification and/or identification purposes. Accordingly, as used herein, the terms "His tag" and "hexahistidine tag" encompass all suitable sequence tags and protein tags as known in the art and indicated in this paragraph.

[0074] The dNTPs includes, for example, dATP, dGTP, dCTP, and dTTP. In leading and lagging strand RPA, ATP, GTP, CTP, and UTP may also be included for synthesis of RNA primers. In addition, ddNTPs (ddATP, ddTTP, ddGTP and ddGTP) may be used to generate fragment ladders. The dNTP may be used at a concentration of between 1 μM to 200 μM of each NTP species. A mixture of dNTP and ddNTP may be used with ddNTP concentrations at $\frac{1}{100}$ to $\frac{1}{1000}$ of that of the dNTP (1M to 200 μM).

[0075] The crowding agents used in the RPA include polyethylene glycol (PEG), dextran and ficoll. The crowding agent may be at a concentration of between 1% to 12% by volume or 1% to 12% by weight of the reaction. While all PEGs are useful, preferred PEGs include PEG1450, PEG3000, PEG8000, PEG10000, PEG compound molecular weight 15000-to 20,000 (also known as Carbowax 20M), and a combination thereof.

[0076] The buffer solution in an RPA may be a Tris-HCl buffer, a Tris-Acetate buffer, or a combination thereof. The buffers may be present at a concentration of 10 to 100 mM. The buffered pH may be 6.5 to 9.0. The buffer may further contain Mg ions (e.g., in the form of Mg Acetate) at a concentration of 1 to 100 mM with a concentration of 5 to 15 mM being preferred. One preferred Mg concentration is 10 mM (Mg concentration or Mg Acetate concentration).

[0077] Reducing agents to be used include DTT. The DTT concentration may be between 1 mM and 10 mM.

[0078] The ATP or ATP analog may be any of ATP, ATP-γ-S, ATP-β-S, ddATP or a combination thereof. A preferred ATP or ATP analog concentration is between 1 and 10 mM.

[0079] Recombinase loading protein may include, for example, T4uvsY, *E. coli* recO, *E. coli* recR and derivatives and combinations of these proteins. One preferred concentration of these proteins is between 0.2 and 8 μM.

[0080] The primers used may be made from DNA, RNA, PNA, LNA, morpholino backbone nucleic acid, phosphorothiorate backbone nucleic acid and a combination thereof. Combinations thereof in this application can refer to single nucleic acid molecules which may contain one or more of one base connected to one of more of another base. Preferred concentration of these molecules may be in the range of between 25 nM to 1000 nM. In one preferred embodiment, the primers may contain a non-phosphate linkage between the two bases at its 3' end and is resistant to 3' to 5' nuclease activity. In another embodiment, the primers may contain a locked nucleic acid at its 3' ultimate base or 3' penultimate base. For example, in a nucleic acid of the sequence 5'-AGT-3', the T is the 3' ultimate base and the G is the 3' penultimate base. The primers may be at least 20 bases in length or at least 30 bases in length. In one preferred embodiment, the primers are between 20 to 50 bases in length. In another preferred embodiment, the primers are between 20 to 40 bases in length such as between 30 to 40 bases in length.

[0081] The primers may contain additional 5' sequence that is not complementary to the target nucleic acid. These 5' sequence may contain, for example a restriction endonuclease recognition site. The primers may be partly double stranded with a single stranded 3' end.

[0082] In addition, any nucleic acid of any of the methods of the invention may be labeled with a detectable label. A detectable label includes, for example, a fluorochrome, an enzyme, a fluorescence quencher, an enzyme inhibitor, a radioactive label, and any combination thereof.

[0083] The target nucleic acid may be single stranded or double stranded. It is known that single stranded nucleic acids would be converted to double stranded nucleic acid in the methods of the invention. The target nucleic acid may be supercoiled or linear. The sequence to be amplified (target nucleic acid) may be in between other sequences. The sequence to be amplified may also be at one end of a linear nucleic acid. In one embodiment, the target nucleic acid is linear and not connected to non-target nucleic acids. In other words, where the target nucleic acid is linear, it can be in any of the following formats:

[0084] 1. [non-target nucleic acid]-[target nucleic acid]-[non-target nucleic acid]

[0085] 2. [non-target nucleic acid]-[target nucleic acid]

[0086] 3. [target nucleic acid]-[non-target nucleic acid]

[0087] 4. [target nucleic acid]

[0088] It should be noted that the arrangement above is intended to represent both single stranded nucleic acids and double stranded nucleic acids. "1" may be described as a target nucleic acid molecule which is linear with two ends and wherein both ends are linked to a non-target nucleic acid molecule. "2" may be described as a target nucleic acid molecule which is linear with two ends and wherein one end is linked to a non-target nucleic acid molecule. "3" may be described as a target nucleic acid molecule which is a linear nucleic acid molecule (with no non-target nucleic acid).

[0089] In another embodiment, the target nucleic acid may be a single-stranded nucleic acid which is converted to a double stranded nucleic acid by a polymerase or a double stranded nucleic acid denatured by the action of heat or chemical treatment.

[0090] The target nucleic acid may be of any concentration such as less than 10,000 copies, less than 1000 copies, less than 100 copies, less than 10 copies or 1 copy in a reaction. A reaction volume may be 5 μl, 10 μl, 20 μl, 30 μl, 50 μl, 75 μl, 100 μl, 300 μl, 1 ml, 3 ml, 10 ml, 30 ml, 50 ml or 100 ml.

[0091] The reaction may be incubated between 5 minutes and 16 hours, such as between 15 minutes and 3 hours or between 30 minutes and 2 hours. The incubation may be performed until a desired degree of amplification is achieved. The desired degree of amplification may be 10 fold, 100 fold, 1,000 fold, 10,000 fold, 100,000 fold, or 1,000,000 fold amplification. Incubation temperature may be between 20° C. and 50° C., between 20° C. and 40° C., such as between 20° C. and 30° C. One advantage of the methods of the invention is that the temperature is not critical and precise control, while preferred, is not absolutely necessary. For example, in a field environment, it is sufficient to keep the RPA at room temperature, or close to body temperature (35° C. to 38° C.) by placing the sample in a body crevice. Furthermore, the RPA may be performed without temperature induced melting of the template nucleic acid.

[0092] In another embodiment of the invention, the RPA further comprise accessory agents. These accessory agents include helicase, topoisomerase, resolvase, and a combination thereof which possess unwinding, relaxing, and resolving activities respectively on DNA. The accessory agents may also include RuvA, RuvB, RuvC, RecG, PriA, PriB, PriC, DnaT, DnaB, DnaC, DnaG, DnaX clamp loader, polymerase core complex, DNA ligase and a sliding clamp and a combination thereof. The sliding claim may be E. coli β-dimer sliding clamp, the eukaryotic PCNA sliding clamp, or the T4 sliding clamp gp45 and a combination thereof. The accessory agents may include, in addition, DNA Polymerase III holoenzyme complex consisting of β-Clamp, DnaX Clamp Loader, and the Polymerase Core Complex. These latter accessory agents would allow the performance of leading and lagging RPA.

[0093] In another embodiment, the RPA may be performed in the presence of a RecA/ssDNA nucleoprotein filaments stabilizing agent. Examples of such stabilizing include RecR, RecO, RecF and a combination thereof. These stabi-lizing agents may be present at a concentration of between 0.01 μM to 20 μM. Other examples of stabilizing agents include the T4 uvsY protein which stabilizes uvsX/ssDNA nucleoprotein complexes.

[0094] Other components of RPA include a system for ATP regeneration (convert ADP to ATP). Such system may be, for example, phosphocreatine and creatine kinase.

[0095] The RPA reaction may also include a system to regenerate ADP from AMP and a to convert pyrophosphate to phosphate (pyrophosphate).

[0096] In one preferred embodiment, the RPA reaction as listed above is performed with E. coli components completely by using recA, SSB, recO, recR, and E. coli polymerase.

[0097] In another preferred embodiment, the RPA reaction is performed with T4 components by using uvsX, gp32, uvsY, and T4 polymerase.

[0098] In one preferred embodiment, RPA may be performed by combining the following reagents: (1) a uvsX recombinase at a concentration of between 0.2 to 12 μM; (2) a gp32 single stranded DNA binding protein at a concentration between 1 to 30 μM; (3) a Bacillus subtilis DNA polymerase I large fragment (Bsu polymerase) at a concentration between 500 to 5000 units per ml; (4) dNTPs or a mixture of dNTPs and ddNTPs at a concentration of between 1-300 μM; (5) polyethylene glycol at a concentration of between 1% to 12% by weight or by volume; (6) Tris-acetate buffer at a concentration of between 1 mM to 60 mM; (7) DTT at a concentration of between 1 mM-10 mM; (8) ATP at a concentration of between 1 mM-10 mM; (9) uvsY at a concentration of between 0.2 μM-8 μM; (10) a first primer and optionally a second primer, wherein said primers are at a concentration of between 50 nM to 1 μM; and (11) a target nucleic acid molecule of at least one copy. After the reaction is assembled, it is incubated until a desired degree of amplification is achieved. This is usually within 2 hours, preferably within 1 hour, such as, for example, in 50 minutes.

[0099] One advantage of the invention is that the reagents for RPA, with the possible exception of the crowding agent and buffer, may be freeze dried (i.e., lyophilzed) before use. Freeze dried reagent offer the advantage of not requiring refrigeration to maintain activity. For example, a tube of RPA reagents may be stored at room temperature. This advantage is especially useful in field conditions where access to refrigeration is limited.

[0100] In one embodiment, the RPA reagents may be freeze dried onto the bottom of a tube, or on a bead (or another type of solid support). To perform an RPA reaction, the reagents are reconstituted in a buffer solution and with a crowding reagent, or simply a buffered solution or water, dependant on the composition of the freeze-dried reagents. Then a target nucleic acid, or a sample suspected to contain a target nucleic acid is added. The reconstitution liquid may also contain the sample DNA. The reconstituted reaction is incubated for a period of time and the amplified nucleic acid, if present, is detected.

[0101] Detection may be performed using any method, such as, for example, using electrophoresis on an agarose or PAGE gel followed by ethidium bromide staining.

[0102] In any of the methods of the invention, the reagents that can be freeze dried before use would include, at least, the recombinase, the single stranded DNA binding protein, the DNA polymerase, the dNTPs or the mixture of dNTPs and ddNTPs, the reducing agent, the ATP or ATP analog, the recombinase loading protein, and the first primer and optionally a second primer or a combination of any of these.

[0103] In one preferred embodiment, the reagents are assembled by combining the reagents such that when constituted, they will have the following concentrations: (1) a uvsX recombinase at a concentration of between 0.2 to 12 μM; (2) a gp32 single stranded DNA binding protein at a concentration between 1 to 30 μM; (3) a T4 gp43 DNA polymerase or Bsu polymerase at a concentration between 500 to 5000 units per ml; (4) dNTPs or a mixture of dNTPs and ddNTPs at a concentration of between 1-300 μM; (5) DTT at a concentration of between 1 mM-10 mM; (6) ATP at a concentration of between 1 mM-10 mM; (7) uvsY at a concentration of between 0.2 μM-8 μM. Optionally, a first primer and optionally a second prime may be added where their concentration would be between 50 nM to 1 μM when reconstituted. The reagents are freeze dried before use. Stabilizing agents such as trehalose sugar may be included in the freeze dried mixture, for example at 20 mM to 200 mM and most optimally 40 mM to 80 mM in the reconstituted reaction, in order to improve freeze-drying performance and shelf life. If desired, the freeze dried reagents may be stored for 1 day, 1 week, 1 month or 1 year or more before use.

[0104] In use, the reagents are reconstituted with buffer (a) Tris-acetate buffer at a concentration of between 1 mM to 60 mM; and (b) polyethylene glycol at a concentration of between 1% to 12% by weight or by volume, or (c) with water. If the primers were not added before freeze drying, they can be added at this stage. Finally, a target nucleic acid, or a sample suspected of containing a target nucleic acid is added to begin the reaction. The target, or sample, nucleic acid may be contained within the reconstitution buffer as a consequence of earlier extraction or processing steps. The reaction is incubated until a desired degree of amplification is achieved.

[0105] Any of the RPA reaction conditions discussed anywhere in this specification may be freeze dried. For example, the following reagents can be assembled by combining each reagent such that when constituted, they will have the following concentrations: (1) 100-200 ng/μl uvsX recombinase; (2) 600 ng/μl gp32; (3) 20 ng/μl Bsu polymerase or T4 polymerase; (4) 200 μM dNTPs; (5) 1 mM DTT (6) 3 mM ATP or an ATP analog; (7) 16 ng/μl to 60 ng/μl uvsY; (8) 50 nM to 300 nM of a first primer and 50 nM to 300 nM of a second primer; (9) 80 mM Potassium acetate; (10) 10 mM Magnesium acetate; (11) 20 mM Phosphocreatine; (12) 50 ng/μl to 100 ng/μl Creatine kinase. The reagents may be freeze dried onto the bottom of a tube or in a well of a multi-well container. The reagent may be dried or attached onto a mobile solid support such as a bead or a strip, or a well.

[0106] In use, the tube with the reagent may be reconstituted with (1) Tris-acetate buffer at a concentration of between 1 mM to 60 mM and polyethylene glycol at a concentration of between 1% to 12% by weight or by volume. If the reagents were dried or attached to a mobile

solid support, the support may be dropped in a tube and reconstituted. As discussed above, the primers may be dried as part of the reagent or added after reconstitution. Finally, a target nucleic acid, or a sample suspected of containing a target nucleic acid is added to begin the reaction. The reaction is incubated until a desired degree of amplification is achieved.

[0107] As another example, the following reagents can be assembled by combining each reagent such that when constituted, they will have the following concentrations: (1) 100-200 ng/μl uvsX recombinase; (2) 300-1000 ng/μl gp32; (3) 10-50 ng/μl Bsu polymerase or T4 polymerase; (4) 50-500 μM dNTPs; (5) 0.1 to 10 mM DTT; (6) 3 mM ATP or an ATP analog; (7) 16 ng/μl to 60 ng/μl uvsY; (8) 50 nM to 1000 nM of a first primer and 50 nM to 1000 nM of a second primer; (9) 40 mM to 160 mM Potassium acetate; (10) 5 mM to 20 mM Magnesium acetate; (11) 10 mM to 40 mM Phosphocreatine; (12) 50 ng/μl to 200 ng/μl Creatine kinase. These reagents are freeze dried and stored. In use, the reagents are reconstituted with Tris-acetate buffer at a concentration of between 1 mM to 60 mM and polyethylene glycol at a concentration of between 1% to 12% by weight or by volume. The primers, item 8 above, may be omitted before freeze drying and added after reconstitution. To initiate the RPA, a target nucleic acid, or a sample suspected of containing a target nucleic acid is added. The reaction is incubated until a desired degree of amplification is achieved.

[0108] Another embodiment of the invention comprises a kit for performing RPA. The kit may comprise any of the reagents discussed above for RPA in the concentrations described above. The reagents of the kit may be freeze dried. For example, the kit may contain (1) 100-200 ng/μl uvsX recombinase; (2) 300 ng/μl to 1000 ng/μl gp32; (3) 10 ng/μl to 50 ng/μl Bsu polymerase or T4 polymerase; (4) 50 μM to 500 μM dNTPs; (5) 0.1 to 10 mM DTT; (6) 1 mM to 5 mM ATP or an ATP analog; (7) 16 ng/μl to 60 ng/μl uvsY; (8) 50 nM to 1000 nM of a first primer and 50 nM to 1000 nM of a second primer (optional); (9) 40 mM to 160 mM Potassium acetate; (10) 5 mM to 20 mM Magnesium acetate; (11) 10 mM to 40 mM Phosphocreatine; (12) 50 ng/μl to 200 ng/μl Creatine kinase.

[0109] In a preferred embodiment, RPA is performed with several auxiliary enzymes that can promote efficient disassembly of Recombinase-agent/dsDNA complexes after DNA synthesis initiation. These auxiliary enzymes include those that are capable of stimulating 3' to 5' disassembly and those capable of supporting 5' to 3' disassembly.

[0110] Auxiliary enzymes include several polymerases that can displace RecA in the 3' to 5' direction and can stimulate 3' to 5' disassembly of Recombinase-agent/dsDNA complexes (Pham et al., 2001). These DNA polymerase include E. coli Poly and homologous polymerase of other species. Normally in the life of E. coli, displacement of RecA in the 3' to 5' direction occurs as part of SOS-lesion-targeted synthesis in concert with SSB, sliding clamps and DNA polymerase. The polymerase essential for this activity in E. coli is Poly, a member of the recently discovered superfamily of polymerases including UmuC, DinB, Rad30, and Rev1, whose function in vivo is to copy DNA lesion templates. Critical to RPA, the in vitro 3' to 5' disassembly of RecA filaments cannot be catalyzed by PolI, PolIII, or PolIV alone. Only Poly, in concert with SSB, has measur-

able ATP-independent 3' to 5' RecA/dsDNA disassembly activity. In effect, Poly pushes and removes RecA from DNA in a 3' to 5' direction ahead of the polymerase (Pham et al., 2001; Tang et al., 2000). Inclusion of Poly or a functional homologue may improve the amplification efficiency.

[0111] Other auxiliary enzymes include a class of enzymes called helicases that can be used to promote the disassembly of RecA from dsDNA. These promote disassembly in both the 5' to 3' and 3' to 5' directions. Helicases are essential components of the recombination process in vivo and function to move the branch points of recombination intermediates from one place to another, to separate strands, and to disassemble and recycle components bound to DNA. After the first round of invasion/synthesis has occurred in RPA, two new DNA duplexes are "marked" by the presence of RecA bound over the site to which primers must bind for additional rounds of synthesis. In such a situation dsDNA tends to occupy the high affinity site in RecA, or homologs, until it is actively displaced, either by ATP hydrolysis-dependent dissociation in the 5' to 3' direction, which may be limiting, or by 3' to 5' dissociation by some other active process. An ideal helicase complex for stimulating disassembly of RecA from intermediates consists of the *E. coli* proteins RuvA and RuvB. The RuvAB complex promotes branch migration, and dissociates the RecA protein, allowing RecA to be recycled (Adams et al., 1994). Normally, the RuvAB complex is targeted to recombination intermediates, particularly Holliday junction-like structures. As it works the RuvAB complex encircles DNA and forces RecA from the DNA in an ATP-driven translocation (Cromie and Leach, 2000; Eggleston and West, 2000). This RecA dissociation activity has been demonstrated using supercoiled dsDNA bound with RecA, which does not even possess Holliday junctions (Adams et al., PNAS 1994). The RuvAB complex can recognize branched structures within the RecA coated DNA. Incorporation of RuvAB into the RPA mixture will promote the dissociation of RecA from dsDNA following strand exchange and displacement, allowing renewed synthesis of the duplicated template from the same site. Additionally, the RuvAB complex can act in concert with RuvC, which finally cuts and resolves Holliday junctions. With RuvC added to the RPA reaction mixture, complicated structures such as Holliday junctions formed at invasion sites, can be resolved. Resolvase activity, such as that provided by RuvC, is particularly important when the targeting oligonucleotides are partially double-stranded. In such situations reverse branch migration can generate Holliday junctions, which can then be resolved by the RuvABC complex, to generate clean separated amplification products.

[0112] Still other auxiliary enzymes include the *E. coli* RecG protein. RecG can stimulate disassembly of branch structures. In vivo this protein functions to reverse replication forks at sites of DNA damage by unwinding both leading and lagging strands driving the replication fork back to generate a 4-way junction (Cox et al., 2000; Dillingham and Kowalczykowski, 2001; Singleton et al., 2001). In vivo such junctions function as substrates for strand switching to allow lesion bypass. In vitro RecG will bind to D-loops, and will lead to a decrease in D-loop structures by driving reverse branch migration. RecG prefers a junction with double-stranded elements on either side, hence partly double-stranded targeting oligonucleotides, homologous to the targeting site in both single-stranded and double-stranded regions, would be ideal. This would stimulate

reverse branch migration and formation of a Holliday junction, which can be resolved by the RuvABC complex. In vivo RecG and RuvAB may compete to give different outcomes of recombination since branch migration will be driven in both directions (McGlynn and Lloyd, 1999; McGlynn et al., 2000). In both cases the proteins target junction DNA coated with RecA, and disassemble it in an active manner.

[0113] Other auxiliary enzymes useful in an RPA reaction mixture are those that allow continual generation of RecA nucleoprotein filaments in the presence of ATP and SSB. In order to allow removal of RecA at the appropriate moments, it is preferred to use ATP rather than ATPγS in an RPA reaction. Unfortunately RecA/ssDNA filaments formed with ATP spontaneously depolymerize in the 5' to 3' direction, and in the presence of SSB, as required here, repolymerization will not occur at significant rates. The solution to this problem is the use of the RecO, RecR, and possibly RecF proteins. Alternatively the uvsY protein may be employed to stabilize the T4 uvsX nucleoprotein filaments in a similar manner. In the presence of SSB and ATP, RecA/ssDNA filaments dissociate (Bork et al., 2001; Webb et al., 1995; Webb et al., 1997; Webb et al., 1999). If RecA/ssDNA is incubated in the presence of RecO and RecR proteins this dissociation does not occur. Indeed the RecR protein remains associated with the filament and stabilizes the structure indefinitely. Even if ssDNA is bound by SSB, in the presence of RecR and RecO, filaments of RecA can reassemble displacing SSB. In the T4 phage system similar properties are attributed to the uvsY protein. Thus it is possible to obviate the use of ATPγS, if necessary, by using ATP in the presence of RecO and RecR to maintain RecA/ssDNA filament integrity, or uvsY to maintain the uvsX/ssDNA filament integrity. The RecF protein interacts with the RecO and RecR system in a seemingly opposing manner. RecF competes with RecR tending to drive filament disassembly in vitro. It is likely that all three components in vivo function together to control the generation of invading structures, while limiting the extent of RecA coating of ssDNA. In another preferred embodiment, RecF is included in RPA reactions at an appropriate concentration to recapitulate the dynamics of the in vivo processes. In addition, RecF may facilitate dissociation of RecA-coated intermediates after invasion has occurred.

[0114] As described, the use of ATP rather than ATPγS, and/or the use of displacing polymerases and helicases (e.g. the RuvA/RuvB complex), RecO, RecR and RecF, or alternatively the T4 uvsX recombinase with the uvsY protein, should permit exponential amplification of double-stranded DNA by driving continual regeneration of targeting sites. This method, however, remains responsive to differences in initiation rate that might occur at the two opposing targeting sites. Such differences may lead to a decrease in amplification efficiency, and to the production of some single-stranded DNA. The PCR method largely avoids these complications because temperature cycling leads to coordinated synthesis from either side. In another embodiment, a situation analogous to the PCR condition just described may be induced by using temperature sensitive (ts) mutants of RecA that are non-functional at 42° C., but function at lower temperatures in the range 25 to 37° C. (Alexseyev et al., 1996; Hickson et al., 1981). In this case, synthesis from either end can be synchronized by periodic lowering to the permissive temperature and then raising the reaction to a

temperature non-permissive for the mutant RecA protein function, but permissive for the other components. By performing RPA with tsRecA mutants in combination with cycling of reaction temperatures, the number of molecules of DNA produced can be controlled. While this will require some mechanism to provide temperature cycling, the temperatures are well below those that would require the use of thermophile-derived proteins. Indeed, a simple chemical-based or portable low-power temperature-cycling device may be sufficient to control such reaction cycles.

[0115] RPA, as all other present-day nucleic acid amplification methods, employs polymerases to generate copies of template nucleic acid molecules. It is a necessity of most nucleic acid polymerases that incorporation requires a free 3'-hydroxyl moiety on the terminal sugar of a short stretch of double-stranded nucleic acid adjacent to the site of new synthesis. This stretch of double-stranded nucleic acid is typically formed on a template by a short complementary sequence, called a primer, which serves as an initiation site for the polymerase synthesis reaction. In some cases a 3' modification, such as a sulfydryl, may utilized to prime the synthesis reaction. The primer nucleic acid, which is base-paired with the template and extended by the polymerase, can be RNA or DNA. In vivo during genomic DNA replication, RNA primer sequences are synthesized de novo onto template DNA by primase enzymes. Typically, for in vitro reactions the primer is supplied as a short, often chemically synthesized, single-stranded DNA (or modified DNA or RNA), and is usually referred to as an oligonucleotide primer. The primer is often of a specific sequence, although random primers can also be used. The primer is targeted to complementary sequences by virtue of its specific base-pairing capacity. Formation of hybrids between the oligonucleotide primer and target nucleic acid are typically formed by incubation of the two in solution under conditions of salt, pH, and temperature that allow spontaneous annealing.

[0116] In the case of the PCR the oligonucleotide primer is usually in vast excess for two main reasons. First, the high concentration will drive rapid annealing. Second, as the reaction proceeds through rounds of melting, annealing and extension the primer is consumed and becomes limiting. PCR targeted nucleic acids are often initially double stranded in character, and if not, become double stranded following the first synthetic cycle. Such double-stranded molecules cannot anneal new oligonucleotides at temperature and solvent conditions appropriate for the catalytic activity and stability of most prokaryotic and eukaryotic proteins. Consequently, in order to allow cycles of amplification the original template and the newly synthesized strands must be first separated before annealing can occur once again. In practice this is achieved by thermal melting. For PCR, temperatures of at least 80° C. are required for thermal melting of most double-stranded nucleic acid molecules of lengths greater than 100 base pairs. In most PCR protocols a temperature of 90 to 100° C. is applied to melt the DNA. Such temperatures allow only rare thermostable enzymes to be used. These polymerases are typically derived from thermophilic prokaryotes.

[0117] The advantage of RPA is that it allows the formation of short stretches of double-stranded nucleic acids bearing a free 3' OH for extension from double-stranded templates without thermal melting. This is achieved by using

the RecA protein from *E. coli* (or a RecA relative from other phyla including the T4 uvsX protein). In the presence of ATP, dATP, ddATP, UTP, ATPγS, and possibly other types of nucleoside triphosphates and their analogs, RecA or uvsX will form a nucleoprotein filament around single-stranded DNA. This filament will then scan double-stranded DNA. When homologous sequences are located the recombinase will catalyze a strand invasion reaction and pairing of the oligonucleotide with the homologous strand of the target DNA. The original pairing strand is displaced by strand invasion leaving a bubble of single stranded DNA in the region.

[0118] RecA protein can be obtained from commercial sources. Alternatively it can be purified according to standard protocols e.g. (Cox et al., 1981; Kuramitsu et al., 1981). RecA homologues have been purified from thermophilic organisms including *Thermococcus kodakaraensis* (Rashid et al., 2001), *Thermotoga maritima* (Wetmur et al., 1994), *Aquifex pyrophilus* (Wetmur et al., 1994), *Pyrococcus furiosus* (Komori et al., 2000), *Thermus aquaticus* (Wetmur et al., 1994), *Pyrobaculum islandicum* (Spies et al., 2000), and *Thermus thermophilus* (Kato and Kuramitsu, 1993). RecA has also been purified from other prokaryotes e.g. *Salmonella typhimurium* (Pierre and Paoletti, 1983), *Bacillus subtilis* (Lovett and Roberts, 1985), *Streptococcus pneumoniae* (Steffen and Bryant, 2000), *Bacteroides fragilis* (Goodman et al., 1987), *Proteus mirabilis* (West et al., 1983), *Rhizobium meliloti* (Better and Helinski, 1983), *Pseudomonas aeruginosa* (Kurumizaka et al., 1994), from eukaryotes e.g. *Saccharomyces cerevisiae* (Heyer and Kolodner, 1989), *Ustilago maydis* (Bennett and Holloman, 2001), including vertebrates e.g. Human Rad51 (Baumann et al., 1997) and *Xenopus laevis* (Maeshima et al., 1996), as well as plants including broccoli (Tissier et al., 1995). We here also show that *E. coli* recA, and T4 uvsX protein, can be purified from overexpression cultures using a hexahistidine tag at the C terminus, and remain biologically active. This is of great utility for the production of recombinant protein.

[0119] For clarity of description, leading strand Recombinase-Polymerase Amplification method (lsRPA) can be divided into four phases.

[0120] Sequence Targeting

[0121] RPA is initiated by targeting sequences using synthetic oligonucleotides coated with RecA, or a functional homologue such as the T4 uvsX protein. In order to permit exponential amplification, two such synthetic oligonucleotides would be employed in a manner such that their free 3'-ends are orientated toward one another. Nucleoprotein filaments comprising these oligonucleotides and recombinase protein will identify targets in complex DNA rapidly and specifically. Once targeted the recombinase protein catalyses strand exchange such that D-loop structures are formed. It may be necessary to use ATP rather than ATPγS in the procedure for efficient amplification. If ATP is used, RecO, RecR, and/or RecF, molecules may prove essential for efficient amplification, or uvsY protein if uvsX recombinase is employed.

[0122] Initiation of DNA Synthesis

[0123] DNA polymerases will detect and bind to the hybrid between the invading oligonucleotides and the template DNA and initiate DNA synthesis from the free 3'-hy-

droxyl exposed in the hybrid. Exposure of this 3'-hydroxyl, and subsequent DNA synthesis, will likely require disassembly of recombinase protein from the double-stranded hybrid formed by strand exchange. To attain this disassembly it will probably be necessary to employ ATP, which can support spontaneous disassembly of recombinase from invasion complexes. Additionally disassembly can be stimulated/enhanced by the use of other proteins contained within the reaction mixture such as RuvA, RuvB, RuvC, recG, other helicases, or other stimulatory components, which can act to strip recombinase from the strand exchange product.

[0124] Strand Displacement DNA Synthesis and Replicon Separation.

[0125] As the DNA polymerases synthesize complementary copies of template DNAs using the free 3'-hydroxyls of invading oligonucleotides, or their partly extended products, the polymerases displace single-stranded DNAs, which may be coated with single strand binding proteins (SSB) included in the reaction. In an ideal configuration, invasion of oligonucleotides at both ends of the target nucleic acid sequence will occur in similar timeframes, such that two polymerases on the same template nucleic acid will initially progress toward one another. When these extending complexes meet one another, the original template should simply fall apart, and the polymerases will continue to synthesize without a need for strand displacement, now copying SSB-bound ssDNA template. Because of steric hinderance, polymerases may become dissociated from the template temporarily when the polymerases meet to permit the separation of the two template strands

[0126] Completion of Synthesis and Re-Invasion.

[0127] Once the template strands have separated, polymerases can complete the extension to the end of the template (or past the sequence acting as the second, facing, targeting site if the initial template is longer than the desired product). To permit exponential amplification it is necessary for new products to be targeted and replicated in a manner similar to the original templates, that is from both targeted ends. The newly synthesized targeted site will be freely available to targeting recombinase/oligonucleotide filaments. The site initially used to prime synthesis should also have been freed as a consequence of the use of conditions in the reaction that favor disassembly of recombinase from strand exchange products. Providing the re-invasion at this latter site occurs in less time than it takes the polymerase to synthesize past the second targeting site, be primed at that second site, and return to the first site, then single-stranded DNA will not be the primary product and exponential amplification will occur. Having multiple synthetic complexes operating on the same template raises the possibility that very short amplification times can be achieved.

[0128] Recombinase-Polymerase Amplification (RPA) Using Simultaneous Leading and Lagging Strand Synthesis

[0129] In our description of (leading strand RPA) lsRPA we detail a multi-component system with the capacity to regenerate targeting sequences thus permitting exponential amplification of double-stranded DNA. Unlike the Zarling method, lsRPA avoids the linear production of single-stranded DNA. There is another approach to solving this problem that completely avoids the possibility of single-stranded products and a requirement for simultaneous end

initiation. This method necessarily involves a more complex reaction mixture. Nevertheless all of the required components are now well understood and should be amenable to assembly into a single system. This system will recapitulate events occurring during the normal replication cycle of cells to permit coupled leading and lagging strand synthesis. This method, leading/lagging strand RPA is described briefly in **FIGS. 1 and 3**.

[0130] During normal replication in vivo, double-stranded DNA is simultaneously separated into 2 strands and both are copied to give 2 new molecules of double-stranded DNA by a replication machine. This 'machine' couples conventional 5' to 3' leading strand synthesis with lagging strand synthesis, in which short RNA primers are synthesized onto template nucleic acids by primase enzymes. During lagging strand synthesis, short fragments of DNA are produced, called Okazaki fragments, which are ligated together to form contiguous lagging strands. This simultaneous leading-strand/lagging-strand synthesis is responsible for duplication of the entire genome of prokaryotic and eukaryotic organisms alike. The essential components of this system have been identified and characterized biochemically. The components can be assembled in vitro to achieve a more efficient amplification than possible using only leading-strand synthesis.

[0131] The essential components of the replication 'machine' are now well characterized for *E. coli* and certain other organisms such as T4 phage. This machine comprises the PolIII holoenzyme (Glover and McHenry, 2001; Kelman and O'Donnell, 1995) and the primosome (Benkovic et al., 2001; Marians, 1999). The PolIII holoenzyme is made up of ten polypeptide components. Each holoenzyme contains two, asymmetrically oriented, core structures, each consisting of a polymerase (α subunit) and two additional core components the ϵ subunit, which possesses 3' to 5' exonuclease activity, and the 0 subunit. In addition to the core complex another set of polypeptides provide the holoenzyme with processivity and couple leading and lagging strand synthesis. The β-dimer sliding clamp encircles the template DNA affixing the complex to the template with extremely high affinity. The sliding clamp loaded onto DNA by the DnaX clamp loader comprising the $\tau_2\gamma\delta\delta'\chi\psi$ polypeptide subunits.

[0132] For clarity of description, the RPA method can be divided into four phases. In reality all phases will occur simultaneously in a single reaction.

[0133] 1) Sequence Targeting

[0134] RPA is initiated by targeting sequences using synthetic oligonucleotides coated with RecA, or T4 uvsX, or a functional homologue. Such nucleoprotein filaments will identify targets in complex DNA rapidly and specifically. Once targeted, the RecA or uvsX protein catalyses strand exchange such that a D-loop structure is formed. It may be necessary to use ATP rather than ATPγS in the procedure for efficient amplification. The linkage of leading and lagging strand syntheses however may obviate the requirement for very rapid recombinase stripping after initiation of synthesis. If ATP is used, RecO, RecR, and RecF may need to be employed with bacterial recA recombinase, or the T4 uvsY, proteins may prove essential for efficient amplification with T4 uvsX protein.

[0135] 2) Primosome Assembly

[0136] Primosomes can be assembled at D-loops. Normally, in *E coli*, D-loop structures are formed by RecA as part of the mechanism to rescue damaged DNA in vivo, or during other forms of recombination. The purpose of the combined action of RecA-mediated strand exchange and primosome assembly is to generate a replication fork. A replication fork is the nucleoprotein structure comprising the separated template DNA strands and the replisome. The replisome consists of the polymerase holoenzyme complex, the primosome, and other components needed to simultaneously replicate both strands of template DNA. Primosomes provide both the DNA unwinding and the Okazaki fragment priming functions required for replication fork progression. Similar primosome assembly occurs at recombination intermediates in T4 phage directed by gp59 and gp41 protein.

[0137] Primosome assembly has been studied intensively through genetic and biochemical analysis in *E. coli*. The minimal set of polypeptides required for this process is well known and exist as purified components. The primosome assembly proteins are PriA, PriB, PriC, DnaT, DnaC, DnaB, and DnaG. These proteins have been shown sufficient to assemble a primosome complex on bacteriophage ΦX174 DNA in vitro (Kornberg and Baker, 1992; Marians, 1992). PriA binds to the primosome assembly site (PAS) on the ΦX174 chromosome. Then PriB, DnaT, and PriC bind sequentially to the PriA-DNA complex. PriB appears to stabilize PriA at the PAS and facilitate the binding of DnaT (Liu et al., 1996). PriC is only partially required for the full assembly reaction. Omission of PriC from the reaction will lower priming 3 to 4 fold (Ng and Marians, 1996a; Ng and Marians, 1996b). The function of PriC in the bacterium is genetically redundant to PriB. DnaC then loads DnaB into the complex in an ATP-dependent fashion. This PriABC-DnaBT complex is competent to translocate along the chromosome. The DnaG primase can interact transiently with the complex to synthesize RNA primers.

[0138] During replication in *E. coli*, DnaB and DnaG function as a helicase and primase respectively. These two components are continually required in association with the PolIII holoenzyme to synthesize primers for the Okazaki fragments. Hence, DnaB and DnaG are the core components of the mobile primosome associated with the replication fork. The other primosome components described are essential for assembly of the primosome onto DNA, and for associating a dimeric polymerase. The primosome assembly proteins are required for the re-establishment of replication forks at recombination intermediates formed by RecA and strand exchange. PriA can initiate assembly of a replisome, competent for DNA synthesis, on recombination intermediates. It is possible to target D-loops in vitro with a mixture of PriA, PriB, and DnaT, which are then competent to incorporate DnaB and DnaC. Once a primosome has been formed at the D-loop, all that remains to initiate replication is to load a holoenzyme complex to the site. Alternatively in the phage T4 system the gp59 helicase loader protein recruits and assembles the gp41 replicative helicase to D-loop structures

[0139] 3) Fork Assembly and Initiation of DNA Synthesis

[0140] Replication forks will assemble at the site of primosome assembly. In *E. coli* the presence of a free 3'-end on

the invading strand of the D-loop stimulates the DnaX clamp loader complex detailed earlier to assemble a β-dimer at this site to act as a sliding clamp. The holoenzyme and 2 core units are joined together by the scaffold τ subunit. The τ subunit also has interaction surfaces for the β-dimer, for the clamp loader, and for the DnaB helicase component of the primosome. These multiple interactions are necessary to coordinate synthesis of both leading and lagging strands using the 2 asymmetrically joined core polymerase complexes. In T4 phage the gp59/41 proteins with uvsY and gp32 proteins, and with other components coordinate assembly of the sliding clamp gp45 aided by gp44 and gp62 proteins initiates replisome assembly.

[0141] In *E. coli* the primosomal primase, DnaG, synthesizes a short RNA primer onto the unwound lagging strand DNA template. In the presence of the holoenzyme, the clamp loader recognizes the RNA/DNA duplex and loads a second β-dimer clamp onto this site. The presence of an active primosome and the interaction of the τ subunit with DnaB are critical to ensure simultaneous leading/lagging strand synthesis. Without this interaction the polymerase will move away from the primosome site without coupling.

[0142] A replication fork is now assembled. Synthesis of both leading and lagging strand will now occur simultaneously, and the DnaB helicase will separate template strands ahead of the oncoming holoenzyme. The lagging strand holoenzyme core will generate Okazaki fragments of 1 to 2 kilobases in length. Once the lagging strand polymerase encounters the previous RNA primer, it dissociates from the β-clamp and synthesis is initiated from a newly assembled clamp loaded in the vicinity of the front of the leading strand. The same lagging strand holoenzyme core will be re-used since it is physically tethered to leading strand core.

[0143] There is a dynamic interaction between β-dimer clamps, core subunits, and clamp loaders. Their affinities can switch depending upon the physical circumstances. The β-dimer that has been 'abandoned' at the end of the Okazaki fragments may be recycled via active removal by clamp loaders, or excess δ subunit that may be present.

[0144] The RNA primers at the ends of Okazaki fragments are removed by the 5' to 3' exonuclease activity of DNA polymerase I. DNA ligase then joins the Okazaki fragments together forming a continuous lagging strand.

[0145] 4) Fork Meeting and Termination

[0146] In RPA, replication is initiated at two distant sites and the replication forks are oriented toward each other. As replication forks converge the two original template strands will dissociate from one another as they become separated entirely both behind, and in front, of each fork. The leading strand core of each fork will then complete synthesis, the remaining RNA primers will be processed, and the final products will be two double-stranded molecules. We can reasonably expect to amplify DNA's on the order of several Megabases (Mb) by such an approach. In this disclosure, megabase also encompasses megabasepairs. Based on the known synthetic rate of the PolIII holoenzyme we can expect the replication forks to proceed at a rate of approximately 1 Mb/1000 seconds, i.e., approximately 15 to 20 minutes per cycle for a 1 Mb fragment.

[0147] The final consideration is the mechanism by which rapid exponential amplification of DNA will be achieved.

The key to this process will be to allow efficient reinvasion of the targeting sites by the use of mixtures of helicases, resolvases and the RecO, RecR, and RecF proteins. Under appropriate conditions reinvasion and primosome assembly should be possible shortly after a holoenzyme has moved away from the fork-assembly site. Continual invasions should present no problems since the DNA will simply become branched at many points. Each branch will naturally resolve as it encounters the oncoming fork. Under these conditions it may be possible to achieve enormous amplification in times similar to the time taken to replicate the DNA only once. It may be critical however to limit the concentrations of targeting oligonucleotides to avoid nucleotide depletion prior to the completion of synthesis.

[0148] In addition to the holoenzyme complex, the replication machine employs another complex known as the primosome, which synthesizes the lagging strand and moves the replication fork forwards. The primosome complex comprises a helicase encoded by DnaB and a primase encoded by DnaG. Finally, in addition to the proteins of the holoenzyme and primosome, replication requires the activity of single-stranded DNA binding protein (SSB), *E. coli* DNA polymerase I and DNA ligase. These latter two components are required to process Okazaki fragments.

[0149] Nested RPA

[0150] In another embodiment, RPA amplification may be performed in a process referred to herein as "nested RPA." A difficulty in detecting a rare sequence is that there can be a high ratio of non-target to target sequence. The ability of a RPA to discriminate between target and non-target DNA and amplify only target sequences is a key aspect of improved sensitivity. Discrimination between non-target and target is a reflection of the specificity of the primers and reaction conditions. The more specific a reaction is the greater the relative amount of the specific target sequence that is produced and the easier that product is to detect. An increase in specificity can, therefore, increase sensitivity as well.

[0151] The need for improved sensitivity and specificity can be addressed by using nested RPA. The nested RPA involves a first RPA of a first region of DNA. Then the reaction mixture is diluted, for example, by 10, 20, 30, 40, 50, 75, or 100 fold or more to reduce the concentration of the first primer pair, and a second primer pair is introduced into the reaction mixture and RPA repeated. According to one embodiment of the invention, the second primer pair is designed to be internal to the first primer pair to amplify a subsequence of the first RPA product. The method increases specific amplification, i.e., reduces non-specific background amplification products and therefore increases sensitivity. Such non-specific amplification products, although they arise by virtue of fortuitous partial homology to the flanking primers, are unlikely to also have sufficient homology to the nested primers to continue to amplify. Detection and specificity of RPA may be further improved by labeling one or both of the second primer pair such that only primers amplified with one or both of the second primer pair is detected.

[0152] Nested RPA is not limited to the use of two sets of primer. Naturally, more sets of primers may be used to increase specificity or sensitivity. Thus, three, four, or five pairs of primers may be used. Furthermore, the different sets

of primers, as another embodiment of the invention, may share common primers as illustrated in **FIG. 4**.

[0153] In **FIG. 4**, the primer sets are designed to be used sequentially. For example, a first RPA is performed with primer set 1, a second RPA using the amplified product of the first RPA is performed with a primer set 2, a third RPA using the amplified product of the second RPA is performed with a primer set 3, a fourth RPA using the amplified sequence of the third RPA is performed with a primer set 4, and finally, a fifth RPA is performed using the amplified product of the fourth RPA is performed with a primer set 5. In this case, primer set 1, 2, and 3, share a common primer-primer (a). Primer 3, 4, and 5 share a common primer-primer (b).

[0154] Nested RPA may be performed using any of the two RPA methods described as well as a combination of the two methods in any particular order. That is, RPA may be performed solely by leading strand RPA, solely by leading and lagging strand RPA, or a combination of leading strand RPA and leading and lagging strand RPA in any particular order.

[0155] One benefit of any of the RPA methods of the invention is the size of the amplified product. While current methods of amplification such as PCR are limited to an upper limit of about 10 Kb, RPA methods are capable of amplifying regions of nucleic acids of up to hundreds of megabases. For leading/lagging strand RPA, the sizes of a target sequence to be amplified can be hundreds of megabases, such as, for example, less than 500 megabases, less than 300 megabase, less than 100 megabase, less than 70 megabase, less than 50 megabase, less than 25 megabase, less than 10 megabase, less than 5 megabase, less than 2 megabases, less than one megabase, less than 500 kb, less than 200 kb, less than 100 kb, less than 50 kb, less than 25 kb, or less than 10 kb, less than 5 kb, less than 2 kb, less than 1 kb. For lsRPA, the sizes of a target sequence can be in the megabase range such as, less than 5 megabase, less than 2 megabases, less than one megabase, less than 500 kb, less than 200 kb, less than 100 kb, less than 50 kb, less than 25 kb, or less than 10 kb, less than 5 kb, less than 2 kb, less than 1 kb.

[0156] General Considerations for Reconstituting and Enabling Recombinase-Mediated Amplification Reactions

[0157] Both lsRPA and leading/lagging RPA rely on similar use of recombinase proteins to target oligonucleotide primers, however they differ in the mode by which the new daughter duplexes are formed during amplification. In leading/lagging RPA a full replication fork is established that simultaneously synthesises leading and lagging strands so that two new duplexes are concomitantly formed. In leading strand RPA (lsRPA), only leading strand synthesis occurs so that synthesis generates one duplex and one displaced single-stranded DNA as products.

[0158] In RPA DNA synthesis initiated after strand exchange is accomplished by a polymerase. During extension of the newly synthesised strand the polymerase must be able to displace the outgoing strand, either alone or in combination with a helicase capable of mediating outgoing strand displacement. Extension of the invading primer results eventually in the release of the outgoing strand as a single-stranded DNA. To ensure that geometric amplifica-

tion occurs, and that the reaction produces a vast majority of double-stranded DNA, it is necessary for this displaced single-stranded DNA to serve as template for DNA synthesis from the opposite direction. This is a central consideration for amplification using lsRPA. Two other central considerations are the polymerase species used, and the existence of a stable, dynamic, recombinase system that functions efficiently in the presence saturating levels of single-strand binding proteins. These considerations are important for both the leading/lagging RPA and lsRPA.

[0159] A) Ensuring Generation of Double-Stranded DNA from the Displaced Strand

[0160] The generation of the second strand of DNA in lsRPA can be achieved in one of several ways:

[0161] 1) The displaced single-stranded DNA can simply hybridise to the complementary strand which has been displaced from invasion and extension of a second 'facing' targeting oligonucleotide. Alternatively the displaced single-stranded DNA can hybridise directly with the second 'facing' oligonucleotide. Such hybridisation events may occur spontaneously, or may be mediated by the strand assimilating activities of DNA binding proteins such as recombinases or single-stranded DNA binding proteins. Following hybridisation a polymerase will extend from the free 3'end to generate a double-stranded product. Note that for this to occur efficiently the reaction environment must enable hybridisation of complementary single-stranded DNAs, a situation not always compatible with the other aspects of the RPA reaction. In some circumstances a hybridising oligonucleotide with a modified backbone, unable to interact with most DNA binding protein, could be used.

[0162] 2) If strand-displacement synthesis begins simultaneously from opposing oligonucleotide primer on the same template then the two converging replication complexes will eventually meet somewhere in the middle of the template. Provided that these converging complexes are able to pass one another the template strands will separate and each complex will complete replication by copying a single-stranded rather than double-stranded template with no further need for strand displacement.

[0163] 3) If the outgoing strand possesses the capacity to form a hairpin then self-priming second strand synthesis may occur. This activity would result in a covalently linked duplex with a hairpin at one end, which could become a target for further invasion/extension reactions. This situation is not ideal for many applications, as it will generate products with variable lengths and structures. This may, however, be acceptable for detection assays, such as some diagnostic tests. Furthermore it may be possible to engineer primers such that after the first few rounds of invasion/extension most outgoing strands are capable of self-priming. This mode of duplex DNA formation may be very efficient.

[0164] Which of these three general processes dominate in an lsRPA reaction will depend on many factors. The most important factors are the distance separating the two oligonucleotides primers in the target, the invasion rate, and the sequence of the oligonucleotides and template.

[0165] In the second general format of RPA, leading/lagging RPA, the generation of substantial single-stranded DNA is avoided by establishing a full replication fork at the invasion site. A full replication fork permits the simulta-

neous copying of both leading and lagging strands (which would be equivalent to the outgoing strand). Leading/lagging RPA is elegant in its avoidance of the generation of single-stranded DNA, however a larger number of distinct proteins is required to generate full replication forks. Nevertheless most aspects of optimisation for RPA reactions apply to both lsRPA and leading/lagging RPA.

[0166] B) Choice of Polymerase, or Polymerase/Helicase System

[0167] The lsRPA method is similar in some respects to PCR. Both processes use of pairs of oligonucleotide primers orientated with 3' ends pointed toward one another in the target DNA and both methods achieve geometric amplification through the use of reaction products as targets for subsequent rounds of DNA synthesis. There are, however, fundamental differences in the reaction configurations. For example, in RPA target DNA is double-stranded prior to synthesis, whereas in PCR it is single-stranded after thermal separation of strands. In RPA, DNA synthesis must necessarily use DNA polymerases or polymerase complexes capable of strand displacement. Furthermore, because partially copied strands are physically associated with displaced strand through the template, there is a risk that if the polymerase dissociates temporarily from the template the 3' end of the new strand, and eventually the whole new strand, will be lost by the action of branch migration or another phenomenon known as bubble migration. This suggests that ideally processive polymerases will be used for RPA reactions. It is also important to consider that if converging replication complexes cannot readily pass one another without polymerase dissociation then some processive polymerases may inhibit the RPA reaction on some templates. In summary the ideal choice of polymerase will depend on the precise format and objective of the particular RPA reaction, in particular the size of the product to be amplified.

[0168] C) Establishment of a Stable Persistent Active Recombinase Activity in a Noise-Suppressed Environment

[0169] A third consideration is how to establish a stable, but dynamic, recombinase activity, while silencing the noise generated by aberrant primer annealing seen at low temperatures. This means establishing a reaction environment that balances several seemingly incompatible requirements. For efficient RPA recombinase proteins must remain active while assembled into oligonucleotide/recombinase filaments scanning for target double-stranded DNAs. These complexes must also disassemble after completing strand exchanges to permit DNA synthesis. This must happen in an environment rich in single-stranded DNA proteins. These proteins are needed to stimulate recombination and DNA synthesis while preventing aberrant oligonucleotide behaviour by melting secondary structures. Fundamentally, the recombinases and single-stranded binding proteins are in competition for oligonucleotide binding. While the single-strand binding proteins are necessary to enable efficient strand displacement during synthesis, they suppress recombination activity because they have a higher affinity for single-stranded DNA and bind with more cooperativity than do recombinases.

[0170] Establishment of a functional recombinase/replication reaction environment requires nucleotide cofactors. RecA, and other recombinases, require nucleotide co-factors, such as ATP, to assemble filaments onto single-stranded

DNA, perform homology searches, and complete strand exchange. We have surmised that non-hydrolysable analogues such as ATP-γ-S would be incompatible with RPA because the extremely high stability of the 3-stranded DNA/recA intermediate formed in the presence of with ATP-γ-S would prevent reinvasion at primer targets and would thus prevent efficient amplification. They may even prevent any useful access of polymerase to the recombination intermediate. Earlier attempts to amplify DNA using E. coli recA (Zarling et al) were probably limited by the ATP-γ-S in the described reactions.

[0171] The requirement for ATP in the reaction and the fact that recombinase-complexes will be dynamically forming and disassembling introduces additional complexities, primarily due to complex interactions and competition between key reaction components. In particular single-stranded binding proteins, such as the E. coli single-stranded binding proteins, such as E. coli SSB or T4 phage gp32 protein, are necessary to stimulate recombination by recA and homologues, due both to their capacity to collect the outgoing strand, and to melt secondary structures in single-stranded DNAs thus enhancing recombinase loading. In RPA it is likely that single-stranded binding proteins will further stimulate DNA synthesis by binding and stabilising the displaced DNA strand, preventing undesirable branch migration.

[0172] Despite the clear requirement for single-stranded binding proteins, these proteins generally have a considerably higher affinity for single-stranded DNA than recombinases such as recA, or uvsX, and can inhibit nucleation of recombinase/DNA filaments. Moreover, as filaments formed in the presence of ATP undergo end-dependant disassembly (Bork, Cox and Inman J. Biol. Chem. 2001 Dec. 7;276(49):45740-3), such filaments are likely to be rapidly saturated with single-stranded binding proteins and inactivated soon after initiation of the reaction. Thus for efficient RPA conditions that prevent inactivation of the reaction components are key in establishing robust amplification.

[0173] We have predicted a potentially stable reaction composition using E. coli recA protein in the presence of ATP and the E. coli single-stranded binding protein SSB, or the T4 uvsX protein in the presence of gp32. We suggested the presence of recO, recR, and possibly recF proteins (Bork, Cox and Inman EMBO J. 2001 Dec. 17;20(24):7313-22), could lead to an environment in which pre-loaded recA filaments were stabilised, and in which recA could nucleate successfully onto SSB-bound oligonucleotides. A similar recombinase loading system has been described in other organisms including the recombination/replication/repair system of bacteriophage T4. The T4 recombinase uvsX can be loaded onto single-stranded DNA coated with the T4 single-stranded DNA binding protein gp32 by the action of a third component, the uvsY protein (Morrical and Alberts J. Biol. Chem. 1990 Sep. 5;265(25):15096-103). Interestingly a principal role of this recombination system in vivo is to permit recombination-dependant DNA synthesis by assembling replication components at recombination intermediates such as D-loops (Formosa and Alberts Cell. 1986 Dec. 5;47(5):793-806). This process is similar to what should happen in RPA driven from D-loops made by the invasion of synthetic oligonucleotides. In addition to interactions between the three components uvsX, uvsY, and gp32, there are also interactions between these components and the

replication machinery such as the polymerase, clamp loader, primase/helicase, and dda helicase (Reddy, Weitzel and Von Hippel, Proc Natl Acad Sci USA. 1993 Apr. 15;90(8):3211-5, Hacker and Alberts, J. Biol. Chem. 1992 Oct. 15;267(29):20674-81). Taken together these facts suggest that the components of the T4 recombination/replication machinery would perhaps be even more ideal for RPA than the E. coli equivalents.

[0174] In addition to the use of recombinase loading proteins, such as recO and recR, or uvsY, there are other ways to create an appropriate balance between recombinase activity and the activity of single-stranded DNA binding proteins. The DNA binding and/or cooperativity behaviour of recombinases and single-stranded DNA binding proteins can be modulated by mutation. In addition, recombinases from different sources have distinct properties (Eggler, Lusetti and Cox, J. Biol. Chem. 2003 May 2;278(18):16389-96. Epub 2003 Feb. 20, Villemain et al., J. Biol. Chem. 2000 Oct. 6;275(40):31496-504). This suggests that a range of recombinase and single-strand DNA binding protein activities could be explored. The use of mutated proteins or proteins from different species, in a set of optimisation experiments could lead to the identification of an optimal ratio of the competing recombinase and single-stranded binding activities. Ultimately the activities would be balanced such that DNA association/dissociation for the two DNA-binding species permits sufficient recombinase activity together with sufficient DNA melting activity of the single-strand DNA binding protein to perform its necessary functions also. In addition, reduction of noise due to mispriming may be achieved through the optimisation of such parameters as oligonucleotide sequence design, reaction buffer, the use of partly modified oligonucleotides, the use of part duplex oligonucleotides or the addition of other specific reaction components detailed below.

[0175] Here we provide the results of experiments that validate the RPA method. In particular, we provide a description and demonstration of reaction compositions capable of supporting DNA amplification. We demonstrate that relatively short synthetic oligonucleotides can be used to target specific sequences and support initiation of DNA synthesis. We describe the requirements for particular types and concentrations of certain recombinases, single-stranded binding proteins, ATP, and oligonucleotide concentrations. We further describe the optimisation and modulation of the reaction environment, which supports an active and dynamic recombination system with desired rate behaviour, through the inclusion of crowding agents (such as polyethylene glycols), recombinase loading factors and/or mutated proteins with altered biochemical activities. We establish that in the presence of distributive polymerases at least (e.g. the E. coli DNA polymerase I Klenow fragment), there are substantial improvements in amplification efficiency when the distance between the amplification priming sites is optimised. We establish that a balance between polymerase exonuclease activity and oligonucleotide protecting agents must be employed to avoid non-specific degradation of oligonucleotide primers. We show that amplification of sequences embedded within linear (or relaxed) DNA substrates is relatively inefficient (at least with very distributive polymerases such as Klenow), whereas amplification reactions directed toward the ends of linear DNA substrates are most effective. We provide methods to prepare target DNA to be more efficiently amplified in an lsRPA reaction, including

the methods of thermal or chemical melting or restriction enzyme digestion. We also provide evidence that the nature of the single-stranded binding protein is critical to establish efficient RPA reactions, and provide a rationale for this. Furthermore we suggest improvements and novel approaches to reduce noise and optimise amplification reactions performed at relatively low, or ambient, temperatures by the use of part-double-stranded oligonucleotides, or oligonucleotide wholly or partly lacking a phosphate backbone. We also provide evidence that other enzymes and proteins involved in DNA metabolism can influence RPA reactions, and some may be configured to improve reaction efficiency and specificity. These include topoisomerases, which can relax recombination/replication intermediates and may aid targeting of embedded sequences, as well as helicases such as T4 dda helicase or T4 gp41 which can improve the polymerase initiation and elongation efficiency, particularly if non-strand displacing polymerases are used. Finally we show that priA and the ruvA/B helicases have activities that might be used to optimise amplification efficiency.

[0176] Selection of RPA Reagents and Reaction Parameters

[0177] The details of leading strand RPA, leading and lagging strand RPA, and nested RPA were listed above. This section will describe the selection of reagents and parameter for any of the three methods discussed above.

[0178] One benefit of RPA is that the amplified product of RPA is double stranded DNA that could be used for other molecular biology procedures. Thus, RPA may be combined with other methods in molecular biology. For example, the starting material for RPA may be a PCR amplified fragment. Alternatively, the product of an RPA may be used for PCR.

[0179] If necessary, the RPA products in any of the methods of the invention may be purified. For example, in the nested RPA method, the amplified product may be purified after each RPA step before a subsequent RPA step. Methods of purification of nucleic acids are known in the art and would include, at least, phenol extraction, nucleic acid precipitation (e.g., with salt and ethanol), column chromatography (e.g., size exclusion, ionic column, affinity column and the like) or any combination of these techniques.

[0180] As discussed, the primers used in RPA may be "double stranded" or "capable of forming double stranded structures." These terms refer to DNA molecules that exist in a double stranded condition in a reaction solution such as a RPA reaction solution or a PCR reaction solution. The composition of a PCR solution is known. The composition of a RPA reaction is listed in this detailed description section and in the Examples.

[0181] The primers may have a single stranded region for hybridization to the target DNA in the presence of a recombinase agent. The single stranded region may be, for example, about 10 bases about 15 bases, about 20 bases, about 25 bases, about 30 bases, about 40 bases, and about 50 bases. Even longer regions such as about 75 bases, about 100 bases, about 150 bases or more may be used but it is not necessary. The choice of single stranded regions will depend on the complexity of the starting nucleic acid so that for example, a human genome may require a longer primer while a plasmid may require a much shorter primer.

[0182] The two strands of nucleic acid in a double stranded DNA need not be completely complementary. For example, the double-stranded region of a double-stranded DNA may differ by up to 1% in sequence. That is, the sequence of two strands of nucleic acid may differ by one base in one hundred bases and would still exist in a double stranded condition in solution. Nucleic acids with 1% difference in their complementary sequence are contemplated as double stranded DNA for the purposes of this disclosure.

[0183] In addition, the target nucleic acid (i.e., the nucleic acid to be amplified by the RPA methods of the invention) may be partially double stranded and partially single stranded.

[0184] For example, nucleic acid in any of the configuration of FIG. 5 would be suitable as a target nucleic acid of the invention. As discussed, the target nucleic acid may be RNA. RNA can be converted to double-stranded cDNA using known methods and the double-stranded cDNA may be used as the target nucleic acid. As shown if FIG. 5, the template nucleic acid may have any combination of ends selected from 3' overhang, 5' overhang, or blunt ends.

[0185] The lsRPA method of the invention comprises at least the following steps. First, a recombinase agent is contacted to two nucleic acid primers (referred to herein as a first and a second primer) to form two nucleoprotein primers (referred to herein as a first nucleoprotein primer and a second nucleoprotein primer).

[0186] Second, the first and second nucleoprotein primers are contacted to the template nucleic acid to form a double stranded structure at a first portion of the first strand and a second double stranded structure at a second portion of the second strand. The two primers are designed so that when hybridized, they are pointed at each other as illustrated in FIG. 6A. Alternatively, primer 1 and primer 2 may hybridize different target nucleic acids as illustrated in FIG. 6B.

[0187] Third, the nucleoprotein primers are extended at their 3' ends to generate a first and a second double stranded nucleic acid (FIG. 7A). Where the primers are hybridized to different target nucleic acids, the elongation of the primers will generate displaced strands (FIG. 7B). In this case, the two displaced strands that result from primer elongation may hybridize and form a new double stranded template nucleic acid (FIG. 7C).

[0188] Step two and three are repeated until the desired degree of amplification is reached. The process is a dynamic process in that primer hybridization to the target nucleic acid and elongation are allowed to proceed continuously. One advantage of this invention is that the amplification is performed continuously without the need for temperature cycling or enzyme addition after initiation of the reaction.

[0189] In an embodiment, steps two and three are repeated at least 5 times. Preferably, it is repeated at least 10 times. More preferably, it is repeated at least 20 times, such as at least 30 times. Most preferably, the two steps are repeated at least 50 times. For multiple repetitions of the amplification step (e.g., step 2 and 3) a RPA of the invention is preferably started with a primer to target nucleic acid ration of at least 100 to 1, preferably at least 300 to 1, and most preferably at least 1000 to 1. That is, there are at least 100, 300 or 1000 copies of the primer per copy of a target nucleic acid.

[0190] In an optional step, after a sufficient round of amplification, additional components may be added to the

reaction after a period of time to enhance the overall amplification efficiency. In one embodiment, the additional components may be one or more of the following: recombinase agents, one or more primers, polymerase, and one or more of the additional agents (discussed in a separate section below).

[0191] In a preferred embodiment, a small fraction of a first RPA reaction is used as a supply of template DNA for subsequent rounds or RPA amplification. In this method, a first RPA amplification reaction is performed on a target nucleic acid. After the first RPA reaction, a small fraction of the total reaction is used as a substitute of the target nucleic acid for a subsequent round of RPA reaction. The fraction may be, for example, less than about 10% of the first reaction. Preferably, the fraction may be less than about 5% of the first reaction. More preferably, the fraction may be less than 2% of the first reaction. Most preferably, the fraction may be less than 1% of the initial reaction.

[0192] The primer used in RPA is preferably DNA although PNA, and RNA are also suitable for use as primers. It is noted that in fact, in DNA replication, DNA polymerases elongate genomic DNA from RNA primers.

[0193] Synthetic oligonucleotides may serve as DNA primer and can be used as substrates for formation of nucleoprotein filaments with RecA or its homologues. Sequences as short as 15 nucleotides are capable of targeting double-stranded DNA (Hsieh et al., 1992). Such oligonucleotides can be synthesized according to standard phophoroamidate chemistry, or otherwise. Modified bases and/or linker backbone chemistries may be desirable and functional in some cases. Additionally oligonucleotides may be modified at their ends, either 5' or 3', with groups that serve various purposes e.g. fluorescent groups, quenchers, protecting (blocking) groups (reversible or not), magnetic tags, proteins etc. In some cases single-stranded oligonucleotides may be used for strand invasion, in others only partly single stranded nucleic acids may be used, the 5' stretch of sequence of an invading nucleic acid being already hybridized to an oligonucleotide.

[0194] In another embodiment of the invention, the primers may comprise a 5' region that is not homologous to the target nucleic acid. It should be noted that the processes of the invention should be functional even if the primers are not completely complementary to the target nucleic acid. The primers may be noncomplementary by having additional sequences at their 5' end. These additional sequences may be, for example, the sequence for a restriction endonuclease recognition site or the sequence that is complementary to a sequencing primer. The restriction endonuclease recognition site may be useful for subsequent cleavage of the amplified sequence. The use of restriction endonuclease that cleaves nucleic acid outside the restriction endonuclease recognition site is also contemplated. The sequence that is complementary for a sequencing primer may allow rapid DNA sequencing of the amplified product using commercially available primers or commercially available sequencing apparatus.

[0195] Formation of nucleoprotein filaments can be performed by incubation of the primer (oligonucleotides) with RecA protein or its homologues in the presence of ATP, and auxiliary proteins such as RecO, RecR and RecF, or uvsY in the case of T4 proteins. When incubated at 37° C. in RecA buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl$_2$, 2 mM ATP,

2 mM DTT and 100 μg/ml Bovine Serum Albumin), RecA will form helical filaments on ssDNA with 6 protomers per turn. The DNA is located within the interior of the protein helix. In the presence of dsDNA, the RecA/ssDNA nucleoprotein filament can scan DNA at rates of at least 10^7 bp per hour. The mode of scanning is unclear but it is at a speed (>10^3 bp per second) that it may involve only the initial few base pairs that can be easily accessed along one face of the major groove. Successful binding may result in a transition to a triple-helical intermediate, which is then followed by strand invasion and displacement to form a D-loop. Such joint molecules can be formed under similar condition to those described above for formation of helical filaments, and hence in the presence of ssDNA, the homologous dsDNA, RecA, ATP, auxiliary proteins and suitable buffer and temperature conditions, joint molecules will form spontaneously. If ATP is used the assembly is reversible and will reach equilibrium, but RecA/ssDNA filaments can be stabilized, even in the presence of SSB, by the auxiliary proteins RecO and RecR. Alternatively the T4 uvsX protein may be stabilized in the presence of uvsY protein. In the case of thermostable proteins the temperature of incubation can be higher. If a renewable supply of ATP is required a standard ATP regeneration system can be included in the reaction.

[0196] DNA polymerases can use the free 3'-hydroxyl of the invading strand to catalyze DNA synthesis by incorporation of new nucleotides. A number of polymerases can use the 3'-hydroxyl of the invading strand to catalyze synthesis and simultaneously displace the other strand as synthesis occurs. For example E. coli polymerase II or III can be used to extend invaded D-loops (Morel et al., 1997). In addition, E. coli polymerase V normally used in SOS-lesion-targeted mutations in E. coli can be used (Pham et al., 2001). All of these polymerases can be rendered highly processive through their interactions and co-operation with the β-dimer clamp, as well as single stranded DNA binding protein (SSB) and other components. Other polymerases from prokaryotes, viruses, and eukaryotes can also be used to extend the invading strand.

[0197] In another embodiment of the invention, the primer may be partially double stranded, partially single stranded and with at least one single stranded 3' overhang. In this embodiment, the primer may comprise a invading strand and a non-invading strand as shown in FIG. 8A. In this case, after the invading strand is hybridized to the target DNA and elongated, it serves as a target nucleic acid for a second primer as shown in FIG. 8B. The elongation of the second primer would displace the noninvading strand as shown in FIG. 8C. In this embodiment, as the target nucleic acid is amplified, the non-invading strand of primer 1 is displaced. If both primer one and primer two are partly double stranded primers, then the non-invading strands of both primer one and primer two will accumulate in solution as the target nucleic acid is amplified.

[0198] In one embodiment of the invention, at least two of the primers in a RPA reaction are partially double stranded and partially single stranded each generated by the hybridization of an invading strand and a non-invading oligonucleotide strand, which possess sequences of sufficiently complementary that they form a double stranded region. Preferably, the two oligonucleotide strands are sufficiently complementary over the relevant region that they can form a double stranded structure in RPA reaction conditions.

[0199] In an embodiment of the invention, the primers, including single-stranded and partially double-stranded primers, are labeled with a detectable label. It should be noted that a fluorescence quencher is also considered a detectable label. For example, the fluorescence quencher may be contacted to a fluorescent dye and the amount of quenching is detected. The detectable label should be such that it does not interfere with an elongation reaction. Where the primer is partially double stranded with an invading strand and a non-invading strand, the detectable label should be attached in such a way so it would not interfere with the elongation reaction of the invading strand. The non-invading strand of a partially double stranded primer is not elongated so there are no limitations on the labeling of the non-invading strand with the sole exception being that the label on the non-invading strand should not interfere with the elongation reaction of the invading strand. Labeled primers offer the advantage of a more rapid detection of amplified product. In addition, the detection of unincorporated label, that is, labeled oligonucleotides that have not been extended, will allow the monitoring of the status of the reaction.

[0200] Monitoring a RPA reaction may involve, for example, removing a fraction of an RPA reaction, isolating the unincorporated fraction, and detecting the unincorporated primer. Since the size of an unincorporated primer may be less than 50 bp, less than 40 bp, less than 30 bp or less than 25 bp, and the size of the amplified product may be greater than 1 Kb, greater than 2 Kb, greater than 5 Kb, or greater than 10 Kb, there is a great size difference between the incorporated and unincorporated primer. The isolation of the unincorporated primer may be performed rapidly using size exclusion chromatography such as, for example, a spin column. If a primer is labeled, a monitor procedure comprising a spin column and a measurement (e.g., fluorescence or radioactivity) can be performed in less than one minute. Another alternative for separating elongated primers from unelongated primers involve the use of PAGE. For example, the elongated primer may be separated from the unelongated primer by gel electrophoresis in less than 5 minutes. Yet another alternative for separating elongated primers involves the use of immobilized oligonucleotides. For example oligonucleotides homologous to sequences found uniquely within the amplified DNA sequence can be used to capture nucleic acids produced by primer elongation specifically. These capturing oligonucleotides can be immobilized on a chip, or other substrate. Capture of the elongated oligonucleotides by the capturing oligonucleotides can be performed by RecA protein mediated methods, or by traditional solution hybridizations if necessary.

[0201] In another embodiment of the invention, a double stranded primer may be labeled such that the separation of the two strands of the primer may be detected. As discussed above, after multiple rounds of elongation, the invading strand and the noninvading strands of a partially double stranded primer is separated. After this separation, the non-invading strand does not participate in the RPA reaction. This characteristic may be used to detect and monitor a RPA reaction in a number of ways.

[0202] In this application, the detectable label may be a fluorescent label or an enzyme and the label quencher (also referred to as the label inhibitor) may be a fluorescence quencher or an enzyme inhibitor. In these cases, the label is detected by fluorescence or enzyme inhibition. The delec-

tability of the label would be the fluorescence if a fluorescent label is used or enzyme activity if an enzyme is used.

[0203] In the first method, the invading strand may be labeled with a label and the non-invading strand may be labeled with a detectable label quencher. The label, in the proximity of the label quencher (label inhibitor) on the partially double stranded primer would not be highly detectable. After RPA, the invading strand would be separated from the noninvading strand and thus, the label and the label quencher would be separated. The separation would cause the label to be more detectable. Thus, measuring the increases in the amount of detectable label may monitor RPA reactions.

[0204] The second method is similar to the first method except that the invading strand is modified with a label quencher while the noninvading strand is modified with a label. Then RPA is allowed to proceed with the result (same as method 1) of the label being separated from the label quencher. Thus, the overall delectability of the label would increase.

[0205] The third method involves labeling the noninvading strand of one double stranded primer with a label. In addition, the noninvading strand of a second double stranded primer is labeled with a label quencher. The two non-invading stands are designed to be complementary to each other. In this configuration, the RPA reaction is initially fluorescent. As the RPA reaction progresses, the two non-invading strands are displaced into solution and they hybridize to each other because they are designed to be complementary. As they hybridize, the label and the label quencher are brought into proximity to each other and the fluorescence of the reaction is decreased. The progress of the RPA reaction may be measured by monitoring the decrease in label detectability.

[0206] In a fourth method, the noninvading strands of a first and second double stranded primers are labeled with a first label and a second label. The two noninvading strands are also designed to be complementary to each other. As in the third method, after RPA, the two noninvading strands are hybridized to each other and the proximity of the two labels will be a reflection of the progress of the RPA reaction. The proximity of the two labels may be determined, for example, by direct observation or by isolation of the non-invading strands. As discussed above, isolation of primers and other small nucleic acids can be accomplished by size exclusion columns (including spin columns) or by gel electrophoresis.

[0207] In another embodiment of the invention, the non-invading strand of one or both of the primers is homologous to a second region of nucleic acid such that the primer can hybridize to and primer DNA synthesis at the second region of nucleic acid. Using this method, a second RPA reaction using the noninvading stand from the primer of a first RPA may be started. The product of the second RPA may be monitored to determine the progress of the first RPA.

[0208] In yet another embodiment of the invention, the non-invading strand is detected by a biosensor specific for the sequence of the non-invading strand. For example, the biosensor may be a surface with a nucleic acid sequence complementary to the non-invading strand. The biosensor may monitor a characteristic that results from the binding of the non-invading strand. The characteristic may be a detectable label.

[0209] Suitable detectable labels for any of the methods of the invention include enzymes, enzyme substrates, coenzymes, enzyme inhibitors, fluorescent markers, chromophores, luminescent markers, radioisotopes (including radionucleotides), and one member of a binding pair. More specific examples include fluorescein, phycobiliprotein, tetraethyl rhodamine, and beta-gal. Bind pairs may include biotin/avidin, biotin/strepavidin, antigen/antibody, ligand/receptor, and analogs and mutants of the binding pairs.

[0210] The recombinase agent of the invention may be RecA, uvsX, RadA, RadB, Rad 51 or a functional analog or homologues of these proteins. If desired, the recombinase may be a temperature-sensitive (referred to herein as "ts") recombinase agent. If a ts recombinase is used, the RPA reaction may be started at one temperature (the permissive temperature) and terminated at another temperature (the non permissive temperature). Combinations of permissive temperatures may be, for example 25° C./30° C., 30° C./37° C., 37° C./42° C. and the like. In a preferred embodiment, the ts protein is reversible. A reversible ts protein's activity is restored when it is shifted from the nonpermissive temperature to the permissive temperature.

[0211] In a preferred embodiment, the RPA is performed in the presences of ATP, an ATP analog, or another nucleoside triphosphate. The ATP analog may be, for example, ATPγS, DATP, ddATP, or another nucleoside triphosphate analog such as UTP.

[0212] Other useful reagents that may be added to an RPA reaction include nucleotide triphosphates (i.e., dNTPs such as dATP, dTTP, dCTP, dGTP and derivatives and analogs thereof) and a DNA polymerase. Other useful reagents useful for leading/lagging RPA include NTPs (ATP, GTP, CTP, UTP and derivatives and analogs thereof). One advantage of the RPA reaction is that there is no limit on the type of polymerase used. For example, both eukaryotic and prokaryotic polymerases can be used. Prokaryotic polymerase include, at least, *E. coli* pol I, *E. coli* pol II, *E. coli* pol III, *E. coli* pol IV and *E. coli* poly. Eukaryotic polymerases include, for example, multiprotein polymerase complexes selected from the group consisting of pol-α, pol-β, pol-δ, and pol-ε.

[0213] In another embodiment of the invention, the RPA process is performed in the presence of an accessory component to improve polymerase processivity or fidelity. Both eukaryotic and prokaryotic accessory components may be used. Preferably, the accessory component is an accessory protein is from *E. coli*. Useful accessory proteins include single-strand binding protein, helicase, topoisomerase, and resolvase. Other useful accessory proteins include a sliding clamp selected from the group consisting of an *E. coli* β-dimer sliding clamp, a eukaryotic PCNA sliding clamp and a T4 sliding clamp gp45. Other accessory components include a DNA Polymerase III holoenzyme complex consisting of β-Clamp, DnaX Clamp Loader, and the Polymerase Core Complex. Still other accessory components include RuvA, RuvB, RuvC, and RecG. The properties endowed by the use of additional components will likely enable the amplification of large DNAs not previously successfully targeted by current methods such as PCR.

[0214] In another embodiment, the RPA is performed in the presence of agents used to stabilize recombinase/ssDNA nucleoprotein filaments. For example, the agent may be RecR, RecO, RecF, or a combination of these proteins, or T4 uvsY protein if T4 components are used. Molecular crowding agents may also be employed to modulate biochemical interactions in a favourable manner. Other useful agents include PriA, PriB, DnaT, DnaB, DnaC, and DnaG.

[0215] One benefit of the present invention is that the RPA reaction may be performed at reduced temperatures compared to a PCR reaction. For example, the RPA process may be performed between 20° C. and 50° C. Preferably, the RPA process is performed at less than 45° C. More preferably, the RPA process may be performed at less than 40° C. Even more preferably, the RPA process may be performed at less than 35° C. Most preferably, the RPA process may be performed at less than 30° C. One of the reasons that the RPA process can be performed at these reduced temperatures is because RPA may be performed without temperature induced melting of the template nucleic acid. Further, unlike PCR, absolute temperature control is not required and the temperature can fluctuate without adversely affecting RPA. For example, the amount of fluctuation may be anywhere within the temperatures specified above. The temperature necessary for melting of double stranded DNA also contribute to premature enzyme inactivation, a disadvantage absent in the methods of this invention.

[0216] RPA may be performed to test for the presences or absences of a genotype. The genotype tested may be associated with a disease or a predisposition to a disease. Alternatively, the genotype may be associated with a normal phenotype or a phenotype that confers special resistance to a disease. The genotype as disclosed above may be any standard genetic variant such as a point mutation, a deletion, an insertion, an inversion, a frameshift mutation, a crossover event, or the presence or absences of multiple copies of a genetic sequence (e.g., the presences of minichromosomes).

[0217] One method of detecting a genotype is to detect the distance between a primer pair in an RPA reaction. The distance between a primer pair is reflected by the size of the amplified sequence. In that method, the two primers are selected such that it spans a target region such as, for example, a gene. Then RPA is performed using the primer pair and the RPA product is analyzed. The analysis may involve determining the size or sequence of the amplified product. Methods of determining the size of a DNA sequence, including at least techniques such as agarose gels, PAGE gels, mass spectroscopy, pulsed field gels, gene chips, sucrose sedimentation and the like are known. There are many DNA sequencing methods and their variants, such as the Sanger sequencing using dideoxy termination and denaturing gel electrophoresis (Sanger, F., Nichlen, S. & Coulson, A. R. Proc. Natl. Acad. Sci. U.S.A. 75, 5463-5467 (1977)), Maxam-Gilber sequencing using chemical cleavage and denaturing gel electrophoresis (Maxam, A. M. & Gilbert, W. Proc Natl Acad Sci USA 74, 560-564 (1977)), pyrosequencing detection pyrophosphate (PPi) released during the DNA polymerase reaction (Ronaghi, M., Uhlen, M. & Nyren, P. Science 281, 363, 365 (1998)), and sequencing by hybridization (SBH) using oligonucleotides (Lysov, I., Florent'ev, V. L., Khorlin, A. A., Khrapko, K. R. & Shik, V. V. Dokl Akad Nauk SSSR 303, 1508-1511 (1988); Bains W. & Smith G. C. J. Theor. Biol 135, 303-307(1988); Drnanac, R., Labat, I., Brukner, I. & Crkvenjakov, R. Genomics 4, 114-128 (1989); Khrapko, K. R., Lysov, Y., Khorlyn, A. A., Shick, V. V., Florentiev, V. L. & Mirzabekov, A. D. FEBS

Lett 256. 118-122 (1989); Pevzner P. A. J Biomol Struct Dyn 7, 63-73 (1989); Southern, E. M., Maskos, U. & Elder, J. K. Genomics 13, 1008-1017 (1992)).

[0218] One method of detecting a genotype is to use primers that are specific for a particular genotype. For example, a primer may be designed to efficiently amplified one genotype but inefficiently or not amplify another genotype at all. In an embodiment, the primer may comprise a 3' sequence that is complementary to one genotype (e.g., a genetic disease genotype) but not to another genotype (e.g., a normal genotype).

[0219] The genotype to be determined may be indicative of a disease such as, for example, the presence of an activated oncogene; the presence of the gene for Huntington's disease or the absence of an anti-oncogene.

[0220] The 3' bases of the primers are especially important in determining the specificity and efficiency of an RPA reaction. A primer may be designed so that the 3' base is complementary to one genotype and not complementary to another genotype. This will allow efficient RPA of one genotype and an inefficient RPA (if any) of the second genotype. It is noted that the method is effective if only one primer of the primer pair can differentiate between different phenotypes (by having different efficiencies of amplification). In a preferred embodiment, both primers in an RPA reaction can differentiate between different genotypes. In this above example, the primers are complementary to one genotype and are not complementary to a second genotype by one base at its 3' end. In a preferred embodiment, the primer is not complementary to the second genotype by at least one base at its 3' end. Preferably, the primer is not complementary to the second genotype by at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 bases at its 3' end. Most preferably, the primer is completely non-complementary or cannot hybridize to the second genotype while it can hybridize to the first genotype.

[0221] In some of the methods discussed, the presence or absence of an amplified product provides the indication of the presence or absence of a genotype. In these cases, the RPA reaction may be monitored by the methods discussed throughout the specification.

[0222] In a preferred embodiment, an RPA reaction for genotyping will amplify a sequence regardless of the genotype of the patient. However, the genotype of a patient will alter a characteristic of the amplified sequence. For example, the amplified sequence may be a different size, or sequence for one genotype than for another genotype. In that way, the RPA reaction will contain an internal control to indicate that the amplification reaction was performed successfully. Naturally, a method of RPA, which includes one or more additional pairs of primers as controls for the performance of the RPA reaction, is also envisioned.

[0223] In another embodiment, an RPA reaction may be used to determine the presence or absences of a nucleic acid molecule. The nucleic acid molecule may be from any organism. For example, the microbial composition of a sample may be determined by using a battery of RPA reactions directed to the nucleic acid of different microbes. RPA is especially useful for the detection of microbes. In one embodiment, the pathogens are selected from viruses, bacteria, parasites, and fungi. In further embodiments, the pathogens are viruses selected from influenza, rubella, varicella-zoster, hepatitis A, hepatitis B, other hepatitis viruses, herpes simplex, polio, smallpox, human immunodeficiency virus, vaccinia, rabies, Epstein Barr, retroviruses, and rhinoviruses. In another embodiment, the pathogens are bacteria selected from *Escherichia coli, Mycobacterium tuberculosis, Salmonella, Chlamydia* and *Streptococcus*. In yet a further embodiment, the pathogens are parasites selected from *Plasmodium, Trypanosoma, Toxoplasma gondii,* and *Onchocerca*. However, it is not intended that the present invention be limited to the specific genera and/or species listed above.

[0224] Here we present data that help to define reaction conditions that permit efficient amplification of DNA by RPA.

[0225] Single-Stranded DNA Binding Protein

[0226] Single-stranded DNA binding proteins are required for RPA reactions. These proteins bind to single-stranded DNA, melt secondary structure, facilitate outgoing strand displacement, and suppress branch migration. In RPA their activity is required during several distinct phases. We have investigated the activities of two single-stranded DNA binding proteins, *E. coli* SSB and bacteriophages T4 gp32. The T4 gp32 has proven to be most useful in our hands. Furthermore we have generated a number of distinct forms of this protein by including hexahistidine (His) peptide tags at the N or C termini, as well as investigating several previously described point mutations. Activities of gp32 variants are depicted schematically in **FIG. 21**.

[0227] Variant Forms of gp32

[0228] T4 gp32 protein possesses several features that are of potential utility to RPA reactions. Foremost gp32 has a relatively small DNA binding site (8-10 nucleotides), displays similar binding properties under a wide range of salt concentrations, and displays high (unlimited) cooperativity between monomers (Scheerhagen et al., J Biomol Struct Dyn. 1986 April;3(5):887-98; Kuil et al., Biophys Chem. 1988 December;32(2-3):211-27). In contrast, *E. coli* SSB protein has several distinct DNA binding modes that vary with salt concentration all of which possess relatively large DNA binding sites (32, 56 or 65 nucleotides)(Ferrari et al., J. Mol. Biol. 1994 Feb. 11;236(1):106-23) and there is complex cooperativity behaviour (Lohman and Ferrari, Annu Rev Biochem. 1994;63:527-70). Because the initial size of the outgoing strand is small when synthetic oligonucleotides are employed, we reasoned that the properties of the gp32 protein would be optimal for RPA. We expressed and purified gp32 possessing an N-terminal His tag (gp32(N)). In initial experiments we found gp32(N) to function at least as well as the *E. coli* SSB protein, even when combined in a heterologous system with the *E. coli* recA recombinase **(FIG. 12)**. This was surprising result as gp32 is reported to display extremely high cooperativity between monomeric subunits and it seemed unlikely that recA would be able to compete effectively for oligonucleotide binding in its presence. When we compared the behaviour of gp32(N) to untagged gp32, however, we discovered that the two proteins did not behave equivalently. As the N-terminal His tag is directly adjacent to the 'B' domain of the gp32 protein, which is required for cooperativity between monomers, we reasoned that gp32(N) must have attenuated cooperativity. We therefore generated a gp32

protein possessing a C-terminal His tag (gp32(C)), as well as point mutant forms of gp32(C) in accordance with previously published mutants having a lysine to alanine change at position 3 (K3 to A), or an arginine either glutamine (R4 to Q) or threonine at position 4 (R4 to T) (FIG. 21). These three point mutant proteins exhibit progressively less cooperativity (FIG. 22)(Villemain, et al. J. Biol. Chem. 2000 Oct. 6;275(40):31496-504). We tested the capacity of these proteins at two different concentrations to support invasion/extension reactions on a linearized template in combination with the bacteriophage T4 uvsX protein and the Klenow fragment of *E. coli* DNA polymerase I (FIG. 22). Firstly we note that gp32(C) yields much less product than other gp32 variants at either concentration (FIG. 22 compare with gp32(N)) and that the products are almost exclusively full-length, in contrast to gp32(N). When we compare these results to those obtained with the point mutant allelic series we note that the gp32(N) protein most closely resembles the profile obtained with gp32(C)R4 to T, which has been reported to be significantly attenuated in cooperativity. This suggests that the N terminal His tag of gp32(N) interferes with the function of the B domain in a manner similar to the point mutations.

[0229] There are several other relevant observations. Firstly, the proteins believed to demonstrate the highest cooperativity, i.e. gp32(C)>gp32(C)K3A seem to produce less product. Secondly, for gp32(C) and gp32(C)K3A, more amplified product is generated when less single-stranded binding protein is used, in contrast to gp32(N) and gp32(C)R4T which generate more product in run-on assays when more proteins is used. Taken together these observations suggest an explanation. If the gp32 species is progressively more cooperative then it will form progressively more stable filaments on the oligonucleotides and make it progressively more difficult for recombinase to load. Consequently when the most cooperative gp32 species are employed there is a significant limitation on the availability of recombinase-loaded filaments. If the concentration of gp32 is raised, recombinase loading is further suppressed by gp32 single-stranded DNA binding activity. Consistent with this, as the cooperativity of the gp32 is progressively decreased the amount of amplified product increases. This is consistent with a substantial increase in recombinase-coated filament formation. As relatively un-cooperative gp32 monomers are less likely to coat oligonucleotides and permit the recombinase, uvsX, to seed instead. We note that in the case of gp32(N) and gp32(C)R4T, more product is generated with increased gp32(N) or gp32(C)R4T in DNA run-on assays. This contrasts results using the more cooperative gp32 variants. One possibility is that during and following the strand exchange reaction gp32 is required to stabilise the recombination and synthesis intermediates. This may happen because gp32 cooperativity is so attenuated that it may no longer participate in those aspects of the reaction, or because the recombinase out-titrates gp32 and halts the reaction. The contrasting results of amplification versus run-on assays suggest that the optimal amount of cooperativity may be less than that possessed by the most cooperative gp32 variants. Thus for RPA mutant gp32 variants, with attenuated cooperativity, may be the most appropriate as these will permit higher levels of recombinase loading. Attenuation of gp32 cooperativity must, however, be balanced against noise generated by mispriming events as may be encountered in environments with less gp32 activity.

[0230] Finally, there is a substantial difference in the quantity of product generated in RPA using 7.5 μg versus 15 μg of gp32(C)K3A. The 7.5 μg level more closely resembles results with more weakly cooperative mutants. Most likely in some cases during the course of the reaction, which was held at 37° C. for 2 hours, the amount of single-stranded run-on product increases to the point that gp32 no longer effectively saturates single-stranded DNA in the reaction. In such a situation two things would occur. First there would be a rapid increase in the number of homology-searching filaments leading to a significant rate increase in invasion. Then the lack of gp32 to stabilise outgoing strands would lead to many partial extensions by Klenow not being stabilised, and possibly a greater rate of bubble migration separating the new strand from the template. Hence many more synthesised strands would not achieve full length.

[0231] A Model of gp32 Function in Multiple Invasion/Extension Reactions

[0232] We had earlier noted that in a heterologous system involving recA and gp32(N), polyethylene glycol was required to permit more than one cycle of invasion and extension from a given template. Here we describe a model to rationalise these observations and explain why targeting DNA ends multiple times requires specific types of single-stranded DNA binding protein. It is clear gp32 activity is required to stabilise the outgoing strand during the strand exchange process. If this does not occur then as the recombinase disassembles the outgoing strand re-hybridises with its complementary strand and displaces the invading oligonucleotide. Following strand exchange the outgoing strand can exist in one of two states, which likely place different demands on the single-stranded DNA binding protein. These two states arise as a consequence of the relationship between the single-stranded searching DNA and the duplex target DNA. If the recombination event can extend to an end of a linear duplex DNA, and it is possible for the outgoing strand to be completely removed from its complement at one end, then the outgoing strand is un-constrained at one end. Under this un-constrained condition the new duplex involving the incoming DNA strand and its complement is able to rewind to form B form DNA. This is necessary because during the pairing reaction the target DNAs are under wound by the activity of the invading recombinase filament. Un-constrained outgoing strands can readily bind single-stranded DNA binding proteins, which will prevent branch migration from occurring and allowing the invading strand to be removed.

[0233] Alternatively if the recombination event does not extend to the end of the target duplex, as would occur if an embedded sequence were targeted, then the outgoing strand is topologically constrained because it is physically joined to the complementary strand upstream and downstream of the recombining region. Such intermediates are highly unstable because the newly formed duplex cannot rewind without making the outgoing strand also wind around it. And since the outgoing strand is constrained at both ends this is energetically unstable, and the new hybrid is placed under considerable strain. Consequently a far higher demand is placed on the activity of single-stranded DNA binding proteins in this context because there is substantial drive to eject the incoming strand and rewind the original duplex (FIG. 6).

[0234] In our efforts to establish effective conditions for repeated strand invasion and extension of linear DNA targets we have noted that only oligonucleotides targeted to the ends of linear sequences are readily extended to full template length, at least when using a distributive polymerase such as the Klenow fragment of *E. coli* DNA polymerase I. We have found that under certain conditions, for example when using recA with gp32(N) or *E. coli* SSB, only one round of invasion and extension can readily occur on each target template. These observations may be understood by considering the outcome of invasion events occurring under several different circumstances leading to either fully released outgoing strands (plectonemic joints) or topologically constrained intermediates (paranemic joints). Finally, we have identified certain other conditions that are permissive for multiple rounds of invasion and extension from a single template, a situation ideally suited to the RPA reaction. We propose a model based on our own data to consolidate these observations, and forms a framework around which optimisation of the RPA reaction can be designed. This model takes into account the behaviour of gp32 protein under different reaction environments and justifies gp32 behaviours with the effects of other reaction components (**FIG. 29**).

[0235] The model schematically describes the nature and outcome of recombination events between the end of a target duplex and an invading oligonucleotide that initially possesses a 5' overhang relative to the target. This situation typifies the experimental circumstance we have studied. Despite this starting situation all but the initial cycle involves the invading oligonucleotides having their 5' extreme flush to the 5' extreme of the target duplex DNA. This is the case because the target DNA complementary strand possesses a 3'-end that can be extended during the first round to copy the overhang region of the invading primer, which we term backfire synthesis. Experiments performed with gp32(N) protein (in the absence of PEG), suggest that once the target is flush at the 5'-end to the oligonucleotide i.e. after the first round, then subsequent invasion/extension cycles are very inefficient or do not occur at all. Early experiments showed only a roughly equimolar quantity of run-on product to start template. How does this occur?

[0236] We believe that this block to re-invasion/extension arises because invading oligonucleotides are rarely fully coated by recombinase. Thus complete exchange to the 5' flush end of a target will also occur very rarely and corresponding outgoing strands will remain in a constrained state (**FIG. 13**). The exchange is initially incomplete and the intermediate is unstable due to an inability to allow the new duplex to relax into a B-DNA helix in the presence of the topologically constrained outgoing strand. Such unstable intermediates will have a tendency to rewind the original duplex and eject the invading strand as the recombinase disassembles.

[0237] We found, however, that if a crowding agent such as polyethylene glycol is included in the presence of gp32(N), subsequent invasion/extension occurs. One possibility is that under these conditions unstable intermediates are temporarily stabilised by gp32(N), such that elongation can occur. It is possible that polyethylene glycol acts to partly rescue the poor cooperativity of the compromised gp32(N) allowing it to stabilise these otherwise unstable intermediates. This conclusion is supported both by our data showing that N-terminally his-tagged gp32 is cooperatively

attenuated, and the known capacity of crowding agents to enhance the effectiveness of interactions between molecules, thus partly making up for the poorer interaction between monomers. We initially believed that this experiment might be best explained by suggesting that recA filaments were more abundant in the presence of PEG, as previously reported elsewhere (Layery P E, Kowalczykowski S C. J. Biol. Chem. 1992 May 5;267(13):9307-14), however we feel that this does not adequately account for the observed switch from a dead-end only one round of invasion to productive multiple rounds of invasion/extension.

[0238] The difference between PEG stimulation of full-length multiple run-ons at end-directed targets versus the far poorer activity at truly embedded targets (**FIG. 15**), suggests that the temporary stabilisation of an intermediate, as suggested above, is insufficient alone to generate substantial elongation. If this were the case for run-on assays at embedded targets it should work as well in reinvasion at end-directed targets, but this is not the case (**FIG. 15**). Alternatively the difference between efficiencies may be explained by supposing that gp32 temporarily stabilises an intermediate in which the 5' extent of the incoming oligonucleotide is not paired but free, and possibly even coated with gp32 dependent on the length. Provided that this unexchanged segment is not coated with gp32, or that the gp32 can readily dissociate, as would be the case without cooperativity, then the 5'-most portion of the oligonucleotide could become paired via rapid branch migration (**FIG. 29**, scenario 1). In fact there is a difference between truly embedded sequences and re-invasion at DNA ends (**FIG. 15**). For embedded targets, secondary branch migration leading to unconstrained release of the outgoing strand can never occur because the ends are too distant.

[0239] We would therefore conclude that the small DNA binding size, and high cooperativity between subunits permits gp32 proteins permits multiple invasion and extension reactions by stabilising the constrained paranemic joint structures for a sufficient time. There are various possible outcomes (**FIG. 29**). Either the last small section of oligonucleotide at the 5' end that was not initially exchanged becomes hybridised through a branch migration event, or disassembly of gp32 on the outgoing strand permits the invading/extending oligonucleotide to be lost through branch migration. Alternatively, loading of recombinase onto the outgoing strand and re-invasion ejects the incoming strand (bubble migration). If hybridisation via branch migration occurs then an unconstrained structure arises that can be readily stabilised and extended. If either gp32 disassembly or bubble migration occur then there is a substantial risk that the new extending strand will be lost before it is fully extended. If a single stranded DNA binding protein with a large binding site, like *E. coli* SSB, or one with poor cooperativity, like. gp32(N), is used in the absence of PEG, then no temporary stabilisation occurs and the invading oligonucleotide is ejected without being extended.

[0240] Consequently based on this model, and the earlier conclusions about the frequency of recombinase-loaded filaments in the presence of various g32 forms, we conclude that a balance between the activities of recombinases and gp32 molecules must be struck that best meets the various requirements of the amplification reactions. We can summarise the needs and effects of single-stranded DNA binding proteins in different phases of the RPA reaction as follows.

[0241] Phase 1

[0242] Single-stranded DNA binding proteins help to prepare single-stranded DNAs for recombinase loading by melting secondary structure so that recombinase loading can occur consistently. Thus the melting activity of single-stranded DNA binding proteins is desirable and also plays a part in silencing non-specific annealing of primers. Despite this, however, excessive levels of protein and excessive cooperativity can significantly reduce number of recombinase-loaded filaments available for invasion.

[0243] Phase 2

[0244] Single-stranded DNA binding proteins collect the outgoing strand and prevent spontaneous ejection of the incoming oligonucleotide as the recombinase disassembles. The instability of paranemic joints means that invasions occurring on embedded sequences, including the case that oligonucleotide ends are flush to the duplex ends (as would occur during most cycles of an amplification), means that significant cooperative activity may be required for many situations. In general this phase of the reaction will benefit from a surplus of highly cooperative single-stranded DNA binding proteins.

[0245] Phase 3

[0246] Single-stranded DNA binding proteins bind to the displaced strand that forms during DNA synthesis. As in phase 2 this displaced strand may be unconstrained, or topologically constrained, and these two circumstances place different demands on the single-stranded DNA binding protein.

[0247] Phase 4

[0248] In certain configuration of the RPA reaction the displaced single-stranded outgoing strand must hybridise to a partner oligonucleotide to permit subsequent generation of a new duplex. Many single-stranded DNA binding proteins prevent complementary strands from annealing, however T4 gp32 protein aids re-annealing of complementary DNA. Therefore bacteriophage T4 gp32 protein is an ideal protein for this phase.

[0249] Oligonucleotide Length:

[0250] There is little published evidence to support how effectively recA, or other recombinases, might be used with relatively the short synthetic oligonucleotide primers used for RPA. It is also unclear whether a stable reaction environment can be generated in which such short DNA oligonucleotides remain actively loaded with recombinase. Most studies performed with recA utilise comparatively large substrates such as single-stranded and double-stranded forms of the bacteriophage M13 genome (many thousands of residues) as donor and acceptor DNAs. Experimental assays for recombinase activity often consist of the formation of intermediate, or completed, recombination events measured by electrophoretic migration or electron microscopy (Harris L D, Griffith J. J. Biol. Chem. 1987 Jul. 5;262(19):9285-92). A few experiments have been described using short oligonucleotides. Sequences as short as 15 nucleotides have been shown to assemble functional homology-searching complexes with recA in the presence of the non-hydrolysable cofactor analogue ATP-γ-S, but investigations combining short oligonucleotides and ATP are ambiguous (Hsieh P, Camerini-Otero C S, Camerini-Otero R D.

Proc Natl Acad Sci USA. 1992 Jul. 15;89(14):6492-6). The homology-searching function of recombinases is not necessarily sufficient to complete strand exchange and to release from the invasion complex to allow access by other DNA metabolising proteins such as polymerases. Indeed, studies have shown that recA shows transitions between low ATP hydrolysis rate (a useful indicator of combined DNA binding activity and functional recombinase activity) and high hydrolysis rate at oligonucleotide lengths substantially longer than the 15 nucleotides required for searching. Furthermore the type of nucleotide cofactor seems to influence on the length at which such hydrolysis transitions occur (Katz F S, Bryant F R. Biochemistry. 2001 Sep. 18;40(37):11082-9). The bacteriophage T4 recombinase uvsX has also been shown to exhibit variable properties on short oligonucleotides, and shows some sensitivity to the base composition (Formosa T, Alberts B M. J. Biol. Chem. 1986 May 5;261(13):6107-18). Despite this, both uvsX and recA are capable of performing recombination events with single-stranded substrates of roughly 30 base pairs or more in the presence of hydrolysable nucleotides like ATP, suggesting that the use of such short synthetic targeting oligonucleotides is reasonable (Salinas F, Jiang H, Kodadek T. J. Biol. Chem. 1995 Mar. 10;270(10):5181-6; Formosa T, Alberts B M. J. Biol. Chem. 1986 May 5;261(13):6107-18).

[0251] An oligonucleotide bearing 33 residues of homology with the end of a linearized DNA target can form a pairing intermediate capable of elongation by the Klenow fragment of *E. coli* (FIG. 9). This experiment and others demonstrate that homology lengths as short as 33 nucleotides are sufficient to direct recombinase/ssDNA filaments to appropriate targets in the presence of ATP and to permit complete strand exchange. Similar results are found when using the bacteriophage T4 uvsX protein.

[0252] In addition to a requirement for a minimal oligonucleotide length there may be a progressive loss of invasion/extension efficiency if the oligonucleotide is extended significantly beyond the minimal length required for recombination, at least when distributive polymerases are used. One possibility lies in the nature of recombinase/ssDNA filaments. Filaments coated with recombinases have varying 5' limits to their coating, as recombinases seed randomly and then extend the filament in a 5'-3' direction, there will be a distribution of 5' extents of coating. If less than roughly 25-30 nucleotides are coated then little recombination can occur because there is insufficient nucleoprotein filament for strand exchange. If more than this is coated it is potentially beneficial from the point of view of recombination, but if greater than 10-20 residues is added beyond the minimal length required for exchange there is a possibility that progressively more active filaments will posses recombinase of a sufficient length of DNA to permit exchange, but retain sufficient 5' uncoated DNA for gp32 to bind, which through cooperative binding of the 5' extreme could inhibit the branch migration phase and prevent the outgoing strand from being un-constrained. Consistent with this notion, we note that stimulation of multiple invasion events was less apparent with the oligonucleotide the longer oligonucleotide primer Tester1 bio compared to Tester3 bio (FIG. 15). The only difference between these oligonucleotides is that the first has 25 additional overhanging nucleotides beyond the initial 33 residues of homology, while the second has only

15 additional residues. Regardless of the explanation this experimental observation argues that an optimal maximal length may exist.

[0253] There are other reasons to suspect shorter oligonucleotides will be best for efficient RPA. At the relatively low temperatures used in RPA reactions there is a substantial increase in stable secondary structures of oligonucleotides as well as a greater probability of inappropriate hybridisation between primer pairs. Despite an overall excess of single-stranded DNA binding proteins, the dynamic nature of the reaction suggested by the instability of nucleoprotein filaments formed with recombinases in the presence of ATP means that there is likely to be a constant cycling of proteins on and off oligonucleotides, and a steady-state concentration of uncoated, unprotected, oligonucleotides. Consequently the use of short oligonucleotides should reduce the likelihood of undesirable intra- and intermolecular interactions.

[0254] Our data indicate that optimal length of oligonucleotide lay between 30 nucleotides and 50 nucleotides, and that progressively larger oligonucleotides can decrease the rate of invasion/extension. It may, however, be desirable to extend the length of the oligonucleotide to accommodate a duplex region in the 3' or 5' region of the searching oligonucleotide. Thus, in one aspect of the invention, the preferred primer length is between about 30 to about 50 bases. Examples of primers sizes that would fit at least one of these criteria includes primers of between 30 to 45 bases, between 30 to 40 bases, between 30 to 35 bases, between 35 to 40 bases, between 40 to 45 bases, and between 45 to 50 bases. It may be possible, however, to identify a recombinase and/or single-stranded binding protein with an optimum primer length of less than 30 bases.

[0255] Oligonucleotide Composition, Sequence and Single-Stranded/Duplex Character:

[0256] Composition and Sequence:

[0257] Software to design oligonucleotides for use in vitro DNA synthesis reactions is well established, particularly for use in PCR. The considerations for the RPA method are similar and include the optimisation of the melting temperature of the oligonucleotide, avoidance of hairpin formation within an oligonucleotide and selection against complementarity with other oligonucleotides present in a given reaction. As RPA is performed at relatively low temperatures such considerations are potentially more important. We have observed the accumulation of extended primer products in RPA reactions, which are apparently template-independent and dependent on the combination of primers used. The sizes of the aberrant products generated suggest they are primer dimers, or the consequence of self-priming of a single oligonucleotide. These undesirable primer artifacts are well known for other methods such as PCR. It is therefore important to design oligonucleotide primer pairs to avoid undesirable side reactions. We have observed that oligonucleotides capable of forming hairpins can erroneously self-prime in an RPA reaction.

[0258] Besides optimising oligonucleotide sequence design there are additional approaches to reduce or eliminate primer dimer formation. We have observed that reaction noise can be significantly reduced by utilising polymerases lacking 3'-5' exonuclease activity. This suggests mispriming may result from oligonucleotides that have been shortened by the 3'-5' exonuclease activity of polymerases. Consequently 3'-5' exonuclease editing activity, pyrophosphorylysis, or any other similar editing activity can be a source of noise. In addition to using polymerases lacking exonuclease activity and the removal of pyrophosphate with pyrophosphatase, use of synthetic oligonucleotides with a non-hydrolysable backbone at the ultimate and/or penultimate link may be beneficial to reduce reaction noise. Alternative backbones could be selected from the considerable range of chemistries available such as phosphorothiorate, morpholino, locked nucleic acid, or peptide nucleic acid.

[0259] Single-Stranded/Duplex Character:

[0260] Deterring aberrant extension of oligonucleotide 3' ends may also be achieved by designing, and including, short competitor oligonucleotides that can efficiently compete for the formation of hybrids with the 3' region of the targeting oligonucleotides. Such an approach could be configured in various ways.

[0261] 1) A short independent oligonucleotide comprising a perfect complement to the most 3' residues of the targeting oligonucleotide could be employed. This oligonucleotide would be sufficiently long that at the reaction temperature it would be likely to form a hybrid with the targeting oligonucleotide moderately efficiently. This might be between 6 and 15 residues in length. The short oligonucleotide will be non-extendable by having a blocking group at its 3' terminus, such as a dideoxy sugar, or other 3' blocking group. This oligonucleotide also may require a non-phosphate linkage at the ultimate and/or penultimate backbone link to deter removal of bases in the 3'-5' direction by editing activities. Although a large proportion of non protein-coated targeting oligonucleotides may form duplexes with this short oligonucleotide this should not significantly decrease the rate and efficiency of the RPA reaction. Firstly, because at the low RPA reaction temperature there is likely to be an equilibrium between hybridised and unhybridised oligonucleotides there will always be a pool of free melted targeting oligonucleotides available. As the oligonucleotide is a better competitor than other random sequences in the reaction, it will be favoured against other transient interactions. Secondly, single-stranded DNA binding proteins such as recA, uvsX, gp32, and E. coli SSB, tend to melt duplex DNA and thus even if hybrids are relatively stable at the reaction temperature when no proteins are bound, when they bind to the single-stranded part of the oligonucleotide and extend cooperatively to the region of duplex they are likely to enhance its melting, thus the duplex state will tend only to exist on naked oligonucleotides. Finally, recombinases have the capacity to extend strand exchange initiated between a single-stranded DNA region and duplex target into regions in which both DNAs are duplex. This appears to be an ATP-dependant branch migration activity. Taken together these considerations suggest that the short duplex region should not significantly reduce the rate of the RPA reaction, but instead act to suppress the formation of primer dimer or other artifacts generated from non protein-coated oligonucleotides by being a better competitor for binding to the 3' region of the targeting primer than other oligonucleotide sequences available in the reaction. If exonuclease deficient polymerases are used, it may be optimal to design this oligonucleotide to have its 5' most base pairing to the

penultimate, rather than ultimate, 3' nucleotide as many polymerases tend to add an additional base to a perfectly blunt end.

[0262]   2) In a second approach, the targeting oligonucleotide possesses a 5' overhang not present in the initial target DNA, and this overhang is the precise reverse complement to the sequence to the 3' end of the same targeting oligonucleotide, perhaps with the most 3' base only unpaired (FIG. 35 part D). The length of this complementary oligonucleotide should be relatively short, but long enough to be a far better competitor than other oligonucleotide sequences present in the reaction. For example between 6 and 10 nucleotides might be optimal. As described for the first approach this arrangement is likely to lead to any uncoated oligonucleotides forming hairpin structures to themselves far more efficiently than to any other sequences. As the design will place the 3' base, or penultimate 3' base, of the oligonucleotide in a perfect base-pairing environment, but at the 5' end of the targeting oligonucleotide, it cannot be extended, apart from the addition of the single residue often catalysed by many polymerases if the end is blunt. In this context it may be fine to leave the editing activity of polymerases intact. Such hairpin forming oligonucleotides may suppress erroneous activity of naked oligonucleotides without deterring the activity of protein-loaded filaments.

[0263]   There are however some important considerations to taking this approach. Firstly, if the recombinase loading is initiated directly on the partly duplex naked oligonucleotide without initial melting of the duplex section, recombinase may not extend as close to the 5' end of the targeting oligonucleotide as might be optimal. Secondly, as the amplification reaction continues beyond the first round there will be active displacement of products generated from previous rounds of synthesis and if a complete displacement occurs then the very 3' end of a displaced strand, which is complementary to immediately adjacent sequences, may be able to hairpin and rapidly self-primer. Such a rapid self-priming event would result in DNA synthesis and formation of a novel double-stranded DNA with both strands joined by a hairpin at one end. This could be a substrate for further rounds of invasion/synthesis and result in formation of dimer-like products, and possibly more complex products (see FIG. 35). We anticipate that this situation may be perfectly acceptable for diagnostic tests. As the amplified sequences are all dependant upon the presence of bona fide target DNA, and will contain the unique inter-oligonucleotide sequences, and because the self-priming event may be engineered to function efficiently, then this may prove an ideal format for diagnostic assays. We have already experienced the generation of greater than unit length amplified DNA fragment apparently generated from specific targets and suspect that this mechanism may operate in the absence of specific oligonucleotide design. A similar activity has been described although in this case the activity was initiated in a totally different manner using a single very large single-stranded DNA (Morrical S W, Wong M L, Alberts B M. J. Biol. Chem. 1991 Jul. 25;266(21):14031-8).

[0264]   3) In a final approach separate short oligonucleotides with blocked 3'-ends would be employed as described in approach 1. In this case however a linkage is engineered between the 5' end of the targeting oligonucleotide and the 5' or 3' end of the short competitor oligonucleotide (FIG. 35 parts B and C). This approach is similar to approach 1,

except that by tethering the competitor oligonucleotide in the close vicinity of the targeting oligonucleotide one ensures efficient competition of this oligonucleotide with any other sequences in the reaction.

[0265]   Polymerase Choice:

[0266]   There are many DNA polymerases that might be used for RPA. There are, however, a number of criteria that should be considered when designing the optimal RPA format for a given application. We have identified a number of different polymerases with activity in RPA reactions, and deduced which properties confer specific advantages for different circumstances. One exciting conclusion is that polymerases from heterologous systems can be used effectively. We discuss below the polymerase activities most relevant to RPA.

[0267]   Polymerase Processivity

[0268]   Polymerase processivity is measured as the typical number of incorporation events catalysed on each individual interaction with a DNA template. Many of the polymerase enzymes used in molecular biology applications are not highly processive, often because they are functional analogues of the of E. coli DNA polymerase I whose primary role is DNA repair and Okazaki fragment processing. Processivity is a more critical consideration for RPA than for PCR. Because RPA uses a double-stranded template, distributive polymerases will produce partially copied strands possessing a joint, which could be ejected by branch migration. In addition, we have evidence to suggest that bubble migration may occur in a variety of RPA configurations. In bubble migration parent strands of the template DNA re-hybridises shortly behind the replication complex leading to ejection of the newly synthesised strand, which is freed as a single-stranded DNA instead of the outgoing strand of the original recombination event. This re-hybridisation has been previously described in the T4 recombination/DNA synthesis system and thought to involve coating of the outgoing strand with the uvsX recombinase and subsequent re-invasion. Alternatively, in the presence of uncooperative single-stranded DNA binding protein, monomers may be lost progressively from one end of the outgoing strand and may lead to progressive branch migration running in a 5'-3' direction, chasing the replication complex.

[0269]   Thus if the polymerase dissociates prematurely from the template, bubble migration or branch migration may result in the newly synthesised strand being separated from the template as an incomplete single strand. This could be catastrophic for RPA reactions, because if such truncated products are too short to form productive hybrids with similar products generated from the opposing side, these undesired short products will accumulate linearly. Thus there is a need to use relatively processive polymerases, or polymerase complexes, to efficiently amplify larger DNA fragments. Several now commonly used polymerases such as Phi-29 DNA polymerase, Bst DNA polymerase and T7 DNA polymerase in complex with thioredoxin are known to be processive. We also surmise that related Pol I enzymes from the Bacilli, such as Bacillus subtilis PolI (Bsu), will likely demonstrate optimal characteristics by virtue of sharing with Bst polymerase processivity and exonuclease minus status, but retaining optimal solubility and activity profiles at temperatures in the 30-37° C. range. Additionally, multi-subunit replication complexes incorporating sliding clamps

may be used such as that from bacteriophage T4, *E. coli*, and others. In all cases it is assumed that the stable interaction between the polymerase and the template will override bubble migration and branch migration activities. We have found that Phi-29 polymerase and Bst polymerase are capable of extending recombination intermediates generated in our studies and that they show a different product length distribution in comparison with those of the Klenow fragment of *E. coli* DNA polymerase I. We have also purified the Polymerase I from *B. subtilis* which is related to Bst polymerase. This polymerase is readily overproduced and purified from *E. coli* with an N-terminal hexhistidine tag, and appears to possess ideal biochemical attributes for RPA.

[0270] Finally, there are some situations where a distributive polymerase may be more appropriate for RPA. Because in many configurations DNA synthesis is initiated from opposing ends and replication complexes move toward one another, there is the possibility of a collision between complexes resulting in a stalemate in which neither progresses further. This requires either that one polymerase temporarily dissociates from the template, or that the polymerases used are able to pass one another effectively without dissociation.

[0271] 3'-5' Exonuclease Activity Present Associated with the DNA Polymerase:

[0272] Many DNA polymerases possess 3'-5' exonuclease activity, and some also possess 5'-3' exonuclease activity, which is probably undesirable in RPA as it results in digestion of one DNA strand progressively as the polymerase moves forward, rather than displacement. The 3'-5' exonuclease has potential advantages as well as its obvious disadvantages. On the one hand 3'-5' exonuclease activity increases the fidelity of the replication reaction, and can also prevent stalling of polymerases at points of misincorporation. High fidelity amplification is desirable for many DNA applications. The 3'-5' exonuclease activity may also be appropriate for amplification of larger DNA fragments where stalling due to misincorporation could inhibit effective amplification.

[0273] Despite these clear advantages of 3'-5' exonuclease activity there are some disadvantages. We have observed that the free oligonucleotides can be subject to end-dependant degradation when polymerases possessing 3'-5' exonuclease are employed. This can be suppressed to a large extent by using saturating amounts of relatively cooperative gp32 protein with some polymerases such as the Klenow fragment, but with enzymes possessing aggressive exonucleases such as T4 DNA polymerase or Phi-29 DNA polymerase, gp32 appears to be insufficient and the oligonucleotides appear to be completely degraded. These data argue that it is advantageous to at least limit to some extent the efficacy of an exonuclease activity in the reaction.

[0274] We have found that 3'-5' exonuclease activity of some polymerases may contribute substantially to noise in the reaction. At the relatively low temperatures used in RPA reactions there is a significant tendency for uncoated single-stranded DNA molecules to form inappropriate hybrids, at low complementarity, with other DNAs in the reaction. Such hybrids will prime DNA polymerases elongation. For extension to occur the last base or two must be paired correctly with its complement. While poorly complementing segments within or between oligonucleotides can form weak hybrids at low temperatures these will rarely be combined with a good hybrid match at the very 3' end. Regardless, in the presence of a 3'-5' exonuclease activity the unpaired 3' most bases will be excised until a correctly paired 3' end is formed, as happens normally when an incorrect base is inserted by the polymerase. Our data suggest that partly extended strands that have been displaced by branch migration or bubble migration can fold back onto themselves leaving an unpaired 3' end, which is trimmed thus promoting inappropriate polymerase elongation. There are therefore good reasons to limit or remove exonuclease activities from polymerases used in RPA. There are other methods to inhibit oligonucleotide degradation that may also be used.

[0275] Access to 3' Ends

[0276] The recombination intermediate formed after invasion must be accessible to the DNA polymerase. The structure near the 3'-end of the targeting oligonucleotide, is not equivalent to the 3'-end of an oligonucleotide hybridised to otherwise single-stranded DNA, which is the situation in PCR. Instead, the outgoing strand, which is hybridised to the template strand immediately, or shortly, downstream of the 3'-end of the invading oligonucleotide may block polymerase loading. Moreover, whether of the outgoing strand is constrained or unconstrained may affect the capacity of certain polymerases to load successfully. Whether a particular polymerase can function effectively in these situations must be addressed experimentally. We find that the Klenow fragment of *E. coli* DNA polymerase I, as well as the Bst DNA polymerase purified from *Bacillus stearothermophilus*, can load onto and extend such recombination intermediates. Helicases such as the T4 dda helicase and T4 gp41 helicase may also function to process recombination intermediates and separate the template and outgoing strands downstream of the exchange event permitting other polymerases to be used. Finally, it may be beneficial to use mixtures of polymerases, acting synergistically in the RPA reaction, for example one polymerase efficient at accessing the 3' ends of recombination intermediates, and the other possessing processive, strand-displacing synthetic activity.

[0277] Cooperative Interactions

[0278] We have demonstrated that enzymatic components from heterologous systems can be combined together effectively in various RPA formats. For example, both the Klenow fragment of *E. coli* DNA polymerase I, the Bst DNA polymerase of *Bacillus stearothermophilus*, and the large fragment of *Bacillus subtilis* polymerase I, can extend recombination products generated with the uvsX recombinase of bacteriophage T4 in the presence of the bacteriophage T4 gp32 protein. Nevertheless, there are likely to be additional synergistic effects when proteins from the same organism are employed together. For example there are known to be physical interactions between the bacteriophage T4 components such as uvsY and uvsX, as well as gp32. T4 polymerase functionally interacts with the gp41 helicase, and physically with gp32 (Formosa and Alberts PNAS 80, 2442-2446, 1983). We suspect that using components from the same organism will enhance RPA efficiency.

[0279] Resolution of Replication Complex Collisions

[0280] In some formats of RPA, such as when a large DNA product is desired, a processive polymerase would be the optimal choice. Under these conditions, however, there is

significant likelihood that replication complexes will converge with one another on the same template. There is a danger that replication complexes will become locked head-to head so that neither can pass. Most useful in this situation are polymerases that are both processive and able to resolve collisions as has been demonstrated for Phi29 DNA polymerase (Elias-Arnanz M, Salas M. EMBO J. 1997 Sep. 15;16(18):5775-83).

[0281] Recombinases

[0282] We have assayed both *E. coli* recA protein, and bacteriophage T4 uvsX protein in RPA experiments. Both these proteins share some limited protein sequence homology and are believed to have evolved from a common progenitor. Crystallographic and electron microscope studies of nucleoprotein filaments of these proteins, which show a conserved filament structure, in terms of the pitch of the helices formed in both ATP and ADP bound states, suggest a remarkable similarity in their mechanism of action. Furthermore, all prokaryotes possess proteins highly homologous to recA, which suggests that the principle activities of recombinases have been conserved throughout evolution. Hence, what is learned from one recombinase may be applied to, or substituted by, another.

[0283] In addition to their similarities, however, there are differences between recA and uvsX relevant to RPA and as additional components of recombination/replication machinery are used in RPA reactions organism-specific protein-protein interactions may have a significant effect on reaction efficiency. The nucleotide hydrolysis rate of uvsX is 10-20 times higher than that of recA, suggesting that it might perform recombination reactions at an accelerated rate (Formosa T, Alberts B M. J. Biol. Chem. 1986 May 5;261(13):6107-18.). An increased hydrolysis rate could be beneficial for RPA reactions in several ways.

[0284] 1) More dynamic turnover of uvsX on oligonucleotides could increase the overall regeneration of nucleoprotein filaments leading to near complete recombinase coverage to the most 5' end of invading oligonucleotides.

[0285] 2) More rapid completion and disassembly of recombinase from successful recombination events will permit more efficient polymerase access

[0286] 3) A more active recombinase will produce a more flexible nucleoprotein filament.

[0287] Another major difference between uvsX and recA is that uvsX hydrolyses ATP to ADP plus phosphate, and to AMP plus pyrophosphate whereas recA and other recombinases do not (Formosa T, Alberts B M. J. Biol. Chem. 1986 May 5;261(13):6107-18.). The biological significance of this difference is not known but the activity might affect RPA efficiency. For instance, the pitch of nucleoprotein filaments formed with ATP and ADP is different and the hydrolysis of ATP to ADP is associated with overall filament flexibility. It may be that AMP-bound uvsX can adopt a different pitch and possess a distinct flexibility.

[0288] The bacteriophage T4 recombinase uvsX stimulates a mode of DNA displacement following synthesis known as bubble migration. In bubble migration uvsX assembles onto the outgoing strand and mediates a re-invasion of the outgoing strand thus displacing the newly synthesised strand. This mode may have biological signifi-

cance because by displacing the newly synthesised strand, the length of region with topological constraint is limited. This process has not been described for recA, although it may occur. Nevertheless several aspects of bubble migration suggest real differences between uvsX and recA. For example, the bubble migration model suggests that it is possible that uvsX bound DNA extends to the end of the invading or partially extended DNA, but that this structure is still accessible by polymerases for elongation (Formosa T, Alberts B M. Cell. 1986 Dec. 5;47(5):793-806). This has certainly not been observed for recA, and if anything there is evidence that may be to the contrary (Xu L, Marians K J. J. Biol. Chem. 2002 Apr. 19;277(16):14321-8). It is unclear how similar uvsX and recA filaments are in their respective abilities to promote polymerase loading at 3' ends with or without recombinase dissociation. We have found differences between uvsX(C) and recA(C) consistent with the notion that their differences have can affect RPA efficiency. Contrary to our findings with recA, uvsX can mediate multiple invasions to an end structure target even when N-terminally tagged gp32 is used in the absence of polyethylene glycol (**FIG. 23**). Thus uvsX may be more optimal for use in RPA.

[0289] The bacteriophage T4 uvsX recombinase has a well-characterised interaction with its partner loading protein uvsY. Despite reports suggesting that *E. coli* recO and recR may be functional analogues of uvsY we have not observed significant improvement for RPA reactions. This may be due to problems with our protein preparations, or due to the use of the heterologous gp32 rather than *E. coli* SSB.

[0290] Finally uvsX is likely to behave better with gp32, which we find to be an optimal single-stranded DNA binding protein, because they have evolved to function in concert and may have relevant interactions. Indeed uvsX and other components of the bacteriophage T4 recombination dependant replication machinery, such as the dda helicase, have known protein-protein interactions that may useful for establishing an optimal RPA reaction (Hacker K J, Alberts B M. J. Biol. Chem. 1992 Oct. 15;267(29):20674-81).

[0291] Despite the apparent advantages of uvsX for RPA, there are features of *E. coli* recA that may be useful. It has been reported that recA nucleoprotein filaments are more stable, which could be of utility in some circumstances. The lower ATP hydrolysis rate places less strain on establishing a durable ATP regeneration system, and the lack of generation of AMP and pyrophosphate obviates the need to regenerate and mop-up these side-products. It may also be the case that other recA homologues possess activities that are optimal or RPA.

[0292] Establishment of a Dynamic Recombination System:

[0293] To make RPA robust, it is critical to configure the reaction to provide a sufficient number of active, coated, homology-searching recombinase/DNA filaments. In addition, following completion of homology-searching, filaments must efficiently disassemble, or be otherwise processed, to permit loading DNA polymerase and other components. It is also essential that a sufficient quantity of single-stranded DNA binding protein be present both to facilitate oligonucleotide melting and to collect displaced outgoing strands. And finally, robust RPA requires will

require processive strand-displacing DNA synthesis. Underlying these requirements is a competition between two the recombinase and the single-stranded DNA binding protein.

[0294] It is widely known that of recombinase-loaded DNA filaments are unstable in the presence of single-stranded DNA binding proteins. Coupled to the finding that nucleotide cofactor hydrolysis is not strictly required for homology searching, led to the use of non-hydrolysable nucleotide analogues of nucleotides such as ATP-γ-S, to load recA onto filaments and produce stable homology-searching complexes. A recA-mediated amplification method using ATP-γ-S has been described (Zarling, et al.), however, has not been widely used. We previously identified a flaw in the method, which we can now observe in our experimental results. The Zarling, et al. method probably fails because recombinase-loaded filaments needs to be dynamic and capable of disassembly as well as other ATP-hydrolysis dependant events to complete strand exchange and permit loading of DNA polymerase other components to the 3' end of the invading oligonucleotide. The use of ATP-γ-S, as well as other modifications such as removing acidic sequences from the C terminus of recombinases, leads to a constant general high affinity for DNA that likely prevents strand exchange and dissociation from the invasion complex. Thus ATP-γ-S-loaded recA filaments become effectively locked onto the target site in the recombination event for an abnormally long time. Consequently non-hydrolysable nucleotide analogues are not generally permissive for recombinase-mediated replication and amplification. Instead, ATP, or other hydrolysable nucleotides capable of supporting recombinase loading, must be employed. In ATP the recombinases are constantly associating with and dissociating from oligonucleotide and are in competition with single-stranded DNA binding proteins. We have addressed the problem this competition poses in two general ways; first by including a recombinase loading protein specific for uvsX, the uvsY protein, and secondly by modulating the cooperative behaviour of gp32, and the recombinases uvsX and recA, by mutation and/or inclusion of crowding agents. It is possible however that limited quantities of non-hydrolysable analogues such as ATP-γ-S, or of non-phosphorylatable analogues such as ADP-O—S, may be included to modulate the global loading/unloading activity of the recombinase.

[0295] Additional Reaction Components

[0296] A number of specific reaction components have a significant influence on RPA reaction efficacy.

[0297] Polyethylene Glycol

[0298] Polyethylene glycols (PEGs) have a profound effect on recombination/DNA synthesis. Firstly, we find that PEGs influence the number of multiple invasion/extension cycles that occur, for example when recA is combined with gp32(N). We have also found that PEGs stimulate amplification reactions configured in several different ways (FIG. 15, Example 3). We also know that in some configurations PEGs alter the length distribution of products formed (FIG. 28). In summary polyethylene glycols, and presumably other similar crowding agents may affect the cooperativity of gp32 and recombinases, affect polymerase processivity and affect the hybridisation rate and behaviour of oligonucleotides in solution. Furthermore the chain length of the polyethylene glycol appears to be critical. We find PEGs of average

molecular weight 1450 and 15-20,000 (PEG 'compound') produce the best results. The PEGs in aiding gp32 function, particularly gp32 variants with attenuated cooperativity, has been detailed above. PEG is also likely to increase the stability of recombinase-loaded filaments and the increased persistence may increase RPA efficacy.

[0299] ATP Regeneration System Components

[0300] An ATP regeneration system is crucial to permit persistent recombination reactions as recombinases have an extremely high rate of ATP hydrolysis when bound to nucleic acids. In particular, the uvsX protein has a hydrolysis rate 10-20 times higher than recA and can consume 200 molecules of ATP per minute per monomer. A number of systems are available and we have routinely used the creatine kinase/phosphocreatine system. When uvsX is employed the AMP that is produced must be converted into ATP. We have used chicken myokinase, which converts a molecule of AMP and one of ATP to two molecules of ADP. ADP is then converted to ATP using the creatine kinase/phosphocreatine system. Poor regeneration of ATP will reduce the reaction rate and possibly stop the reaction.

[0301] Pyrophosphatase

[0302] Pyrophosphate (PPi) accumulates during DNA synthesis and when uvsX is employed, as it hydryolyses ATP to AMP+PPi. PPi accumulation in the reaction can have several detrimental consequences. Significant pyrophosphate accumulation will permit an unacceptable rate of pyrophosphorylysis, whereby the synthetic reaction of a polymerase is driven into reverse and removes the 3'-most nucleotides from duplex templates. This could lead to unacceptable levels of primer noise, or enhanced levels of undesired self-priming of outgoing strands, because editing activities of the polymerase tend to trim 3'-ends back until a suitable duplex region is revealed to permit rapid elongation. Additionally pyrophosphatase accumulation will lead to inhibition of the recombinase, and to a slowing of the polymerase synthetic reaction.

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EXAMPLES

[0371] As shown herein, we have developed an in vitro DNA amplification system that couples recombinase-driven sequence targeting with strand-displacement synthesis. This permits DNA amplification without global thermal, chemi-

cal, or enzymatic template melting. Reactions are sensitive, specific and operate at 37° C. with no pre-treatment of sample DNA. As much as 1012-fold amplification is observed within 1-1½ hours. Less than 10 copies of a given target DNA can be detected in a complex sample with a simple single-step reaction. This method is an ideal alternative to PCR for a variety of applications and will enable highly portable DNA diagnostic systems. The examples are presented in order to more fully illustrate the preferred embodiments of the invention. These examples should in no way be construed as limiting the scope of the invention, as encompassed by the appended claims.

Example 1

An example of a Leading Strand Recombinase-Polymerase Amplification (lsRPA)

[0372] DNA sequences can be amplified using leading strand synthesis according to the Recombinase-Polymerase amplification (RPA) method depicted in **FIG. 1**. **FIG. 1** shows RecA/primer loading. Prior to the addition of template DNA and/or Polymerase, RecA and SSB will compete for binding to single-stranded oligonucleotide primers. In the presence of a RecR and RecO, RecA is selectively stabilized onto the single-stranded primers forming RecA nucleoprotein filaments in a complex with RecO and RecR. This complex is competent to invade double-stranded DNA to form a D-loop at sites homologous to the oligonucleotide primers. Alternatively, RecA, RecO and RecR can be pre-loaded onto oligonucleotide primers prior to the introduction of SSB to the reaction mixture.

[0373] The following details the likely composition of an RPA reaction assembled with *E. coli* recA and *E. coli* recO and recR stabilizing agents:

Component	Concentration
D-LOOP FORMATION/RESOLUTION COMPONENTS:	
RecA	20 μM
Single-stranded oligonucleotide primers	0.25 μM
ATP	3 mM
RecF	0.1 μM
RecO	0.13 μM
RecR	0.5 μM
Single-stranded Binding protein (SSB)	1 to 10 μM
DNA polymerase V	5 units
POLYMERASE/HELICASE/RESOLVASE MIX:	
DNA Polymerase	5 units
RuvA	0.5 μM
RuvB	0.5 μM
RuvC	0.5 μM
RecG	10 nM
REACTION BUFFER:	
MgCl2	2 to 10 mM
TrisHCl pH 7.2	50 mM
DTT	0 to 10 mM
KCl	0 to 50 mM
Deoxyribonucleotide triphosphates	0.2 mM
Bovine serum albumin (BSA)	0 to 10 μg per ml

[0374] The reaction is assembled so that the final concentration satisfies the D-Loop Formation/Resolution Components, Polymerase/Helicase/Resolvase Mix, and Reaction

Buffer with the DNA polymerase and/or template added last if necessary. For example, a 2× concentrated solution of D-Loop Formation/Resolution Components and of the Polymerase/Helicase/Resolvase Mix may be made in 1× reaction buffer. The reaction may be initiated by mixing an equal volume of each of the two components (each in 1× reaction buffer). Optionally, and as stated above, the DNA polymerase or template (target DNA) may be added last. The reaction is incubated for a sufficient time of until the reactants are exhausted. Typical incubation times would range from 1 hour, 2 hours, 3 hours, 5 hours, 10 hours or overnight (about 16 hours). Unlike PCR, which requires small volumes for rapid temperature change, there is no limit to the reaction volume of RPA. Reaction volumes of 25 μl, 50 μl, 100 μl, 1 ml, 10 ml and 100 ml or larger may be performed in one vessel. Incubation temperature may be a typical laboratory temperature such as 25° C., 30° C., or 37° C.

[0375] Prior to the addition of template DNA and/or Polymerase, recombinase and SSB will compete for binding to single-stranded oligonucleotide primers. In the presence of a RecR and RecO, RecA is selectively stabilized onto the single-stranded primers forming RecA nucleoprotein filaments in a complex with RecO and RecR. This complex is competent to invade double-stranded DNA to form a D-loop at sites homologous to the oligonucleotide primers. Alternatively, RecA, RecO, and RecR can be pre-loaded onto oligonucleotide primers prior to the introduction of SSB to the reaction mixture (**FIG. 1**).

[0376] The invading strands will be extended by the polymerase in a 5' to 3' direction. As D-loops are formed and synthesis proceeds, displaced single stranded DNA becomes coated with SSB. RecA release from double-stranded DNA can occur via ATP hydrolysis in a 5' to 3' direction or as a result of helicase/resolvase or polymerase activity (**FIG. 2A, B**). New rounds of invasion/synthesis will continuously occur. The third round of strand-invasion/synthesis will release discrete products released whose ends correspond to the two facing primer sites. These fragments will soon become the dominant reaction product and will accumulate to high levels. As each synthetic complex processes to the end of the template RecA protein is displaced either by polymerase activity or by the activity of helicases, such as RuvAB or resolvases, such as RuvC. Once primers, ATP, deoxynucleoside triphosphates, or any other limiting component is exhausted, the reaction will stop.

[0377] The inclusion of temperature-sensitive recombinase mutants will allow the controlled initiation of DNA synthesis. In such a situation, the initiation reaction is performed at 25 to 37° C. permitting the formation of D-loops. Elongation reactions are performed at 42° C., which is non-permissive for RecA mediated double-strand invasion. The number of cycles will determine the amount of reaction product. Extended elongation phases will permit the amplification of extremely long DNAs without interference of re-invasion.

Example 2

Nested RPA

[0378] The RPA reaction is performed as described in Example 1. A fraction of one tenth (⅒) and one hundredth

(1/100) of the reaction is removed and used in place of the DNA template in a second round of RPA. LsRPA, leading/lagging RPA, and combinations thereof may be used for nested RPA.

Example 3

Simultaneous Leading and Lagging Strand Recombinase-Polymerase Amplification

[0379] DNA sequences can be amplified using simultaneous leading and lagging strand synthesis according to the Recombinase-Polymerase amplification (RPA) method depicted in **FIG. 2**. This figure specifically illustrates lsRPA. **FIG. 2A** shows that RecA/primer nucleoprotein filaments invade double stranded template DNA preferentially associating with homologous target sites. As D-loops are formed and synthesis proceeds, displaced single stranded DNA becomes coated with SSB (**FIG. 2A**). RecA release from double-stranded DNA can occur via ATP hydrolysis in a 5'-3' direction or as a result of helicase/resolvase or polymerase activity (**FIG. 2A**). As synthesis continues (**FIG. 2B**), polymerases encounter SSB bound, displaced single-stranded template. Double-stranded target sites are re-invaded by RecA/primer nucleoprotein filaments. Subsequent rounds of lsRPA proceed from re-invaded sites (**FIG. 2B**).

[0380] The following details likely components of a replisome-mediated amplification utilizing components from *E. coli*. A reaction is assembled with the following composition:

Component	Concentration
D-LOOP FORMATION/RESOLUTION COMPONENTS	
RecA	20 μM
Single-stranded oligonucleotide primers	0.25 μM
ATP	3 mM
RecF	0.1 μM
RecO	0.13 μM
RecR	0.5 μM
Single-stranded Binding protein (SSB)	1 to 10 μM
DNA polymerase V	5 units
HELICASE/RESOLVASE MIX	
RuvA	0.5 μM
RuvB	0.5 μM
RuvC	0.5 μM
RecG	10 nM
PRIMOSOME COMPLEX	
PriA	20 nM
PriB	20 nM
DnaT	100 nM
DnaB	100 nM
DnaC	200 nM
DnaG	200 nM
DNA POLYMERASE III HOLOENZYME COMPLEX	
β-Clamp	2 μM
DnaX Clamp Loader	500 nM
Polymerase Core Complex	500 nM
LAGGING STRAND MIX	
DNA polymerase I	5 units
DNA ligase	2 units

Component	Concentration
-continued	
REACTION BUFFER	
MgCl₂	2 to 10 mM
TrisHCl pH 7.2	10 to 60 mM
DTT	0 to 10 mM
KCl	0 to 50 mM
Deoxyribonucleotide triphosphates	0.2 to 0.4 mM
Bovine serum albumin (BSA)	0 to 10 μg per ml

[0381] The reaction is assembled so that the final concentration of all the reagents is as listed above. Thus, for example, a 5 fold concentrated solution of each of the components (D-loop Formation/Resolution Components, Helicase/Resolvase Mix, Primosome Complex, DNA Polymerase III holoenzyme Complex, Lagging Strand Mix) is made in 1x reaction buffer. Then, the five solutions are mixed together in equal volumes to initiate the reaction. The reaction is incubated for a sufficient time of until the reactants are exhausted. Typical incubation times would range from 1 hour, 2 hours, 3 hours, 5 hours, 10 hours or overnight (about 16 hours). As stated above, there is no limit to the reaction volume of RPA. Reaction volumes of 25 μl, 50 μl, 100 μl, 1 ml, 10 ml and 100 ml or larger may be performed in one vessel. Incubation temperature may be a typical laboratory temperature such as 25° C., 30° C., or 37° C.

[0382] **FIG. 3** shows initiation (**FIG. 3A**), synthesis (**FIG. 3B**), and polymerase amplification (**FIG. 3C-3D**). First, the primosome loads onto the D-loop formed by RecA nucleoprotein filament invasion (**FIG. 3A**). The primosome synthesizes a stretch of RNA primer. Finally, the primosome recruits the clamp loader, which recruits both the sliding clamp dimer and the asymmetric DNA polymerase core (**FIG. 3A**). Synthesis occurs simultaneously in both the leading and lagging directions. Eventually lagging strand synthesis stops and the lagging strand clamp is unloaded (**FIG. 3B**). Synthesis of the leading strand continues until a new site of lagging stand synthesis is formed (**FIG. 3B**). While leading strand synthesis continues, a new site of lagging stand synthesis is formed. Lagging strand synthesis continues back to the previous Okazaki fragment where the lagging strand clamp is unloaded (**FIG. 3C**). DNA Polymerase I removes the RNA primer, and fills in the gap while DNA ligase connects the two Okazaki fragments forming a continuous lagging strand (**FIG. 3D**).

Example 4

Establishment of an Amplification Environment Using the Assembly of Heterologous Components *E. coli* recA(C) and T4 gp32(N)

[0383] **FIG. 18** shows the results of an experiment in which recA(C) has been combined with gp32(N) in the presence of pairs of oligonucleotides, Tester3bio (possessing a 5' biotin label) and Sizer1, Sizer2, Sizer3, Sizer4 or Tester2. These latter unbiotinylated oligonucleotides were positioned progressively further away from the common Tester3bio oligonucleotide. The template was a linear DNA fragment, approximately 300 bp, released from a plasmid. Tester3bio was designed to be complementary to one end of this fragment and included a 5' overhang relative to this sequence.

[0384] The reaction buffer included Magnesium acetate at 10 mM, required to support recA binding to DNA, and 3 mM ATP. Also included was an ATP regeneration system, comprising phosphocreatine and creatine kinase, as well as dNTPS at 200 μM, and the Klenow fragment of *E. coli* DNA polymerase I. PEG compound was employed as shown. Double stranded template DNA (0.5 fmoles), derived from a plasmid carrying the *E. coli* ruvb gene, was used as a starting target. The Sizer1, Sizer2, Sizer3, and Sizer4 oligonucleotides did not recognise the other end of the template. Instead, these oligonucleotides were positioned to face Tester3bio with increasing distance between their relative 3' ends.

[0385] After an incubation of 2 hours at 37° C., there was a substantial amplification of specific fragments of the correct size when Tester2, 3, and 4 were used. In the best conditions (with Sizer2), we estimated that the amplification product were 104 fold greater than the starting template.

Example 5

The Nature of Amplification Products and the Sensitivity of the Reaction Using a Heterologous Assembly of *E. coli* recA(C) and Bacteriophage T4 gp32(N)

[0386] **FIG. 19** shows the results of an experiment in which recA(C) has been combined with gp32(N) in the presence of the pair of oligonucleotides, Tester3bio (possessing a 5' biotin label) and Sizer2, under conditions similar to those used in Example 1. PEG compound or PEG 1450 were employed as shown and 0.5 fmoles of template was used as a starting template amount. In this example, progressive dilution of the template was investigated. Alternatively we explored the use of linearised starting template possessing no end that overlaps the primer (by using a ClaI digest of the *E. coli* ruvB gene carrying plasmid), and dilution of the Klenow fragment. Amplification of correctly sized fragments occurred in all lanes and was strongest in the case of 0.5 fmoles-starting template in the presence of PEG compound.

[0387] When the products of these optimal reactions were electrophoresed on agarose gels and stained with ethidium bromide, a clean band of double-stranded DNA of the correct size was observed. When this sample was treated with BbvC1 restriction enzyme prior to electrophoresis the expected increase in gel mobility occurs consistent with a single cut as expected. Amplification of a product of the correct size was observed with starting template dilutions of 100-fold, or greater, although the product was less abundant and includes a ladder of shorter products below the main band. A similar pattern was observed when uncut template is employed or when no template is employed. We reasoned that the proteins used in these studies were significantly contaminated with *E. coli* genomic DNA (naturally carrying the ruvB gene) as they were purified in single column purifications without the use of nucleases. Consequently we believe this test system generates false positives when the sensitivity is high enough.

Example 6

Establishment of an Amplification Environment Using an Assembly of gp32(N) and uvsX(C)

[0388] **FIG. 24** shows results of an experiment in which uvsX(C) has been combined with gp32(N) in the presence of

the oligonucleotides Tester3bio and Sizer2. The template DNA in this experiment was an EcoRV digestion of the *E. coli* ruvB gene carrying plasmid used in Examples 1 and 2. Tester3bio recognised one end of an approximately 300 base pair fragment and included a 5' overhang relative to the end of the target sequence. Sizer2 recognised the other strand of this template. This oligonucleotide was directed toward embedded sequences such that its 3' end was about three and a half helical turns from the end of Tester3bio.

[0389] In the presence of PEG1450, we observed the amplification of the expected fragment within the 2 hours of the reaction. In the cases where amplification has occurred, almost the entire of population of oligonucleotides was consumed indicating an amplification of $3\text{-}5 \times 10^4$. The reaction components are indicated on **FIG. 24**. Included in some samples were additional components. We found that 200 μM ADP-β-S included in this reaction slightly increased the amount of product formed under these conditions. Conversely, under the conditions used here, inclusion of *E. coli* toposiomerase I was inhibitory to DNA amplification. Under the conditions used, we detected no amplification with uvsX(C)delta protein. However, no PEG1450 was included in these samples and uvsX(C) also failed to amplify under these conditions without PEG1450.

Example 7

Amplification of a Target from Human Genomic DNA Using T4 Recombination Proteins

[0390] **FIG. 30** shows the results of an experiment in which several pairs of primers were employed to amplify a specific DNA fragment from human genomic DNA. The reaction included bacteriophage T4 gp32(C)K3A, uvsX(C) and uvsY(N) proteins, as well as exonuclease deficient Klenow fragment, and proteins comprising the ATP regeneration system to convert ADP and AMP. To detect the specific DNA fragment, we transferred the electrophoretically separated reaction products to nylon membrane, then hybridised a biotinylated probe, which recognised a unique non-primer internal sequence.

[0391] Three primer pairs were employed, and in each case a comparison was made between no input genomic DNA, 10,000 copies of uncut human genomic DNA, and 10,000 copies of HpaII cut genomic DNA (which generates at least one end for the primer pairs). In all cases, specific amplification of the desired DNA sequence occurred, while the efficiency showed variation between primer pairs, and between uncut and cut DNAs. In all cases, prior HpaII digestion of the DNA sample was not absolutely required, but improved the efficiency of amplification. In all cases, input genomic DNA was important. In the best amplification (shown in lane 4), we estimated at least 10^{11} molecules, indicating an amplification of the approximate order 10^7.

Example 8

Sensitivity of lsRPA when Targeting a Complex DNA—Human Genomic DNA

[0392] **FIG. 31** shows the results of an experiment in which several pairs of primer were employed to amplify a specific DNA fragment from human genomic DNA. The reaction included bacteriophage T4 gp32(C)K3A, uvsX(C) and uvsY(N) proteins, as well as an exonuclease deficient

Klenow fragment, and comprising the ATP regeneration system to convert ADP and AMP. To detect the specific DNA fragment, we transferred the electrophoretically separated reaction products to nylon membrane. Then we hybridised a biotinylated probe, which recognises a unique non-primer internal sequence.

[0393] Three primer pairs were employed and in each case a comparison is made between no starting template and approximately 10, 100, 1000, 3000, and 10,000 copies of the genomic target. In all cases, clear amplification was detected when at least 1000 copies of the genomic target were used (a weak signal is seen with the best primer pair at 100 copies). We concluded that during that lsRPA reactions configured in this way were capable of amplifying DNA from very complex targets with a sensitivity of at least 1000 copies, and potentially higher.

Example 9

Competition Between the Accumulation of Bona Fide Product and Primer (Template-Independent) Artifacts During Reactions

[0394] FIG. 32 shows the results of an experiment in which a pair of primers was employed to amplify a specific DNA fragment from human genomic DNA. Employed in the reaction were bacteriophage T4 gp32(C), uvsX(C) and uvsY(N) proteins, as well as an exonuclease deficient Klenow fragment, and proteins comprising the ATP regeneration system to convert ADP and AMP. PEG 1450 was included at 10% w/v. One of the oligonucleotides included a 5'-biotin so that all reaction products could be observed at the end of the amplification. Samples were taken at 1, 2 and 3 hours to observe how the reaction progressed. In one sample, when a minimal amount of uvsY(N) was employed (50 ng/μl), amplification of the correct fragment was observed (see arrow in lane 4). This fragment was cleaved by BstXI to the expected size fragment, indicating it was principally double-stranded. However the fragment was less abundant than apparently template-independent bands that also accumulated during the reaction. The size and template-independent nature of these bands suggested that they were primer artifacts, e.g., primer dimers and/or snapback synthesis products. The absence of amplification of the specific fragment suggested that, at uvsY(N) concentrations greater than 50 ng/μl, the reaction occurred suboptimally. This was borne out by later experiments.

Example 10

Optimisation of Reaction Composition to Severely Limit or Eliminate Primer Artifacts and Enable Sensitive Noise-Free Amplification from Complex Templates

[0395] FIG. 36 shows the results of an experiment in which a pair of primers was employed to amplify a specific DNA fragment from human genomic DNA. Employed in the reaction were bacteriophage T4 gp32(C), uvsX(C) and uvsY(N) proteins, as well as an exonuclease deficient Klenow fragment, or Bst polymerase, and proteins comprising the ATP regeneration system to convert ADP and AMP. One of the oligonucleotides included a 5'-biotin so that all reaction products could be observed at the end of the amplification. Amplified fragments were visualised follow-

ing separation of fragments by size by running a small sample of the reaction on an acrylamide gel. In this experiment, uncut human genomic DNA was titrated from zero copies, 45 copies, and then doublings in target copy number up to 2880. Slightly different conditions were employed in this experiment for each of the two polymerase species with regard to both buffer and temperature. The reaction with the Klenow fragment was performed at 37° C., while that with Bst polymerase was performed at 42° C. The details of the buffer composition are given in the figure description.

[0396] Of note, and important to the efficiency of reactions under these optimised conditions, PEG compound was included at 5% final weight to volume in both cases. Both polymerases have effectively amplified the correct fragment, and in some cases, utilised most of the available primers. Under the conditions used for the Klenow fragment, the sensitivity was so great that a weak signal was observed even in the zero copies lane presumably reflecting contamination with a quantity of human DNA representing less than the 45 copies present in the lane immediately adjacent. At the level of sensitivity that is demonstrated here, it was difficult to eliminate trace levels of contamination from the equipment that was used leading to signals in the negative controls. Routine employment of conditions similar to those utilised in the Klenow-mediated amplification proved effective for noise-free amplification of numerous primer pairs in later experiments. This suggested that these conditions were close to one optimum for reactions involving this set of protein components.

Example 11

Experimental Methods for Production of Clones and Proteins

[0397] All clones have been constructed by cloning PCR amplified products from E. coli, T4 phage, B. subtilis, or Phi-29 phage. All stock organisms used for amplification were obtained from a public source at the DSMZ. Cloned DNA's used for protein expression have in general been cloned into pET vectors (e.g., pET-21) with the insertion of a hexahistidine peptide tag at either the N or C terminus during the PCR amplification of the fragment, or into pQE vectors (e.g., pQE31) in the case of Pol I from B. subtilis (Bsu polymerase). In this disclosure all proteins containing an N terminal tag are referred to as the protein name followed by (N), e.g. gp32(N), or if containing the tag at the C terminus the name is followed by (C), e.g. gp32(C). Additionally we have constructed several clones to produce otherwise modified proteins. These include a recA(C) with a deletion of the last 17 amino acid residues of the native protein, referred to as recA(C)delta17. A similar form of the T4 UvsX(C) protein has been generated and is referred to as UvsX(C)delta 21. We have also constructed mutant forms of gp32, which modify either lysine 3 or arginine4.

[0398] All proteins were overexpressed in E. coli and purified using conventional protocols. Proteins have generally been purified by standard procedures on Nickel resin in 1 M NaCl and phosphate buffer. Proteins were eluted with 250 mM imidazole and dialysed into appropriate buffers. Proteins produced from clones generated in-house include: E. coli recA(C), E. coli SSB(N), E. coli PriA(N), E. coli PriB, E. coli PriC, E. coli DnaB, E. coli DnaC, E. coli DnaC810, E. coli DnaT, E. coli RuvA, E. coli RuvB, T4

phage UvsX(C), T4 phage UvsX(N), T4 gp32(N), T4 gp32(C), T4 gp32(C)K3A, T4 phage gp32(C)R4Q, T4 phage gp32(C)R4T, T4 phage gp32, T4 phage gp32 K3A, T4 phage gp32R4Q, T4 phage gp32R4T, T4 phage UvsY(N), T4 phage UvsY(C), T4 phage gp43, T4 phage gp43(exo–), *E. coli* Klenow fragment, *E. coli* Klenow exo–. Untagged gp32 proteins were purified by a 2-column procedure involving DEAE sepharose anionic exchange followed by binding to single-stranded DNA cellulose matrix.

[0399]   DNAs Used in RPA Reactions.

[0400]   We have employed several different target DNAs in this study, and a number of oligonucleotides. The sequence of the relevant section of the templates, and the sequence of the oligonucleotides is given below.

[0401]   The *E. coli* RuvB Gene Target

[0402]   The sequence of the EcoRV fragment of the RuvB gene is given below.

```
    ATCATGATTGGTGAAGGTCCGGCGGCACGCTCC  (SEQ ID NO:1)

ATTAAAATTGATTTGCCGCCGTTTACCCTGATTGGTG

CAACCACGCGCGCAGGTTCGCTGACATCACCGTTGCG

CGACCGTTTTGGTATTGTGCAACGTCTGGAGTTTTAT

CAGGTGCCGGATCTGCAATATATCGTCAGTCGCAGCG

CACGCTTTATGGGGCTTGAGATGAGTGATGACGGCGC

GCTGGAAGTTGCTCGTCGCGCTCGCGGTACGCCGCGC

ATTGCCAACCGTCTGCTGCGTCGAGTGCGTGATTTCG

CCGAAGTGAAGCACGATGGCACCATCTCGGCAGAT
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[0403]   The sequence of oligonucleotides targeting this template mentioned in this study are given below:

```
    Tester2
CTAGCGATGGTGCCATCGTACAGAATTCCCTCAGCA (SEQ ID NO:2)

TCTGCCGA

    Tester3
CTCACTATACCTCAGCATCATGATTGGTGAAGGTCC (SEQ ID NO:3)

GGCGGCAC

    Tester1bio
5'-biotin-GCTAATACGACTCACTATACCTCAGC (SEQ ID NO:4)

ATCATGATTGGTGAAGGTCCGGCGGCAC

    Tester3bio
5'-biotin-CTCACTATACCTCAGCATCATGATTG (SEQ ID NO:5)

GTGAAGGTCCGGCGGCAC

    Sizer1
CTATGCGAATTCAGCGAACCTGCGCGCGTGGTTGCA (SEQ ID:6)

CCAATCAGGG

    Sizer2
CTATGCGAATTCGGTGATGTCAGCGAACCTGCGCGC (SEQ ID NO:7)

GTGGTTGCA
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                     -continued
    Sizer3
CTATGCGAATTCTCCAGACGTTGCACAATACCAAAA (SEQ ID NO:8)

CGGTCGCGC

    Sizer4
CTATGCGAATTCCGTGCGCTGCGACTGACGATATAT (SEQ ID NO:9)

TGCAGATCC

    Gen2bio
5'-biotin-ATCTGCCGAGATGGTGCC            (SEQ ID NO:10)
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[0404]   The sequence of part of the human angiotensin converting enzyme targeted in this study is shown below:

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AACCAACTCCGCCCCGGGCCACGGCCTCGCTC (SEQ ID NO:11)

TGCTCCAGGTACTTTGTCAGCTTCATCATCCAGTTC

CAGTTCCACGAGGCACTGTGCCAGGCAGCTGGCCAC

ACGGGCCCCCTGCACAAGTGTGACATCTACCAGTCC

AAGGAGGCCGGGCAGCGC
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[0405]   Underlined are HpaII restriction sites that have been targeted with HpaII in the preparation of some DNAs in some experiments.

[0406]   The sequence of oligonucleotides used to target part of the human ACE gene are shown below:

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    Up3
ATTCGTCAGCCTCGCTCTGCTCCAGGTACTTTGTCA (SEQ ID NO:12)

GCTTCATC

    Down1
GCCTCCTTGGACTGGTAGATGTCACACTTGTGC     (SEQ ID NO:13)

    Down2
GCGCTGCCCGGCCTCCTTGGACTGGTAGATGTCACA (SEQ ID NO:14)

CTTGTGC

    Down3
TATGCGAATTGCCTCCTTGGACTGGTAGATGTCACA (SEQ ID NO:15)

CTTGTGC

    Angio1bio
5'-biotin-GCCTCCTTGGACTGGTAGATGTCACA (SEQ ID NO:16)

CTTGTG

    Angio3
GGCCACGGCCTCGCTCTGCTCCAGGTACTTTGTCAG (SEQ ID NO:17)

CTTCATC
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<div align="center">

Example 12

Experimental Results and Analysis

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[0407]   **FIG. 9** shows the results from investigations into the nature of double-stranded DNA targets and targeting oligonucleotides. Experiments using either supercoiled templates or linearised DNAs suggested that recA catalyses the

formation of intermediates capable of supporting polymerase elongation most readily on supercoiled DNA, or the ends of linearised DNA. Shown are the results of an experiment in which the biotinylated oligonucleotide, Tester3bio, has been incubated with either supercoiled target DNA, or a target template linearized with EcoRV, or ClaI. This generated an end that overlapped with the oligonucleotide or embedded sequences respectively. The reaction solution included 20 mM Tris-acetate pH 7.9, 10 mM Mg-acetate, 13 μg recA, 1 μg E. coli SSB, 27 mM phosphocreatine, 1 U creatine kinase, 0.2 μM Tester3bio, 3 mM ATP, 200 μM dG, dC, and dT; 1 mM dA, 50 U Klenow, 0.5 pmoles template, 120 ng recO, 120 ng recR, 0.5 μM dnaB, and 0.5 μM dnaC810. E. coli recO and recR proteins, as well as dnaB and dnaC810 proteins, were included in this experiment although they did not significantly affect the results. After 2 hours of reaction at 37° C., the reaction was precipitated and run on a 6% denaturing gel, transferred to nylon membrane, and incubated with streptavidin-HRP prior to performing ECL to detect reactive material. In each reaction, 0.5 pmoles of template was used. Included on the gel as a control for size and amount was 0.5 pmoles of biotinylated PCR fragment (labeled CON). Other reaction components and conditions are indicated on the figure.

[0408] FIG. 10 shows backfire synthesis. Backfire synthesis occurs when a recombinase-coated targeting oligonucleotide possessing a 5' overhang invades a duplex DNA end in the presence of a suitable polymerase and dNTPs. This new duplex region is stable to subsequent branch migration and can be utilised as a platform for other applications. Forward fire is the elongation of the invading oligonucleotide, which also occurs in these reactions. Shown are the results of experiments to detect the activity of polymerases on intermediates formed when the oligonucleotide Tester3, possessing a 5' overhang relative to the end of a linearized target DNA, is incubated with various templates.

[0409] In part A, the template used is a double-stranded PCR product generated such that the product has a biotin label at the 5' end of the strand complementary to the targeting oligonucleotide. This fragment is otherwise similar to the EcoRV fragment released from a plasmid carrying the E. coli RuvB gene used elsewhere in this study, and which is a target for the Tester3bio oligonucleotide. The reaction solution included 10 mM Mg-acetate, 7.5 μg recA, 1 μg SSB, 27 mM phosphocreatine, 1 U creatine kinase, 0.3 μM Tester3bio, 3 mM ATP, 200 μM dNTPs, 50 U Klenow, 0.5 pmoles biotinylated template. Optionally, we included 0.5 μM ruvA and 0.5 μM ruvB; or 1 μM ruvA and 1 μM ruvB; or 1.5 μM ruvA and 1.5 μM ruvB. The final volume was 30 μl. Incubation was carried out for 1 hour at 37° C. In the presence of recA, the biotinylated strand of the target was extended by 16 bases, as would be expected if a recombination intermediate were accessible by a polymerase to copy the overhang region of the invading oligonucleotide.

[0410] In part B, the reaction is configured in a similar manner except that the template is not biotinylated, and the invading oligonuleotide is biotinylated. Several polymerases were investigated in this experiment, and only unmodified Klenow fragment gave a significant production of product. In this experiment, we also investigated including a small oligonucleotide designed to recognise the target directly downstream of the Tester3 targeting site. The reaction solu-

tion included 10 mM Mg-acetate, 10 μg recA, 1 μg SSB, 27 mM phosphocreatine, 1 U creatine kinase, 0.3 μM Tester3bio, 3 mM ATP, 200 μM dNTPs, 50 U Klenow, 0.5 pmoles unbiotinylated template. Optionally, we included 5 μl preloaded stable ATPγS oligonucleotide. The final volume was 30 μl. Incubation was carried out for 1 hour at 37° C. We pre-incubated with recA in the presence of ATP-γ-S in an effort to load recombinase stably onto it. Pre-load solution included 10 mM Mg-acetate, 2.5 μg recA, 50 μM ATPγS, and 0.15 μM oligonucleotide. The pre-load solution was added into the Tester3bio invasion/extension mixture. In all cases, the yield of product was decreased by inclusion of this premixed material. Based on our data, we believe that the presence of ATP-γ-S (final concentration ~8 μM) in the reaction was mildly inhibitory. The purpose of this experiment was to address whether the presence of a stable 3-stranded hybrid formed immediately downstream of the Tester3 targeting site would stabilise these invasions to branch migration.

[0411] FIG. 11 shows uses of backfire synthesis. Backfire synthesis can be useful because it generates a branch migration resistant platform that can be employed in applications other than straightforward forward fire. Some examples are shown here, including introduction of a nicking enzyme target site, introduction of an RNA polymerase promoter, and the linear generation of short dsDNA fragments through successive invasion/synthesis/cleavage events. If a restriction enzyme site is included in the additional overhang sequence such that after targeting a suitable linearized fragment, backfire synthesis will generate the duplex target for the restriction enzyme. The enzyme can then cut the sequence releasing a short double-stranded DNA, and a longer double-stranded DNA, which is a target for further invasion events.

[0412] In FIG. 11B, the 5' overhang of a targeting oligonucleotide is designed such that should backfire synthesis occur, a target for a nicking endonuclease is generated. In the presence of the nicking endonuclease, for example BbvC1a or b, a suitable polymerase, for example the Klenow fragment, can extend from the nick and displace a DNA strand. Multiple strands may be run-off by successive nicking and elongation from a single template. In FIG. 11C, the 5' overhand that is converted to duplex by backfire synthesis contains the sequence of an RNA polymerase promoter, such as the phage T7 RNA polymerase gene. In the presence of the necessary polymerase and suitable nucleoside triphosphates, transcription can initiate downstream of the promoter to generate an RNA as shown. The presence of a break in the non-template strand is not predicted to prevent successful elongation. RNA products might be used in some form of amplification reaction, or for other purposes.

[0413] FIG. 12 shows that single stranded binding proteins facilitate recombinase invasion and primer extension. Both E. coli SSB and bacteriophage T4 gp32 with an N-terminal His tag (gp32(N)) are able to stimulate recA-mediated invasion/elongation on a linear DNA template. The results of an experiment are shown in which 0.5 pmoles of target template (the EcoRV fragment released from a plasmid carrying the E. coli ruvB gene) was incubated with the Tester3bio oligonucleotide that overlaps one end of the template. Either the E. coli SSB protein, or the T4 gp32(N) protein was included to stimulate the reaction. The reaction solution included 10 mM Mg-acetate, 6 μg recA, 8.8 μg

gp32 or 1 μg SSB, 27 mM phosphocreatine, 1 U creatine kinase, 0.3 mM Tester3bio, 3 mM ATP, 200 μM dNTPs, 50 U Klenow, 0.5 pmoles template. Optionally, we included 120 ng recO and 120 ng recR. The final volume was 30 μl. Incubation was carried out for 1 hour at 37° C. Other reaction components and conditions are indicated in the figure. The figure also shows the general relationship of the primer and target DNA. In the reactions where *E. coli* recO and recR proteins were included, little effect was seen from their addition under these conditions. Invasion and elongation appeared to have proceeded in all cases, and the gp32(N) appeared to have stimulated synthesis even better than *E. coli* SSB, although it was used at higher concentration in this experiment.

[0414] **FIG. 13** shows the requirement for a minimal oligonucleotide length or overhang for invasion and elongation during end targeting of linear templates. The results of an experiment are shown in which 0.5 pmoles of target template (the EcoRV fragment released from a plasmid carrying the *E. coli* ruvB gene) was incubated with the either the Tester3bio oligonucleotide. This oligonucleotide overlaps one end of the template, or the Gen2bio oligonucleotide, which is flush to the other end of the template and is only 18 residues long. The reaction solution included 10 mM Mg-acetate, 27 mM phosphocreatine, 1 U creatine kinase, 0.2 μM Tester3bio or Gen2bio, 10 mM dATP, 3 mM ATP, 200 μM dNTP mixture, 50 U Klenow or Phi29 polymerase, 13 μg recA(C), 1 μg *E. coli* SSB, and 0.5 pmoles template. The final volume was 30 μl. Incubation was carried out for 2 hours at 37° C., and 2 μl or the reaction was loaded in each lane of the gel. Other reaction components, conditions, and the general relationship of the primers and target DNA are indicated on the figure. Invasion and elongation appeared to have proceeded efficiently in the presence of the Klenow fragment, less efficiently with the Phi29 polymerase, and less well with the Gen2bio primer and the Klenow fragment. We concluded that a minimal primer length and/or an overhand relative to the template was required to stimulate efficient invasion and elongation.

[0415] **FIG. 14** shows paranemic (A-E) and pletonemic (F-H) joints. For paranemic joints, the interaction of the recombinase filament with DNA stimulates unwinding (**FIG. 14A**). The unwound region moves with the homology search (**FIG. 14B**). The homology is found (**FIG. 14C**). The recombinase dissociates and a new duplex attempts to rewind (**FIG. 14D**). Because it topologically restrained, the 'outgoing' strand is forced to rewind around the new duplex (**FIG. 14E**). This state is highly unfavorable and unstable, and cannot always be managed by SSBs (**FIG. 14E**). For plectonemic joints, the interaction of the recombinase filament with DNA stimulates unwinding (**FIG. 14F**). If strand exchange overlaps with a DNA end, the 'outgoing' strand is freed and can relax as the incoming oligonucleotide and its complement rewind as the recombinase dissociates (**FIG. 14G**). This forms a deconstrained product, and a single-strand DNA binding protein inhibits branch migration (**FIG. 14H**).

[0416] This figure compares the likely events that occur when a nucleoprotein filament initiates strand exchange with a homologous sequence located at the end of a linearized duplex (right side of figure), or within a duplex which lacks homology on either side (left side of figure). Starting with the left side, once the nucleoprotein filament has located the

correct sequence it will pair the searching DNA to its complement, and one strand of the original duplex becomes unpaired. In fact, the exchange complex consists of 3 strands, which are relatively under-wound and stabilised by the recombinase. As the recombinase begins to disassemble in a 5' to 3' direction, the under-wound 3-stranded intermediate becomes unstable. For the new duplex to regain the normal conformation of relaxed DNA, it must rotate. However, in doing so, it must co-rotate the outgoing strand, as it is linked upstream and downstream to its original partner. This results in over-winding the outgoing strand, as it has to make the same number of turns but take a longer path around the new duplex, and is energetically unfavourable. Consequently there is a requirement for single-stranded binding proteins with very stable DNA interactions to permit such structures to exist for any significant time. Alternatively the right side of the diagram indicates that should the exchange include an end of the duplex then exchange can cause the complete release of the outgoing strand at one end and thus permit it to rotate freely unconstrained by the other strands involved in recombination. This leads to a stable situation in which the new duplex is free to rewind after recombinase disassembly, and single-stranded DNA binding proteins need only deter spontaneous branch migration.

[0417] **FIG. 15** shows the effect of crowding agents. In the presence of polyethylene glycols, gp32(N) and recA recombinase can mediate multiple invasion events on single templates without a requirement for regeneration of the template ends that would permit 5' overhangs in the targeting oligonucleotide. **FIG. 1** SA shows the results of an experiment in which either the Tester1bio, or Tester3bio oligonucleotides (which differ in the length of 5' overhang relative to the template) were incubated with the EcoRV fragment released from a plasmid carrying the *E. coli* ruvB gene, or the ClaI digest of the plasmid, in the presence or absence of 10% PEG 8000. The reaction solution included 10 mM Mg-acetate, 10.6 μg recA, 8.8 μg gp32, 27 mM phosphocreatine, 1 U creatine kinase, 0.3 mM Tester3bio or Tester1bio, 3 mM ATP, 200 μM DNTP mixture, 50 U Klenow, and 0.5 pmoles template (species indicated in figure). Optionally, we included 120 ng recO and 120 ng recR. PEG8000 was included as shown. The final volume was 30 μl. Incubation was carried out for 1 hour at 37° C.

[0418] The diagram shown represents the relationship of the oligonucleotides to the two possible templates. In particular, both oligonucleotides recognised an embedded sequence within the ClaI fragment. In each case, 0.5 pmoles of template was used, other conditions were carried out as indicated. Both primers stimulated invasion/elongation on the EcoRV template. Based on signal intensity, approximately one elongation occurred per target template. However, in the presence of 10% PEG 8000, the intensity of the fully elongated fragment was significantly greater than in its absence and stronger than the 0.5 pmoles of control biotinylated PCR product. The strongest signal was seen with the Tester3bio oligonucleotide. In that case, we estimated at least 10 invasion/run-ons occurred per template.

[0419] In **FIG. 15B**, we compared the stimulation of invasion/elongation in 10% w/v of various commercially available polyethylene glycols. The reaction solution included 10 mM Mg-acetate, 10.6 μg recA, 8.8 μg gp32, 27 mM phosphocreatine, 1 U creatine kinase, 0.3 mM Tester3bio or Tester1bio, 3 mM ATP, 200 μM dNTPs, 50 U

Klenow, and 0.5 pmoles RV template. PEG species were included as shown. There was significant variation observed in the degree of stimulation. PEG compound (MW=15,000 to 20,000) appeared to be the most effective, followed by PEG1450.

[0420] FIG. 16 shows the effect of end targeted amplification using leading strand RPA. Amplification comprising several rounds of invasion and extension was demonstrated, achieving at least a 10 fold amplification from 0.05 pmoles of template. In this experiment, we have employed pairs of oligonucleotide primers to establish an amplification reaction. Shown schematically is the relationship of the oligonucleotides used to the EcoRV fragment from a plasmid carrying the E. coli ruvB gene, which was used as template. The reaction solution included 10 mM Mg-acetate, 6 μg recA, 8.8 μg gp32(N), 27 mM phosphocreatine, 1 U creatine kinase, 0.3 mM Tester3bio, 0.3 mM variable oligonucleotide, 3 mM ATP, 200 μM dNTPs, 10% PEG compound, 50 U Klenow, and 0.5 pmoles template. Additional proteins were used as indicated.

[0421] Tester3bio included a 16-nucleotide overhang relative to the starting template, while Tester2 included a 21-nucleotide overhang and was targeted to the other end of the template. Phospho1 was used as an oligonucleotide with a phosphorothiorate backbone. This oligonucleotide was 15 residues long, and was flush to the target end. Phospho-1 was predicted not to interact with recombinase or single-stranded DNA binding protein as it lacked a phosphate backbone. However, it was predicted to function in straightforward solution hybridisation. A control fragment of biotinylated PCR product was employed to demonstrate the signal intensity of 0.5 pmoles of DNA, and was also the precise size of the starting template. The reaction products were run on a 6% denaturing gel, transferred to nylon, and bound with streptavidin-HRP prior to performing enhanced chemiluminescence to reveal the biotinylated products of the reactions.

[0422] In all cases, successful invasion and elongation with the biotinylated Tester3bio has occurred as seen by presence of fully elongated products. The products were slightly slower mobility that the control, due to the presence of overhangs on the oligonucleotides. Furthermore, there was evidence for several rounds of invasion/run-ons as the signal intensity was at least as great as the 0.5 pmoles control (we initiated the reaction with only 0.05 pmoles). There was a significant accumulation of a product roughly 37 nucleotides larger than the control. This was predicted to arise from Tester3bio elongating on a strand previously copied from, and including the overhang from, the opposing primer. Two exposures of the same gel are shown.

[0423] The inclusion of various different proteins, which are normally involved in DNA metabolism, had varying effects. DNA gyrase, and toposiomerase I (human) decreased the yield of amplification product, and the topoisomerase profoundly reduced the generation of shorter elongation products. Inclusion of E. coli ruvA and ruvB also lead to a general reduction in product formation. E. coli priA increased the amount of product formed, and significantly increased the number of shorter products formed. Inclusion of E. coli dnaB and dnaC810 protein slightly increased the amount of product formed. Note that a significantly stronger signal detected in reactions containing Phospho-1 oligo-

nucleotide in comparison to Tester3bio alone. This suggested that Phospho-1 was able to hybridise with displaced strands and lead to formation of duplex DNA.

[0424] FIG. 17 shows leading strand RPA and Klenow processivity. In this experiment, we have employed pairs of oligonucleotide primers in an effort to establish an amplification reaction in a manner similar to that shown in FIG. 16, except using a further 100-fold dilution of the start template. The reaction solution included 10 mM Mg-acetate, 6 or 12 μg recA, 8.8 or 14.3 μg gp32, 27 mM phosphocreatine, 1 U creatine kinase, 0.3 or 0.9 μM Tester3bio, 0.3 or 0.9 μM Tester2, 3 mM ATP, 200 μM dNTPs, 10% PEG compound, 50 U Klenow, and 0.5 pmoles template. The final volume was 30 μl. Incubation was carried out for 2 hours at 37° C. Shown schematically is the relationship of the oligonucleotides used to an EcoRV fragment from a plasmid carrying the E. coli ruvB gene, which is used as target template. Tester3bio included a 16-nucleotide overhang relative to the starting template, while Tester2 included a 21-nucleotide overhang, is targeted to the other end of the template, and encodes an EcoRI site within the overhang. A control fragment of biotinylated PCR product was employed to demonstrate the signal intensity of 0.5 pmoles of DNA, and was also the precise size of the starting template. The reaction products were run on a 6% denaturing gel, transferred to nylon, and bound with streptavidin-HRP prior to performing enhanced chemiluminescence to reveal the biotinylated products of the reactions.

[0425] The concentration of the oligonucleotides, gp32(N) and the recA(C) were varied, and we have also investigated whether the inclusion of EcoRI restriction enzyme, which can cleave part of the additional sequence incorporated by the Tester2 overhang, has any effect on the reaction. In most cases, there was evidence of some limited degree of amplification of the expected size of fragment, but there was principally generation of shorter DNA fragments. We deduced that the relatively poor accumulation of bona fide full length product may occur at these more dilute template concentrations because the poor processivity of the E. coli DNA polymerase I Klenow fragment (10-50 nucleotides) results in most interactions generating short fragments, which is more significant at low target template concentrations.

[0426] FIG. 18 shows spacing dependence of RPA primers. As a consequence of earlier results, we attempted to establish whether decreasing the distance between primer pairs would result in an increase in amplification efficiency. To test this, we employed a series of oligonucleotides, Sizer1, 2, 3, and 4, which were positioned at increasing distances away from the 3' end of the Tester3bio oligonucleotide. All Sizer oligonucleotides included the EcoRI overhang indicated in the bottom right side of the figure. The sequence of the target DNA, an EcoRV fragment from a plasmid carrying the E. coli ruvB gene, and the position of the oligonucleotides used are shown. The reaction solution included 10 mM Mg-acetate, 6 μg recA, 8.8 μg gp32, 27 mM phosphocreatine, 1 U creatine kinase, 0.3 μM Tester3bio, 0.3 μM variable oligonucleotide, 3 mM ATP, 200 μM dNTPs, 10% PEG compound, 50 U Klenow, 5 U EcoRI, and 0.5 pmoles template. The final volume was 30 μl. The reaction solution was incubated for 2 hours at 37° C. Other reactions conditions are indicated on the figure.

[0427] A control fragment of biotinylated PCR product was employed to demonstrate the signal intensity of 0.5 pmoles of DNA, and was also the precise size of the starting template. The reaction products were run on a 6% denaturing gel, transferred to nylon, and bound with streptavidin-HRP prior to performing enhanced chemiluminescence to reveal the biotinylated products of the reactions. Specific fragments of the expected lengths were efficiently amplified from 0.5 fmoles of starting template when Sizer2, 3, and 4 were employed. The yield of product decreased somewhat however as the length of the product increased. Little or no product of the expected size is produced when Sizer1 was used. Lane 4 was estimated to contain ~4×10^4 fold amplification. This primer included the shortest inter-oligonucleotide distance of only 25 nucleotides. This suggested there is a minimal required distance to separate the ends of the oligonucleotides, although other explanations such as poor primer behaviour could also explain the result. Included in the experiment were several samples containing no template DNA. In the case of Sizer2, a faint band of approximately the expected size is observed even in the absence of exogenous DNA. Based on a variety of data we believe that this arises from contamination of our protein preparations with significant quantities of E. coli genomic DNA.

[0428] FIG. 19 shows that RPA products are largely double stranded. RPA reaction can generate double-stranded DNA products as evidenced by agarose gel electrophoresis and restriction enzyme cleavage. However, under the conditions used here, there were significant decreases in reaction efficiency if the starting template dropped significantly below 0.5 fmoles. Furthermore, signals observed in the water-only control suggested significant genomic contamination of E. coli DNA. In this experiment we have incubated 0.5 fmoles, or more dilute quantities, of the fragment detailed in FIG. 10 with Tester3bio and Sizer2 under the conditions indicated. The reaction solution included 10 mM Mg-acetate, 6 μg recA, 8.8 μg gp32, 27 mM phosphocreatine, 1 U creatine kinase, 0.3 μM Tester3bio, 0.3 μM Sizer2, 3 mM ATP, 200 μM dNTPs, 10% PEG compound, 50 U Klenow, and 0.5 pmoles template or dilution indicated in figure. The final volume was 30 μl.

[0429] We have included a no DNA control, progressive dilution of the template, and investigated initiating the reaction on embedded template (the ClaI fragment detailed on FIGS. 1 and 7), of using PEG 1450, and diluting the Klenow fragment. A fraction (¹⁄₁₀) of the reaction products were run on a 6% denaturing gel, transferred to nylon, and bound with streptavidin-HRP prior to performing enhanced chemiluminescence to reveal the biotinylated products of the reactions (FIG. 19A). A further fraction (³⁄₁₀) was phenol extracted, precipitated, and run on a 2% agarose gel and stained with ethidium bromide (FIG. 19B). A final fraction (³⁄₁₀) was cut with BbvC1 and electrophoresed on a 2% agarose gel alongside equivalent uncut DNA (FIG. 19C).

[0430] Lane 2 (FIG. 19A) was estimated to contain 5×10^4 fold amplification, which corresponded to 10^{13} molecules of final product. In the presence of 0.5 fmoles of starting template, an extremely robust amplification of the expected size fragment was seen as evidenced by denaturing gel electrophoresis. Furthermore, when part of the sample was electrophoresed on agarose a strong clean band of precisely the correct size for a double-stranded DNA product was observed. This product could be cut by BbvC1 to yield a

slightly smaller fragment of the expected size. Dilution of the template by 100-fold or more resulted in a significantly less intense band, and a much larger quantity of fragments shorter than the expected length. This was determined by denaturing gel electrophoresis and by agarose gel electrophoresis. A similar pattern was observed when ClaI cut DNA was used, or if no DNA was used. We believe that when less than a threshold quantity of DNA is used under these conditions, there is a suboptimal amplification reaction, which leads to heterogeneous products, and furthermore that our samples are highly contaminated with E. coli genomic DNA from the single-step purification procedures used in generating our recombinant proteins used here.

[0431] FIG. 20 shows activity of a recA C-terminal truncation mutant. RecA proteins with a deletion of the C-terminal acidic peptide (recA(C)A) are active in promoting strand exchange and extension in a linear template run-on assay. However, there was some suggestion that the optimal protein concentration was lower than that with the recA(C) protein. This experiment addresses whether a C terminal truncated form of the E. coli recA protein, described elsewhere, could be used successfully in invasion/extension reactions. Shown is the result of a single-sided run-on assay using either the E. coli recA(C) protein or the E. coli recAA 17(C) protein, which lacks the 17 C-terminal acidic residues. The relationship between primers and template and other experimental conditions are indicated. The reaction solution included 10 mM Mg-acetate, 27 mM phosphocreatine, 1 U creatine kinase, 0.3 μM Tester3bio, 3 mM ATP, 200 μM dNTPs, 50 U Klenow, 0.5 pmoles RV template, and recA species and amount as indicated in figure. PEG was included as shown. The final volume was 30 μl. The solution was incubated for 2 hours at 37° C. Reactions were performed with or without 10% PEG1450, and with the indicated quantities of the respective recombinase. Both recombinases successfully supported invasion/extension, although under the conditions used here there appears to be a different optimum for the amount of protein required.

[0432] FIG. 21 shows Modified gp32 proteins. Shown is a schematic representation of T4 gp32 proteins used in this study and the position of various modification and mutations.

[0433] FIG. 22 shows activity of gp32 proteins. Significant variations confirm that gp32 cooperativity has a substantial effect on the rate of invasion/extension reactions, and further confirms that gp32(N) displays a significant decrease in cooperativity consistent with interference with the function of the N-terminal B domain. Shown are the results of an experiment in which linear run-ons were generated using as template the EcoRV fragment of a plasmid carrying the E. coli RuvB gene and the Tester3bio oligonucleotide. The reaction solution included 10 mM Mg-acetate, 27 mM phosphocreatine, 100 ng/μl creatine kinase, 400 nM Tester3bio, 3 mM ATP, 200 μM dNTPs, 10 U chicken myokinase, 8 μg C-tag uvsX, 7.5 or 15 μg gp32 (each species), 50 U Klenow, and 0.5 pmoles template; no PEG was included. The final volume was 30 μl. The solution was incubated for 2 hours at 37° C. Reactions contained uvsX(C) and various gp32 forms as indicated. Two concentrations of each gp32 form were used in this experiment. To analyze the reaction products, 2 μl of the total volume (30 μl) was loaded onto the gel.

[0434] In all cases, elongated products of the oligonucleotide were generated that extend up to a length consistent with full length run-ons. We note that in this experiment there was a gel artifact which we occasionally observed. We observed a slower mobility shadow of the bands. This was seen in the PCR control fragment lane, also. The smallest quantity of product was formed when using the gp32(C) protein, which was consistent with it permitting only a low level of recombinase-loaded filaments to be present in the reaction. A smaller quantity of product was formed with 15 μg compared with 7.5 μg, consistent with the notion that higher concentrations decreased the efficiency of recombinase loading.

[0435] The gp32(C)K3A protein was predicted to be the next most cooperative form. Consistent with this, it produced a limited number of fill-length products when 15 μg of protein is used. However, the number of run-ons was greater than that observed with either quantity of gp32(C), indicating that there were more recombinase-loaded filaments in the reaction and a higher rate of invasion/elongation. When 7.5 mg of gp32(C)K3A was used, there was a dramatic change in the quantity of product formed. One explanation is that an increased rate of invasion/elongation in this reaction could lead to the out-titration of the gp32(C)K3A by single-stranded DNA run-offs. Under these conditions, most of the oligonucleotide would become coated with uvsX(C), leading to a high invasion rate and to an inability to stabilise the outgoing strand and coat it with gp32. This would result in shorter truncated products, some of which would be folded back on themselves, self-primed, and formed into a variety of other such products. This suggested that the rate of invasion/elongation of gp32(C)K3A was notably higher for this protein than gp32(C).

[0436] By comparing the intensities of the products produced when using 15 μg of each protein, we estimated that reactions containing gp32(C)K3A have an invasion/elongation rate of approximately 10 times that of gp32(C). All of the other gp32 proteins tested in this experiment produced a pattern similar to that seen when gp32(C)K3A was employed and large amounts of product. This was the case even when 15 μg of the relevant protein was employed, suggesting that they all exhibited lower cooperativity than either gp32(C) or gp32(C)K3A. Notably, however, both gp32(N) and gp32(C)R4T produced significantly less product when only 7.5 μg of protein was used when compared with 15 μg. This contrasted to the situation with the other proteins. On this basis, we suggest that gp32(N) and gp32(C)R4T possess a similar degree of cooperativity. An earlier study has suggested that gp32K3A and gp32R4Q are of similar cooperativity. However, our data would suggest that gp32(C)R4Q lies somewhere between gp32(C)K3A and gp32(C)R4T with regard to its behaviour in supporting invasion/synthesis.

[0437] FIG. 23 shows invasion and extension using uvsX. This experiment addresses whether a C terminal truncated form of the bacteriophage T4 uvsX protein could be used successfully in invasion/extension reactions. Shown are the results of single-sided run-on assays using either the uvsX(C) or the uvsXΔ21(C) protein which lacks the 21 C-terminal acidic residues. The reaction solution included 10 mM Mg-acetate, 27 mM phosphocreatine, 1 U creatine kinase, 0.4 μM Tester3bio, 3 mM ATP, 200 μM dNTPs, 50 U Klenow, 1 U chicken myokinase, uvsX(C) or uvsXΔ21(C), 8.8 μg gp32(N), and 0.5 pmoles RV template. The final volume was 30 μl. The solution was incubated for 2 hours at 37° C., and 2 μl or reaction mixture was loaded onto each lane of the gel. The relationship of the template and oligonucleotides and other experimental conditions are indicated in the figure. Reactions were performed with the indicated quantities of the respective recombinase.

[0438] Both recombinases successfully supported invasion/extension. However, under the conditions used here, there appears to be a different optimum for the amount of protein required. In the case of uvsX(C), the rate of invasion/extension increased progressively with the concentration of protein within the range tested. However for the uvsXΔ21(C) protein, the rate was inhibited at higher concentration and the overall level of product production was lower under these conditions. In contrast to recA-mediated invasion/extension in similar reactions, uvsX(C), appeared to stimulate multiple invasion/extension events without the need for the addition of polyethylene glycol.

[0439] FIG. 24 shows RPA using uvsX(C). In this experiment, uvsX(C) has been combined with gp32(N) in the presence of the oligonucleotides Tester3bio and Sizer2. The template DNA in this experiment was the EcoRV digested plasmid carrying the E. coli ruvB gene used in Example 1. The reaction solution included 10 mM Mg-acetate, 27 mM phosphocreatine, 100 ng/μl creatine kinase, 400 nM Tester3bio, 400 nM Sizer2, 3 mM ATP, 200 μM dNTPs, 50 U Klenow fragment, 10 U chicken myokinase, 10 μg (IX) or 20 μg (2x) C-tag uvsX, 8.8 μg gp32(N). Optionally, we included 0.2 mM ADPβS, 10 μg E. coli topoisomerase I, 10% PEG 1450, and 10 μg uvsXΔ21(C). Tester3bio recognised one end of an approximately 300 base pair fragment and included a 5' overhang relative to the end of the target. Sizer2 recognised the other strand of this template and was directed toward an embedded sequence such that its 3' end is about three and a half helical turns from the end of Tester3bio.

[0440] In the presence of PEG1450, we observed the amplification of the expected fragment within 2 hours. In the cases where amplification occurred, almost the entire of population of oligonucleotides was consumed indicating an amplification of 3-5×10^4. The reaction components are indicated. Included in some samples are additional components. We found that 200 μM ADP-β-S slightly increased the amount of product generated under these conditions. Conversely under the conditions used here, addition of E. coli toposiomerase I inhibited amplification. Under the conditions used, we detected no amplification with uvsXΔ21(C) protein. However, no PEG1450 was included in these samples, and uvsX(C) did not amplify either under these conditions without PEG1450.

[0441] FIG. 25 shows wild-type versus modified gp32. The variant uvsX(C) protein was determined to be qualitatively different to native untagged gp32. The reaction solution included 10 mM Mg-acetate, 27 mM phosphocreatine, 100 ng/μl creatine kinase, 300 nM Tester3bio, 300 nM Sizer2, 3 mM ATP, 200 μM dNTPs, 50 U Klenow fragment, 10 U chicken myokinase, 300 ng/μl uvsX(C), 300 ng/ml gp32 (untagged) or 100, 200, 300, 400, 500, or 600 ng/ml gp32(C), and PEG as indicated. The reaction was incubated for 2 hours at 37° C. A comparison is shown between

amplification reactions performed in the presence of untagged gp32 and a titration of gp32(C), either in the presence or absence of PEG1450. We observed that PEG was required in the reaction for gp32(C) to function, while this is not the case for untagged gp32. However, PEG significantly increased the quantity of product formed during the reaction period in either case. Even in the presence of PEG, untagged gp32 consistently appeared to generate slightly more product than gp32(C) at each point on the titration curve.

[0442] **FIG. 26** shows titration of gp32 and effect of uvsY. Titration of gp32 revealed a requirement for a minimal quantity of gp32, and a requirement for uvsY(N) protein when untagged gp32 was employed. In **FIG. 26A**, the results of an experiment are shown demonstrating that when untagged gp32 was used, uvsY(N) protein was required for amplification. The reaction solution included 10 mM Mg-acetate, 27 mM phosphocreatine, 100 ng/μl creatine kinase, 300 nM Tester3bio, 300 nM Sizer2, 3 mM ATP, 200 μM dNTPs, 10% PEG1450, 50 U Klenow fragment, 10 U chicken myokinase, 300 ng/μl uvsX(C), 300 ng/μl gp32 (untagged), and uvsY(N) as indicated in the figure. The solution was incubated for 2 hours at 37° C. The uvs(Y) protein operated over a range of concentrations shown here (50 to 300 ng/μl). Other experiments demonstrated that higher quantities inhibited the reaction. Thus, an optimum must be established for any given reaction (probably between 5 and 50 ng/μl).

[0443] **FIG. 26B** shows the results of titrating the untagged gp32 protein in the presence or absence of uvsY(N). The reaction solution included 10 mM Mg-acetate, 27 mM phosphocreatine, 100 ng/μl creatine kinase, 300 nM Tester3bio, 300 nM Sizer2, 3 mM ATP, 200 μM dNTPs, 10% PEG1450, 10 U chicken myokinase, 750 ng/μl uvsX(C), 300 ng/μl gp32 (untagged), and 300 ng/μl uvsY(N). The solution was incubated for 2 hours at 37° C. Once again, there is a requirement for uvsY(N) to achieve amplification. Additionally, this experiment demonstrates a need for a minimal amount of gp32. In this experiment, varying the gp32 concentrations between 80 and 160 ng/μl gp32 resulted in a sharp transition from no amplification to efficient amplification. A simple analysis of known binding site size for gp32, the length of the oligonucleotides, and their concentration, suggested that this rise in concentration would represent a transition from substoichiometric levels of gp32 for the primer, to saturating levels. Consequently, the simplest interpretation is that gp32 saturates the oligonucleotides in order to have excess gp32 in the reaction to collect and stabilise the outgoing strand.

[0444] **FIG. 27** shows factors affecting reaction rate and noise. High gp32 cooperativity inhibits recombinase filament formation **(FIG. 27A)**. In addition, uvsY acts to increase recombinase loading in an unfavorable gp32 environment **(FIG. 27B)**. PEG aids the function of cooperatively-disabled gp32 **(FIG. 27C)**. The reaction rate is influenced by both recombinase activity and effectiveness of gp32 **(FIG. 27D)**. Post-invasion phases are enhanced in the presence of cooperative gp32 **(FIG. 27E)**. Cooperative gp32 is more effective at silencing reaction noise **(FIG. 27F)**.

[0445] The predicted effects and interactions of gp32, uvsX, uvsY, and PEG were suggested, with the conclusion that an optimal balance between reaction rate and noise must

be reached. The degree of cooperativity of gp32 is indicated across the top of the figure. High cooperativity favoured efficient binding to single-stranded DNA, which prevented significant reaction noise by inhibiting undesirable priming behaviour. High cooperativity also favoured stabilisation of the outgoing strand during recombination and DNA synthesis. Conversely highly cooperative gp32 reduced the abundance of recombinase-loaded searching filaments, and can affect reaction rate considerably.

[0446] This behaviour could be partly overcome by including uvsY in the reaction. However, whether this could achieve as high a loading rate as desired is yet to be determined. One could employ modified gp32 proteins that are less cooperative. Also, the cooperativity of gp32 proteins and uvsX proteins can be affected by the inclusion of PEGs. PEG may also have beneficial, or sometimes detrimental, consequences on other components of the reaction such as DNA hybridisation behaviour and polymerase processivity. Optimal rate may be acquired at some position away from either extreme, as indicated, as a balance between recombinase loading and gp32 function may need to be reached.

[0447] **FIG. 28** shows the effects of PEG. PEG was able to reduce the average length of linear invasion/run-on products in uvsX-mediated linear run-on experiment in the presence of gp32(C). Shown are the results of a linear run-on experiment utilising the Tester3bio oliognucleotide targeted to the end of the approximately 300 base pair EcoRV fragment of *E. coli* RuvB. This fragment was used throughout the disclosed experiments and is depicted schematically on the right of the figure. Reaction were reconstituted with 8 mg of UvsX(C) in a final reaction volume of 30 ml, in the presence of gp32(C), and in some cases, varying amounts of UvsY(N) or UvsY(C). The reaction solution included 10 mM Mg-acetate, 27 mM phosphocreatine, 1 U creatine kinase, 0.4 μM Tester3bio, 3 mM ATP, 200 μM dNTPs, 50 U Klenow fragment, 1 U chicken myokinase, 8 μg uvsX(C), 7.5 μg gp32(C) or 8.8 μg gp32(N), and 0.5 pmoles template. The final volume was 30 μl. The solution was incubated for 2 hours at 37° C., and 2 μl of the solution was loaded in each lane.

[0448] In the absence of PEG, multiple invasion/run-on cycles appear to have occurred on each template, and there was generation of a significant amount full-length product. However, an even greater amount of slightly shorter fragment was produced, which we believe constitute slightly shorter run-ons which may have folded back on themselves and synthesised a short hairpin. We interpreted all bands not full-length as resulting from some form of bona fide invasion/extension reaction that has not achieved full extension. Both UvsY(N) and UvsY(C) stimulate the quantity of product formed to some extent. In the case of no PEG, UvsY(C) seems to be more effective than UvsY(C). This is in contrast to other geometric amplification data we generated, suggesting that only UvsY(N) supported efficient geometric amplification.

[0449] Most notably, the inclusion of PEG, in contradiction to findings with *E. coli* recA, seemed to decrease the overall amount of product on this gel. It also decreased the average length distribution of products. In order to explain this, we suggest that under these conditions the cooperativity of gp32(C), perhaps already at a maximum, cannot be increased by PEG whilst that of UvsX can be. Thus, rela-

tively hyperactive UvsX behaviour results in rapid loading onto the outgoing stand, reinvasion, and efficient 'bubble migration' which chases the newly synthesised strand and displaces it more readily. Consequently, the average product length is significantly reduced.

[0450] FIG. 29 shows DNA end directed invasion. The first round of invasion/synthesis using end-targeting and oligonucleotide overhang is illustrated (FIG. 29A). Additional ~10 to 15 residues (5' end) and ~30 residues (3' end) approximate the minimal requirement for strand exchange (FIG. 29B). Invasion occurs, followed by release of the deconstrained outgoing strand, and backfire synthesis (FIG. 29C). Most nucleoprotein filaments that are coated enough to complete strand exchange will catalyze complete and deconstrained release of the outgoing strand (FIG. 29D). Subsequent rounds of invasion/synthesis occur (FIG. 29E). Few or no nucleoprotein filaments can exchange to the end of the target (FIG. 29F). One or no gp32 molecules are provided (FIG. 29G). Following this is recombinase loading (FIG. 29H) and branch migration (FIG. 29I).

[0451] This figure describes how targeting oligonucleotides initially possessing an overhang relative to a linear target template might behave during the first and then subsequent attempts to carry out strand exchange. The purpose of this model is to rationalise data suggesting that when such a situation in reconstituted experimentally, there is a significant difference between the first and subsequent invasions. The top of the figure depicts recombinase loaded oligonucleotide filaments, displaying different 5' extents of coverage. The 5 to 3' directional assembly means that most should have coating to the very 3' end. As depicted in the figure, the oligonucleotides all possess a 5' overhang relative to the initial target.

[0452] The first invasion event is likely to result in complete release of the outgoing strand as there is a significant likelihood that recombinase will coat the searching oligonucleotide to a further 5' extent than the sequence that will be involved in the strand exchange. Once the outgoing strand is freed, it is topologically unconstrained and can be easily stabilised by single-stranded DNA binding proteins, presumably even those with relatively poor cooperativity. Furthermore, stability is also generated by polymerases extending the 3' end of the duplex DNA to generate the complement to the very 5' end of the targeting oligonucleotide. We refer to this synthesis as backfire synthesis. As a consequence of backfire synthesis any subsequent invasion will be flush with the extended template.

[0453] Under these circumstances, most oligonucleotides are not completely coated with recombinase to the their very 5' ends. In some cases, there may be one or more gp32 molecules coating the 5' part of the oligonucleotide. When these oligonucleotides perform strand exchange on the now extended target, the outgoing strand is unlikely to be immediately freed. As a consequence, the event initially resembles the topologically constrained event already depicted in FIG. 6. The model suggests that only if, the cooperativity of the single-stranded DNA binding protein is sufficient will these strained unstable intermediates be able to exist for some limited period. In the bottom part of the figure, we explore what might occur to these unstable intermediates.

[0454] In scenario 1, the unexchanged 5' extent of the oligonucleotide undergoes branch migration with the equivalent duplex portion of the target. This could easily lead to complete dissociation of this part of the outgoing strand which would then rapidly rotate to release any stress and be stabilised by single-stranded DNA binding proteins as occurred in the first invasion. These now stable substrates will be ideal and relatively stable assemblies for polymerase elongation. Alternatively, in scenario 2, the single-stranded DNA binding protein disassembles from the outgoing strand and branch migration proceeds in the opposite direction to that in scenario 1, so that the invading DNA is ejected. In scenario 3, the outgoing strand becomes coated with recombinase and re-invades leading to ejection of the oligonucleotide. This process resembles a process described elsewhere as bubble migration. If recombinase loads onto the freed outgoing strand in scenario 1 then bubble migration could also occur. We have experimental data that is most easily reconciled by considering the existence of bubble migration as shown in FIG. 28.

[0455] FIG. 30 shows RPA in a complex sample. In this experiment we have examined the sensitivity of RPA, and need for a DNA-end, in an RPA reaction on a complex DNA target. A schematic representation is given of the DNA sequence, which corresponds to part of the human angiotensin converting enzyme (ACE) gene. Three different combinations of primers were used. The experiment used a mixture of uvsX(C), uvsY(N), and gp32(C)K3A. The reaction solution included 10 mM Mg-acetate, 27 mM phosphocreatine, 100 ng/μl creatine kinase; 300 nM Up3 primer; 300 nM Down1, 2, or 3 primer; 3 mM ATP, 200 μM dNTPs, 10% PEG1450, 50 U Klenow fragment, 10 U chicken myokinase, 300 ng/μl uvsX(C), 300 ng/μl gp32(C)K3A, and 50 ng/μl uvsY(N). The final volume was 30 μl. The solution was incubated for 5 hours at 37° C. Reaction products were electrophoretically separated on an acrylamide gel, transferred to a nylon membrane, and probed with a biotinylated oligonucleotide recognising a unique internal sequence.

[0456] For the RPA reaction, uncut template (genomic DNA) and cut template were compared, and primer pairs were compared. A fragment of the expected size was detected. In all cases, there was no specific product when no genomic DNA is added to the reaction, but a specific product was generated when DNA (equivalent to roughly 10,000 copies of any sequence) was added. Digestion of the DNA prior to RPA with HpaII resulted in the generation of at least one end overlapping with one of the oligonucleotides, and an increase in signal strength. However, there was not an absolute requirement for HpaII digestion for RPA to occur.

[0457] FIG. 31 shows RPA sensitivity. In this experiment, we have examined the sensitivity in an RPA reaction on a complex DNA target. A schematic representation is given of the DNA sequence, which corresponds to part of the human angiotensin converting enzyme (ACE) gene. Three different combinations of primers were used. The experiment used a mixture of uvsX(C), uvsY(N), and gp32(C)K3A. The reaction solution included 10 mM Mg-acetate, 27 mM phosphocreatine, 100 ng/μl creatine kinase; 300 nM Up3 primer; 300 nM Down1, 2, or 3 primer; 3 mM ATP, 200 μM dNTPs, 10% PEG1450, 50 U Klenow fragment, 10 U chicken myokinase, 300 ng/μl uvsX(C), 300 ng/μl gp32(C)K3A, and 50 ng/μl uvsY(N). The final volume was 30 μl. The solution was incubated for 5 hours at 37° C., and probe ACE-hyb was used. Reaction products were electrophoretically separated on an acrylamide gel, transferred to a nylon membrane, and

probed with a biotinylated oligonucleotide recognising a unique internal sequence. A fragment of the expected size was detected. In all cases, there was no specific product when no genomic DNA is added to the reaction, but a specific product was generated when sufficient DNA was added. In all cases, 1000 copies were sufficient to generate a significant signal, and in one case we could detect a very faint signal at 100 copies.

[0458] FIG. 32 shows RPA sensitivity and template independent artifacts. The results of an experiment are shown in which we have investigated the sensitivity in an RPA reaction on a complex DNA target. A schematic representation is given of the DNA sequence, which corresponds to part of the human angiotensin converting enzyme (ACE) gene. A time course of the amplification was performed taking reaction samples at 1, 2 and 3 hours. Reaction products were detected by virtue of the presence of a biotin residues attached to the 5' end of one of the oligonucleotides used in the amplification. In this way, it was possible to visualise all the reaction products involving this oligonucleotide, including any artifacts that might arise. We tested several different concentrations of the uvsY(N) protein. The reaction solution included 10 mM Mg-acetate, 27 mM phosphocreatine, 100 ng/μl creatine kinase; 300 nM Up3 primer; 300 nM Down1 primer; 3 mM ATP, 200 μM dNTPs, 10% PEG1450, 50 U Klenow fragment, 10 U chicken myokinase, 300 ng/μl uvsX(C), 300 ng/μl gp32(C), and 50 ng/μl uvsY(N). The final volume was 30 μl. The solution was incubated for 5 hours at 37° C. At an uvsY(N) concentration of 50 ng/μl, we detected the correct product directly, albeit faintly, after 3 hours of incubation. During this period there was an accumulation of strong bands of roughly twice the length of the oligonucleotide, which accumulated similarly in the template minus sample. These were most likely to be primer artifacts.

[0459] FIG. 33 shows how primer artifacts may arise. Primer artifacts likely initiate by erroneous self-priming events as depicted here. Primers may form hairpins, as occurs in FIG. 33A, or hybridise to a second primer, as occurs in FIG. 33B. If a polymerase can extend such a hairpin a significant stretch of double-stranded DNA may be formed, as seen in A* and B*. These structures might become targets for other recombinase-loaded filaments, titrate active filaments from bona fide targets, and possibly enter into geometric forms of amplification themselves.

[0460] FIG. 34 shows primer artifact suppression. Depicted schematically are several strategies to suppress primer artifact noise. In FIG. 34A, a second short, 3'-blocked oligonucleotide complementary to the 3' sequences of the targeting oligonucleotide is included in the reaction to compete for the formation of secondary structure formation that might result in erroneous priming. In FIGS. 34B and 34C, a similar short blocked oligonucleotide is employed as in (A), but in this case a covalent bridge is engineered between the 5' end of the targeting oligonucleotide and the 5', or 3', end of the competing short primer. In this way, the blocked nucleotide is tethered at its 5'end to the 5' end of the targeting oligonucleotide (FIG. 34A-B) In FIG. 34D, a short sequence complementary to the 3' region of the targeting oligonucleotide is added to the 5' region of the targeting oligonucleotide. It competes efficiently with secondary structure formation.

[0461] FIG. 35 shows use of hairpin oligonucleotides to stimulate self-priming of displaced strands. Depicted is a scheme showing how oligonucleotides whose design includes a 5' section with perfect complementarity to the 3' section might be used to stimulate amplification through self-priming. At the top of the diagram is shown a target DNA, designated A, which has distinct ends which are targets for one of the two targeting oligonucleotides shown in the upper left and right of the figure. Both of these oligonucleotides possess complementarity between their 5' and 3' regions as indicated by short arrows. The target, A, would likely have been generated by earlier invasion/elongation events involving these oligonucleotides and an initial target lacking the 5'-most regions of these oligonucleotides. On the left or right side of the figure we follow the outcome of invasion/elongation events initiated by targeting by the left or right primers respectively. The outcome is similar in both cases, albeit the final products are arranged slightly differently.

[0462] Focusing on the left side of the figure we observe that when target A is subject to invasion and elongation with the left primer the result is formation of a new duplex identical to A and a single-stranded DNA equivalent to the top strand of the initial target, designated B. Due to the presence of the complementarity between the very 3' region and adjacent sequences, B is capable of forming a hairpin which will prime DNA synthesis to generate a largely double-stranded product C. The product C can readily be targeted once again by the left oligonucleotide. However, in this case, no single-stranded displaced strand is formed. Instead, product D is formed with a length that is roughly twice that of the original target. This product is an inverted repeat and contains two sequences that are targets for the left oligonucleotide, and one for the right oligonucleotide located in the middle.

[0463] Subsequent invasion/elongation events, and the possible occurrence of hybridisation events between displaced strands, could easily lead to such 'dimeric' species becoming further enlarged, and the formation generally of more complex products. A similar course of events is shown on the right side of the figure, this time initiated by invasion/elongation by the right primer. The final dimeric product, D', is not equivalent to D as the two end sequences are targets for the right primer, and the central region is a target for the left primer. Processes similar or identical to those shown here are likely to occur with some frequency under some conditions even in the absence of the deliberate design of oligonucleotides to promote it, as there is often some limited capacity for self-priming of single-stranded DNAs.

[0464] FIG. 36 shows conditions that support the amplification of DNA with little or no primer artifacts. The results of an experiment are shown in which we have investigated the sensitivity of an RPA reaction on a complex DNA target. A schematic representation is given of the DNA sequence, which corresponds to part of the human angiotensin converting enzyme (ACE) gene. Oligonucleotides used are the biotinylated Angiolbio primer and the unbiotinylated Angio3 primer whose sequence is given in the experimental methods. These primers amplified a 132 bp double-stranded DNA fragment. Uncut human genomic DNA was titrated from 45 copies up to 2880 copies. Reaction products were detected by virtue of the presence of a biotin residue attached to the 5' end of one of the oligonucleotides used in

the amplification. In this way, it was possible to visualise all the reaction products involving this oligonucleotide, including any artifacts that might arise.

[0465] The reaction was incubated for 2 hours at 37° C. in the case of the Klenow exo–, and 2 hours at 42° C. in the case of the Bst polymerase. The reaction included the following: 50 ng/μl uvsY(N), 300 ng/μl gp32(C), 100 ng/μl uvsX(C), 20 mM phosphocreatine, 3 mM ATP, 25 milliunits/μl myokinase, 100 ng/μl creatine kinase, 200 μM dNTPs, 5% w/v PEG compound, 300 nM Angiolbio primer, 300 nM Angio3 primer, 800 ng/μl Klenow exo– or 1.2 units/μl Bst polymerase. The Klenow-mediated amplification was performed in U2 buffer comprising a final composition of 20 mM Tris acetate pH 7.9, 8 mM magnesium acetate, 120 mM potassium acetate. The Bst polymerase-mediated amplification was performed in U1 buffer comprising a final composition of 20 mM Tris acetate pH 7.5, 6 mM magnesium acetate, 100 mM potassium acetate.

Example 13

DNA Amplification for Point-of-Use Applications

[0466] Clones and proteins were produced as described in Example 11, above.

[0467] DNAs Used in RPA Reactions

[0468] We have employed several different target DNAs in this study, and a number of oligonucleotides. The sequence of the oligonucleotides is given below, and the template target in the experiment shown in **FIG. 39B**. The *E. coli* RuvB gene target was used for linear run-on assay **(FIG. 39B)**. Identical quantities of plasmid template containing this fragment were cut either with EcoRV, releasing a roughly 300 bp fragment, or with ClaI which linearises the DNA. Equal molar quantities of template were used in the run-on experiments (20 nM each template). The sequence of a KpnI/ClaI fragment of this template is given below. The EcoRV fragment is embedded within this sequence, and the sites are highlighted.

```
GGTACCACTTTGCCGGAAGATGTAGCAGATC (SEQ ID NO:18)

GCGCCATTCGCCCCAAATTACTGGAAGAGTATGTTG

GTCAGCCGAGGTTCGTTCACAGATGGAGATTTTCAT

CAAAGCAGCGAAACTGCGCGGCGATGCCCTCGATCA

TTTGTTGATTTTTGGTCCTCCGGGGTTGGGTAAAAC

TACGCTTGCCAACATTGTCGCCAATGAAATGGGCGT

TAATTTACGCACGACTTCTGGTCCGGTGCTGGAAAA

GGCGGGCGATTTGGCTGCGATGCTCACTAACCTTGA

ACCGCATGACGTGCTGTTTATTGATGAGATCCACCG

TCTATCGCCAGTTGTTGAAGAAGTGCTGTACCCGGC

AATGGAAGACTACCAACTG**GATATC**ATGATTGGTGA

AGGTCCGGCGGCACGCTCCATTAAAAATTGATTTGCC

GCCGTTTACCCTGATTGGTGCAACCACGCGCGCAGG

TTCGCTGACATCACCGTTGCGCGACCGTTTTGGTAT
```

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TGTGCAACGTCTGGAGTTTTATCAGGTGCCGGATCT

GCAATATATCGTCAGTCGCAGCGCACGCTTTATGGG

GCTTGAGATGAGTGATGACGGCGCGCTGGAAGTTGC

TCGTCGCGCTCGCGGTACGCCGCGCATTGCCAACCG

TCTGCTGCGTCGAGTGCGTGATTTCGCCGAAGTGAA

GCACGATGGCACCATCTCGGCA**GATATC**GCTGCTCA

GGCGCTGGATATGTTGAATGTCGATGCTGAAGGTTT

CGATTATATGGACCGCAAATTGTTGCTGGCGGTA**AT**

**CGAT**
```

[0469] Oligonucleotide Tester3bio sequence. Bases homologous to the target are in bold.

```
5' biotin-CTCACTATACCTCAGCATC**ATGATTG** (SEQ ID NO:19)

**GTGAAGGTCCGGCGGCAC.**
```

[0470] Human DNAs

[0471] We have used human genomic DNAs from several sources. A mixed population male genomic DNA from Promega was utilised. Also, DNA from individual male samples was studied in **FIG. 38A**. Individual 1 and 2 were father and son. Individual 2 DNA was used in the experiment in **FIG. 39E**, while DNA for the experiment in **FIG. 39F** was another male individual. Sequences of oligonucleotides used for amplifying human and *B. subtilis* sequences are as follows:

[0472] Corresponding to the Human ApolipoproteinB Locus:

```
ApoB4
5' CAGTGTATCTGGAAAGCCTACAGGACACCAA    (SEQ ID NO:20)

AA 3'

Apo300
5' TGCTTTCATACGTTTAGCCCAATCTTGGATA    (SEQ ID NO:21)

G 3'

Apo700
5' TGGTAAACGGAAGTCTGGCAGGGTGATTCTC    (SEQ ID NO:22)

G 3'

Apo800
5' CAATTGTGTGTGAGATGTGGGGAAGCTGGAA    (SEQ ID NO:23)

T 3'

Apo900
5' GAGGTGGTTCCATTCCCTATGTCAGCATTTG    (SEQ ID NO:24)

C 3'

Apo1000
5' GGGTTTGAGAGTTGTGCATTTGCTTGAAAAT    (SEQ ID NO:25)

C 3'
```

-continued

Apo1500
5' TTGAATTTCAAGTTTAGAAAAGTTGAGGGAG   (SEQ ID NO:26)

CCAG 3'

[0473]   Corresponding to the Human SRY Locus:

SRY3
5' AAAGCTGTAACTCTAAGTATCAGTGTGAAA   (SEQ ID NO:27)

C 3'

SRY4
5' GTTGTCCAGTTGCACTTCGCTGCAGAGTAC   (SEQ ID NO:28)

C 3'

[0474]   Corresponding to *B. subtilis* Genomic DNA:

BSA1
5' TTGGGCACTTGGATATGATGGAACTGGCA   (SEQ ID NO:29)

C 3'

BSA3
5' ACAGAAAGCTATTAAAGCAACTGACGGTG   (SEQ ID NO:30)

TGG 3'

BSB3
5' CCATCTTCAGAGAACGCTTTAACAGCAAT   (SEQ ID NO:31)

CC 3'

[0475]   Human STR Marker Primers:

CSF1PO
5' GTTGCTAACCACCCTGTGTCTCAGTTTTCCTAC (SEQ ID NO:32)

CSF1PO
3' AGACTCTTCCACACACCACTGGCCATCTTCAGC (SEQ ID NO:33)

D7S820
5' GAACACTTGTCATAGTTTAGAACGAACTAACG (SEQ ID NO:34)

D7S820
3' GAATTATAACGATTCCACATTTATCCTCATTGA (SEQ ID NO:35)

C

D13S317
5' TTGCTGGACATGGTATCACAGAAGTCTGGGATG (SEQ ID NO:36)

D13S317
3' CCATAGGCAGCCCAAAAAGACAGACAGAAAGA (SEQ ID NO:37)

D16S539
5' AAACAAAGGCAGATCCCAAGCTCTTCCTCTTCC (SEQ ID NO:38)

D16S539
5' ATACCATTTACGTTTGTGTGTGCATCTGTAAGC (SEQ ID NO:39)

D18S51
5' GGTGGACATGTTGGCTTCTCTCTGTTCTTAAC (SEQ ID NO:40)

D18S51
3' GGTGGCACGTGCCTGTAGTCTCAGCTACTTGC (SEQ ID NO:41)

-continued

THO1
5' TACACAGGGCTTCCGGTGCAGGTCACAGGGA   (SEQ ID NO:42)

THO1
3' CCTTCCCAGGCTCTAGCAGCAGCTCATGGTGG   (SEQ ID NO:43)

TPOX
5' ACTGGCACAGAACAGGCACTTAGGGAACCC   (SEQ ID NO:44)

TPOX
3' GGAGGAACTGGGAACCACACAGGTTAATTA   (SEQ ID NO:45)

[0476]   Timecourse Experiment:

APOB600
GCTCACTGTTCTGCATCTGGTCAATGGTTCTG   (SEQ ID NO:46)

APOB300REV
CTATCCAAGATTGGGCTAAACGTATGAAAGCA   (SEQ ID NO:47)

[0477]   Shorter Oligonucleotide Experiment:

APOB500
ATGGTAAATTCTGGTGTGGAAAACCTGGATGG   (SEQ ID NO:48)

APO500-28
TAAATTCTGGTGTGGAAAACCTGGATGG   (SEQ ID NO:49)

APO500-25
ATTCTGGTGTGGAAAACCTGGATGG   (SEQ ID NO:50)

APOB300REV
CTATCCAAGATTGGGCTAAACGTATGAAAGCA   (SEQ ID NO:51)

APOB300REV-28
CCAAGATTGGGCTAAACGTATGAAAGCA   (SEQ ID NO:52)

APOB300REV-25
AGATTGGGCTAAACGTATGAAAGCA   (SEQ ID NO:53)

D18S51
5' GGTGGACATGTTGGCTTCTCTCTGTTCTTAAC (SEQ ID NO:54)

D18S51
5'-28 GACATGTTGGCTTCTCTCTGTTCTTAAC   (SEQ ID NO:55)

D18S51
5'-25 ATGTTGGCTTCTCTCTGTTCTTAAC   (SEQ ID NO:56)

D18S51
3' GGTGGCACGTGCCTGTAGTCTCAGCTACTTGC (SEQ ID NO:57)

D18S51
3'-28 GCACGTGCCTGTAGTCTCAGCTACTTGC   (SEQ ID NO:58)

D18S51
3'-25 CGTGCCTGTAGTCTCAGCTACTTGC   (SEQ ID NO:59)

SRY3
AAAGCTGTAACTCTAAGTATCAGTGTGAAAC   (SEQ ID NO:60)

SRY3-28
GCTGTAACTCTAAGTATCAGTGTGAAAC   (SEQ ID NO:61)

SRY3-25
GTAACTCTAAGTATCAGTGTGAAAC   (SEQ ID NO:62)

SRY4
GTTGTCCAGTTGCACTTCGCTGCAGAGTACC   (SEQ ID NO:63)

SRY4-28
GTCCAGTTGCACTTCGCTGCAGAGTACC   (SEQ ID NO:64)

```
                        -continued
SRY4-25
CAGTTGCACTTCGCTGCAGAGTACC                   (SEQ ID NO:65)
```

[0478] Conditions of standard RPA reactions included: 50 mM Tris pH 8.4, 80 mM Potassium acetate, 10 mM Magnesium acetate, 1 mM DTT, 5% PEG compound (Carbowax-20 M), 3 mM ATP, 20 mM Phosphocreatine, 100 ng/μl Creatine kinase, 600 ng/μl gp32; 109 ng/μl, or 125 ng/μl, or 200 ng/μl uvsX; 16 ng/μl, or 25 ng/μl, or 40 ng/μl, or 60 ng/μl uvsY; 20 ng/μl Bsu polymerase, 200 μM dNTPs, and 300 nM each oligonucleotide. Reaction conditions C_1-C_4 are as above with: C1=109 ng/μl uvsX, 16 ng/μl uvsY; C2=125 ng/μl uvsX, 25 ng/μl uvsY; C3=200 ng/μl uvsX, 40 ng/μl uvsY; C4=200 ng/ml uvsX, 60 ng/μl uvsY.

[0479] Experimental Results

[0480] **FIG. 37** shows a schematic representation of RPA method. In **FIG. 37A**(i), Recombinase protein uvsX binds cooperatively to single-stranded oligonucleotides in the presence of ATP. Nucleoprotein filaments actively hydrolyse ATP to ADP. Spontaneous disassembly can lead to competitive binding of single stranded binding protein gp32, this being deterred and reloading aided by uvsY protein and polyethylene glycol. In **FIG. 37A**(ii), recombinase filaments catalyse strand exchange if homologous DNA is detected. In **FIG. 37A**(iii), strand exchange matches the searching strand with its complement and displaces a strand then bound by gp32. Recombinase disassembles. In **FIG. 37A**(iv), polymerases access the structure and extend the oligonucleotide, displacing more of the original strand. In **FIG. 37B**(i), two opposing targeting nucleoprotein complexes recombine with their respective targets and DNA synthesis is initiated. In **FIG. 37B**(ii), polymerase complexes encounter one another and one of the polymerases dissociates. In **FIG. 37B**(iii), the remaining polymerase continues synthesis freeing the two parent strands, a polymerase re-binds to the free 3'-end thus replication of both strands occurs. In **FIG. 37B**(iv), new targeting events occur. In the second round one targeting primer will displace a free end. In **FIG. 37C**, comparison of the products of strand exchange at a DNA end or at an embedded DNA sequence.

[0481] **FIG. 38A** shows the results of amplification of STR markers from two individuals (1 and 2, father and son) using primer pairs for seven independent markers. RPA conditions C4 were employed (see above). **FIG. 38B** shows titration of reaction components to determine concentrations that support in vitro amplification. Reactions included the primers SRY3 and SRY4 at 0.3 μM (targeting the SRY gene), 80 mM potassium acetate, 50 mM TrisCl pH 8.4, 2 mM DTT, 5% Carbowax-20M, 200 ng/μl uvsX, 60 ng/μl uvsY, 600 ng/μl gp32, 20 ng/μl Bsu polymerase, and 50 copies/μl Y chromosomal DNA, except when a given component was that under investigation. Optimal quantities of gp32, ATP, uvsX, uvsY, PEG, and Bsu polymerase for effective amplification of this particular product were determined. ATP-γ-S and ADP-O—S inhibited the reactions.

[0482] **FIG. 39A** shows the size limits of RPA reactions. Primer ApoB4 was combined with opposing primers capable of generating amplified products of the indicated sizes. Conditions of 125 ng/μl uvsX and 25 ng/μl uvsY (C2) were employed except C1 where 109 ng/μl uvsX and 16 ng/μl

uvsY were used; 15 copies/μl human DNA were used (30 μl reactions). Under conditions C2, some hairpin-mediated product duplication occurred converting some of the 300 bp amplicon to 2× and 3× unit length (*) (L. D. Harris, J. D. Griffith, J Mol Biol 206, 19-27 (Mar. 5, 1989)).

[0483] **FIG. 39B** shows elongation efficiencies from embedded or end sequences. A biotinylated primer was incubated with linearized plasmid DNA. Equal quantities (20 mM final) of templates linearized with either ClaI (lane 3) or EcoRV (lanes 1 and 2) were used, the primer either overlapping the cut end, or the target site being embedded (lane 3). Incubation with recombinase targeting components with (lanes 2 and 3) or without (lane 1) Klenow reveals limited elongation from the embedded site (product 1*), and abundant elongation from the end site (product 2*). Electrophoresed products were transferred to nylon and biotin detected by chemiluminescence. The weak common band (~300 bp, lanes 1 and 3) was an artifact arising from this particular protocol.

[0484] **FIG. 39C** shows the sensitivity of RPA reactions. The indicated copy number of *B. subtilis* genomes was amplified with oligonucleotides BsA1 and BsB3, which amplify a 200 bp fragment. Conditions C1 were employed. **FIG. 39D** shows human DNA of the indicated copy number amplified with primers ApoB4 and Apo300 to generate a 300 bp fragment. Conditions C2 were employed. **FIG. 39E**, F show results from human DNA from single individuals. The DNA was diluted and samples theoretically containing the indicated copy number were amplified with primers D18S51 5' and 3' which amplify an STR of size 300-360 bp. At predicted copy numbers of 2 or 3, a number of samples amplified single alleles (*). Conditions employed were C2 in (E), and C4 in (F).

[0485] **FIG. 40** shows specificity of RPA reactions. Primers BsA3 and BsB3, which amplify a 380 bp fragment from *B. subtilis* genomic DNA were incubated with 1 μg of human DNA, with (+) or without (−) addition of 100 copies of *B. subtilis* DNA (**FIG. 40A**). An asterisk indicates the position of the expected reaction product, and an arrow indicated the position of the genomic DNA. Conditions C3 were employed. To investigate how long it takes RPA to generate detectable reaction products a series of amplification reactions were established with oligonucleotides Apo600bio and Apo300rev generating a 345 bp fragment. A copy number of 60 copies/μl (**FIG. 40B**) or 6 copies/μl (**FIG. 40C**) were used. Individual reactions were stopped at the indicated number of minutes and analysed on a gel. Conditions C4 were employed (**FIG. 40D**).

[0486] For long-term storage of reaction components we lyophilised RPA reactions. Mixtures of reaction components were assembled in the absence of the indicated components, PEG and buffer. The material was freeze-dried, and then reconstituted with PEG and buffer plus the additional omitted components. Primers used are indicated. All components apart from PEG and buffer could be lyophilised and successfully reconstituted in a functional reaction. Target DNA was human male genomic DNA at 150 copies/μl (**FIG. 40E**). Oligonucleotides targeting three independent loci in human genomic DNA were incubated with overlapping primer pairs of 25, 28, or 32 bases as indicated. Only the primers of 32 bases in length successfully amplified targets. Other experiments show that primers of 30 residues are also effective at amplifying target DNAs.

[0487] Discussion

[0488] There is a long-standing need for in vitro methods to amplify specific DNA sequences. Since the late 1980's the polymerase chain reaction (PCR) method has principally met this need (R. K. Saiki et al., Science 239, 487-91 (Jan. 29, 1988)). The requirement for thermal cycling equipment, however, poses a significant barrier to the use of PCR outside of a laboratory setting. As described herein, we have developed a method called RPA, which obviates the need for thermal melting of template DNAs. RPA combines components of the bacteriophage T4 recombination/replication system in vitro to mediate the hybridisation of oligonucleotide primers to template DNAs. Specifically, the bacteriophage T4 recombinase uvsX, single-stranded DNA binding (SSB) protein gp32, and recombinase loading factor uvsY together with molecular crowding agents, allow high fidelity in vitro recombinase-mediated DNA targeting. When this targeting system is combined with strand displacement DNA synthesis, efficient exponential DNA amplification is achieved.

[0489] Any oligonucleotide sequence may be coated by recombinase to form homology searching filaments (FIG. 37) giving RPA a broad utility allowing amplification of virtually any DNA sequence. This feature has been one of the major advantages of PCR over other in vitro DNA amplification methods (G. T. Walker, M. C. Little, J. G. Nadeau, D. D. Shank, Proc Natl Acad Sci U SA 89, 392-6 (Jan. 1, 1992); D. Y. Zhang, M. Brandwein, T. Hsuih, H. B. Li, Mol Diagn 6, 141-50 (June, 2001); M. Vincent, Y. Xu, H. Kong, EMBO Rep 5, 795-800 (August, 2004); J. Compton, Nature 350, 91-2 (Mar. 7, 1991)). Resembling their in vivo role, homology-searching filaments scan duplex DNA for sequences complementary to that of the oligonucleotide (T. Yonesaki, Y. Ryo, T. Minagawa, H. Takahashi, Eur J Biochem 148, 127-34 (Apr. 1, 1985); T. Shibata, C. DasGupta, R. P. Cunningham, C. M. Radding, Proc Natl Acad Sci USA 76, 1638-42 (April, 1979)). On finding a match, the recombinase catalyses several reactions: the primer is paired with its complement, the similar 'outgoing' strand is displaced, and the recombinase dissociates. This establishes a 'D-loop' structure accessible to other reaction components. Exchange events occurring away from a free DNA end generate topologically strained joints, as the outgoing strand is attached on both sides of the exchanged region (FIG. 37C).

[0490] Embedded sequences generate topologically constrained intermediates that are unstable. Joints formed at DNA ends permit free rotation of the displaced strand (P. W. Riddles, I. R. Lehman, J Biol Chem 260, 165-9 (Jan. 10, 1985)). Because these two structures have different stabilities elongation of initial strand invasion events are less efficient than subsequent ones (FIG. 39B). The free 3' end of the oligonucleotide primes synthesis by a strand-displacing DNA polymerase such as the Klenow fragment of E. coli, or the Bacillus subtilis DNA polymerase I (Bsu). The synthetic and strand-displacing activities of the polymerase result in the production of a double-stranded DNA and a displaced single strand. This displaced strand is replicated either by direct hybridisation and elongation of the second oligonucleotide, or by strand displacement synthesis if an invasion event had already occurred from the opposite end. The generation of two complete daughter duplexes completes one round of RPA. Invasions continue to act on

products of previous synthesis reactions with the endtargeted products eventually dominating the reaction.

[0491] In developing the method of the invention, we have found that several important conditions are important for optimal RPA to occur. First, there needs to be saturating quantities of nucleic acid melting proteins present in the reaction especially a SSB such as gp32. Second, there needs to be a sufficient quantity of recombinase-loaded primer to achieve an acceptable invasion/strand-exchange rate. Finally, the recombinase/single-stranded DNA primer filaments need to be dynamic and capable of disassembly. There are competing biochemical activities of the reaction components. For example, in a typical in vitro situation recombinases are usually out-competed by saturating amounts of SSBs such as gp32.

[0492] To overcome this problem, others have used non-hydrolysable ATP analogues such as ATP-γ-S, which stabilises the recombinase/single-stranded primer DNA interaction (S. C. Kowalczykowski, J. Clow, R. Somani, A. Varghese, J Mol Biol 193, 81-95 (Jan. 5, 1987); A. L. Eggler, S. L. Lusetti, M. M. Cox, J Biol Chem 278, 16389-96 (May 2, 2003); T. Shibata, C. DasGupta, R. P. Cunningham, C. M. Radding, Proc Natl Acad Sci USA 77, 2606-10 (May, 1980)). Non-hydrolysable ATP analogues are, however, incompatible with the dynamic activity of recombinase/single-stranded DNA primer filaments needed to complete strand-exchange reactions and allow polymerases access to the D-loops (L. Xu, K. J. Marians, J Biol Chem 277, 14321-8 (Apr. 19, 2002); P. W. Riddles, I. R. Lehman, J Biol Chem 260, 170-3 (Jan. 10, 1985); N. Armes, D. Stemple, in patent application PCT WO 03/072805 (ASM Scientific, Inc., USA, 2003)) (FIG. 38).

[0493] There are other ways the recombinase/single-stranded primer DNA interaction could be stabilised. For example, another bacteriophage T4 protein called uvsY is known to aid loading of uvsX onto gp32-coated DNA (L. D. Harris, J. D. Griffith, J Mol Biol 206, 19-27 (Mar. 5, 1989)). In addition, molecular crowding agents are also known to facilitate loading and stabilisation of the E. coli recA recombinase protein (P. E. Layery, S. C. Kowalczykowski, J Biol Chem 267, 9307-14 (May 5, 1992)). We therefore tested whether uvsY and molecular crowding agents might alleviate the unfavourable competition between uvsX and gp32 in a dynamic, ATP-dependent system.

[0494] Titration of reaction components reveals that defined quantities of T4 gp32, T4 uvsX, T4 uvsY, ATP, and PEG are required for DNA amplification (FIG. 38). Indeed the T4 uvsY recombinase mediator protein and PEG-compound are required to achieve detectable amplification. At reduced concentrations of gp32, amplification efficiency is impaired generating smearing and laddering of reaction products. The recombinase uvsX protein is important for RPA and the reaction rate is accelerated at higher concentrations, although this can also increase artifacts (FIG. 39A). ATP is important for the reaction, and we have found that an ATP regeneration system is needed to reach detectable levels of product for most reactions. Conversely, ATP-γ-S is a powerful inhibitor of amplification.

[0495] To be a useful tool for routine DNA amplification applications such as diagnostic testing, RPA should be sensitive and specific, applicable to diverse sequence targets and be capable of amplifying fragments of sufficient size.

We first investigated the size of products that could be generated. Amplified products up to 1000 base pairs in size could be amplified using standard reaction conditions (**FIG. 38A**). Larger amplified products may also be generated using RPA.

[0496] We tested the sensitivity of RPA under the most stringent conditions, using a single-step RPA reaction, without nesting, and detecting products by conventional ethidium bromide staining of agarose gels. With several independent primer/target sets we routinely detected less than 10 copies of starting duplex template. We observed variability between experiments at lowest detectable copy number but all attempts were successful at detecting less than 10 copies. With proper sample handling, RPA may be used to amplify from single molecules to detectable levels in a single reaction. To explore this possibility, we amplified a polymorphic simple tandem repeat (STR) marker from human DNA diluted until only a few copies should remain. We generated a number of amplified products corresponding to both possible separate alleles present in the sample DNA (**FIG. 39E**, F). This allele separation effect suggests that RPA has single molecule sensitivity and thus surpasses the sensitivity of many other DNA amplification methods.

[0497] Analysis of the amount of amplified product shows that RPA will routinely amplify DNA samples by 1011-12-fold from small quantities of starting template. Final product levels are typically in the range of 10-250 nM, generating more than sufficient quantities of DNA for even the least sensitive detection protocols. To assess specificity of amplification reactions we have analysed many primer pairs, most directed to human DNA sequences. For every primer/template set, we have tested the predicted product sizes, restriction enzyme digestion patterns, or product DNA sequence to show that amplification is specific. We have not observed amplification of non-target sequences from sample DNA. Artifacts we have observed are product or primer related (**FIG. 39A**).

[0498] Often in diagnostic settings, specificity problems arise from the large amounts of nontarget DNA present in a sample. For example, in pathogen detection, human DNA from blood samples can interfere with detection of pathogen DNA. We therefore sought to detect trace quantities of target DNA in a large mass of unrelated DNA. We found that RPA was able to amplify a target to detectable levels from 100 copies of *Bacillus subtilis* DNA in the presence of 1 μg of human DNA (i.e., 108-fold less *B. subtilis* DNA than human DNA by mass). With such a large mass of sample DNA we found we had to increase levels of uvsX and uvsY compared to equivalent reactions without excess competitor DNA to achieve an acceptable reaction rate. This is perhaps due to out-titration of the homology-searching components.

[0499] In time-course experiments with several different primer/template sets, we found that amplification rate was partially dependent on product length. For fragments in the range 150-400 base pairs, however, fairly similar rates were observed, allowing amplification from hundreds to thousands of starting copies to gel detectable levels (~10^{12} copies) in 40 to 60 minutes (**FIG. 40B**, C). We estimate the cycle times to be about 2 minutes on average for such fragments. Using optimally short target sequences and sensitive detection method, we expect that a diagnostic amplification/detection assay could be performed within an hour. Our studies show that for a large number of arbitrary DNA targets in complex samples high-quality primer pairs can be easily designed. We have addressed minimal oligonucleotide length and found that while oligonucleotides of less than 30 nucleotides do not amplify DNA effectively, those of 30-35 nucleotides in length are excellent primers and are short enough for easy synthesis (**FIG. 40**).

[0500] As demonstrated herein, RPA is an excellent general method to amplify specific DNA sequences. Initial experiments indicate that it is easy to lyophilise the components of the RPA reaction for convenient storage and reconstitution (**FIG. 40**). This method, which operates robustly at constant low temperature, can be lyophilised for easy storage and requires no thermal cycling or melting for high sensitivity, nor other complex handling. It offers a significant breakthrough in the development of DNA diagnostic, forensic and other point-of-use applications. Once integrated with portable sample DNA extraction and product detection systems, RPA should enable easy-to-use clinical or domestic testing kits for a variety of pathogens (e.g., *Clamydia* or MRSA) as well as field kits for other applications.

[0501] The details of one or more embodiments of the invention have been set forth in the accompanying description above. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

[0502] In the specification and the appended claims, the singular forms include plural referents unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Unless expressly stated otherwise, the techniques employed or contemplated herein are standard methodologies well known to one of ordinary skill in the art. All patents and publications cited in this specification are hereby incorporated by reference herein, including the previous disclosure provided by U.S. application 60/553,999 filed Mar. 16, 2004, U.S. application Ser. No. 10/371,641 filed Feb. 21, 2003, and U.S. Application 60/358,563 filed Feb. 21, 2002.

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ttgccaacat tgtcgccaat gaaatgggcg ttaatttacg cacgacttct ggtccggtgc  240

tggaaaaggc gggcgatttg ctgcgatgc tcactaacct tgaaccgcat gacgtgctgt  300

ttattgatga gatccaccgt ctatcgccag ttgttgaaga agtgctgtac ccggcaatgg  360
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aagactacca actggatatc atgattggtg aaggtccggc ggcacgctcc attaaaattg        420

atttgccgcc gtttaccctg attggtgcaa ccacgcgcgc aggttcgctg acatcaccgt        480

tgcgcgaccg ttttggtatt gtgcaacgtc tggagtttta tcaggtgccg gatctgcaat        540

atatcgtcag tcgcagcgca cgctttatgg ggcttgagat gagtgatgac ggcgcgctgg        600

aagttgctcg tcgcgctcgc ggtacgccgc gcattgccaa ccgtctgctg cgtcgagtgc        660

gtgatttcgc cgaagtgaag cacgatggca ccatctcggc agatatcgct gctcaggcgc        720

tggatatgtt gaatgtcgat gctgaaggtt tcgattatat ggaccgcaaa ttgttgctgg        780

cggtaatcga t                                                           791
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<210> SEQ ID NO 19
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 19

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<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 20

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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 21

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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 22

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<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 23

caattgtgtg tgagatgtgg ggaagctgga at                                      32
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<210> SEQ ID NO 24
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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 24

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<210> SEQ ID NO 25
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 25

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<210> SEQ ID NO 26
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 26

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<210> SEQ ID NO 27
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 27

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<210> SEQ ID NO 28
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 28

gttgtccagt tgcacttcgc tgcagagtac c   31

<210> SEQ ID NO 29
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 29

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<210> SEQ ID NO 30
<211> LENGTH: 32

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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 30

acagaaagct attaaagcaa ctgacggtgt gg                                32


<210> SEQ ID NO 31
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 31

ccatcttcag agaacgcttt aacagcaatc c                                 31


<210> SEQ ID NO 32
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 32

gttgctaacc accctgtgtc tcagttttcc tac                               33


<210> SEQ ID NO 33
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 33

agactcttcc acacaccact ggccatcttc agc                               33


<210> SEQ ID NO 34
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 34

gaacacttgt catagtttag aacgaactaa cg                                32


<210> SEQ ID NO 35
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 35

gaattataac gattccacat ttatcctcat tgac                              34


<210> SEQ ID NO 36
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide
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<400> SEQUENCE: 36

ttgctggaca tggtatcaca gaagtctggg atg   33

<210> SEQ ID NO 37
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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 37

ccataggcag cccaaaaaga cagacagaaa ga   32

<210> SEQ ID NO 38
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 38

aaacaaaggc agatcccaag ctcttcctct tcc   33

<210> SEQ ID NO 39
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 39

ataccattta cgtttgtgtg tgcatctgta agc   33

<210> SEQ ID NO 40
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 40

ggtggacatg ttggcttctc tctgttctta ac   32

<210> SEQ ID NO 41
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 41

ggtggcacgt gcctgtagtc tcagctactt gc   32

<210> SEQ ID NO 42
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 42

tacacagggc ttccggtgca ggtcacaggg a   31

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<210> SEQ ID NO 43
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 43

ccttcccagg ctctagcagc agctcatggt gg   32

<210> SEQ ID NO 44
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 44

actggcacag aacaggcact tagggaaccc   30

<210> SEQ ID NO 45
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 45

ggaggaactg ggaaccacac aggttaatta   30

<210> SEQ ID NO 46
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 46

gctcactgtt ctgcatctgg tcaatggttc tg   32

<210> SEQ ID NO 47
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 47

ctatccaaga ttgggctaaa cgtatgaaag ca   32

<210> SEQ ID NO 48
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 48

atggtaaatt ctggtgtgga aaacctggat gg   32

<210> SEQ ID NO 49
<211> LENGTH: 28

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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 49

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<210> SEQ ID NO 50
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 50

attctggtgt ggaaaacctg atgg                                          25


<210> SEQ ID NO 51
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 51

ctatccaaga ttgggctaaa cgtatgaaag ca                                 32


<210> SEQ ID NO 52
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 52

ccaagattgg gctaaacgta tgaaagca                                      28


<210> SEQ ID NO 53
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 53

agattgggct aaacgtatga aagca                                         25


<210> SEQ ID NO 54
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 54

ggtggacatg ttggcttctc tctgttctta ac                                 32


<210> SEQ ID NO 55
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide
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<400> SEQUENCE: 55

gacatgttgg cttctctctg ttcttaac   28

<210> SEQ ID NO 56
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 56

atgttggctt ctctctgttc ttaac   25

<210> SEQ ID NO 57
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 57

ggtggcacgt gcctgtagtc tcagctactt gc   32

<210> SEQ ID NO 58
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 58

gcacgtgcct gtagtctcag ctacttgc   28

<210> SEQ ID NO 59
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 59

cgtgcctgta gtctcagcta cttgc   25

<210> SEQ ID NO 60
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 60

aaagctgtaa ctctaagtat cagtgtgaaa c   31

<210> SEQ ID NO 61
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 61

gctgtaactc taagtatcag tgtgaaac   28

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<210> SEQ ID NO 62
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 62

gtaactctaa gtatcagtgt gaaac                                         25


<210> SEQ ID NO 63
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 63

gttgtccagt tgcacttcgc tgcagagtac c                                  31


<210> SEQ ID NO 64
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 64

gtccagttgc acttcgctgc agagtacc                                      28


<210> SEQ ID NO 65
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 65

cagttgcact tcgctgcaga gtacc                                         25


<210> SEQ ID NO 66
<211> LENGTH: 49
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 66

ctcactatac ctcagcatca tgattggtga aggtccggcg gcacgctcc               49


<210> SEQ ID NO 67
<211> LENGTH: 327
<212> TYPE: DNA
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 67

atcatgattg gtgaaggtcc ggcggcacgc tccattaaaa ttgatttgcc gccgtttacc   60

ctgattggtg caaccacgcg cgcaggttcg ctgacatcac cgttgcgcga ccgttttggt  120

attgtcaac gtctggagtt ttatcaggtg ccggatctgc aatatatcgt cagtcgcagc  180

gcacgcttta tggggcttga gatgagtgat gacggcgcgc tggaagttgc tcgtcgcgct  240
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cgcggtacgc cgcgcattgc caaccgtctg ctgcgtcgag tgcgtgattt cgccgaagtg    300

aagcacgatg gcaccatctc ggcagat                                        327


<210> SEQ ID NO 68
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide overhang

<400> SEQUENCE: 68

ctatgcgaat tc                                                         12


<210> SEQ ID NO 69
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Peptide

<400> SEQUENCE: 69

His His His His His His
1               5


<210> SEQ ID NO 70
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Peptide

<400> SEQUENCE: 70

Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu
1               5                   10


<210> SEQ ID NO 71
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Peptide

<400> SEQUENCE: 71

Asp Tyr Lys Asp Asp Asp Asp Lys
1               5


<210> SEQ ID NO 72
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Peptide

<400> SEQUENCE: 72

Glu Asp Gln Val Asp Pro Arg Leu Ile Asp Gly Lys
1               5                   10


<210> SEQ ID NO 73
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Peptide
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<400> SEQUENCE: 73

Glu Glu Thr Ala Arg Phe Gln Pro Gly Tyr Arg Ser
1               5                   10


<210> SEQ ID NO 74
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Peptide

<400> SEQUENCE: 74

Gly Lys Pro Ile Pro Asn Pro Leu Leu Gly Leu Asp Ser Thr
1               5                   10


<210> SEQ ID NO 75
<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Peptide

<400> SEQUENCE: 75

Tyr Thr Asp Ile Glu Met Asn Arg Leu Gly Lys
1               5                   10


<210> SEQ ID NO 76
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Peptide

<400> SEQUENCE: 76

Asp Leu Tyr Asp Asp Asp Asp Lys
1               5


<210> SEQ ID NO 77
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Peptide

<400> SEQUENCE: 77

Tyr Pro Tyr Asp Val Pro Asp Tyr Ala
1               5
```

We claim:

1. An recombinase polymerase amplification process of DNA amplification of a double stranded target nucleic acid molecule, said target nucleic acid molecule comprising a first and second strand of DNA, comprising the steps of

(a) contacting a recombinase agent selected from the group consisting of uvsX and recA, with a first and second nucleic acid primer to form a first and second nucleoprotein primer, wherein said nucleic acid primer comprises a single stranded region at its 3' end;

(b) contacting the first and the second nucleoprotein primer to said double stranded target nucleic acid molecule thereby forming a first double-stranded struc-

ture at a first portion of said first strand and forming a second double stranded structure at a second portion of said second strand such that the 3' ends of said first nucleic acid primer and said second nucleic acid primer are oriented toward one another on the same double-stranded template nucleic acid molecule;

(c) extending the 3' end of said first and second nucleic acid primer with one or more polymerases and dNTPs to generate a first and second double-stranded nucleic acid and a first and second displaced strands of nucleic acid; and

(d) continuing the reaction through repetition of (b) and (c) until a desired degree of amplification is reached.

2. The process of claim 1, wherein in step (c), the first and second displaced strands of nucleic acid are converted to double-stranded DNA by the action of a concurrent recombinase polymerase amplification reaction established from the first or second nucleoprotein primer targeting the displaced strands.

3. The process of claim 1 wherein the first and second displaced strands of nucleic acid are converted to double-stranded DNA by: (a) contacting the first or second nucleoprotein primer to said displaced strand; and (b) extending said first or second primer with a polymerase and dNTPs.

4. The process of claim 1, wherein the first and second displaced strands of nucleic acid are converted to double-stranded DNA by: (a) contacting the first or second nucleic acid primer to said first or second displaced strand; and (b) extending said first or second nucleic acid primer with a polymerase and dNTPs.

5. The process of claim 1, wherein the first or second displaced strand of nucleic acid is converted to double-stranded DNA by: (a) contacting a first or second nucleoprotein primer to said displaced strand of nucleic acid to form a DNA duplex hybrid; and (b) extending said first or second nucleoprotein primer with a polymerase and dNTPs.

6. The process of claim 1 wherein said first and second displaced strands are at least partially complementary to each other hybridize to form a nucleic acid molecule that is double stranded or partially double stranded.

7. The process of claim 6 wherein the partially double stranded nucleic acid is converted to a fully double stranded nucleic acid by a polymerase and dNTPs.

8. The process of claim 1 wherein the first and second displaced strands of nucleic acid comprise two regions of nucleic acid at its 3' end which can form a self-priming hairpin structure.

9. The process of claim 1 wherein said nucleic acid primers are selected from the group consisting of DNA, RNA, PNA, LNA, morpholino backbone nucleic acid, phosphorothiorate backbone nucleic acid, or a combination thereof.

10. The process of claim 1 wherein the recombinase agent is a fusion protein comprising a short tag sequence at the N or C terminus.

11. The process of claim 1 wherein the recombinase agent comprises a C terminal deletion of acidic residues.

12. The process of claim 1 wherein the recombinase agent is present at a concentration of between about 0.2 μM and about 1 μM.

13. The process of claim 1 wherein the recombinase agent is present at a concentration of between about 1 μM and about 4 μM.

14. The process of claim 1 wherein the recombinase agent is present at a concentration of between about 4 μM and about 6 μM.

15. The process of claim 1 wherein the recombinase agent is present at a concentration of between about 6 μM and about 12 μM.

16. The process of claim 1 performed in the presence of one or more accessory agents.

17. The process of claim 16 wherein said accessory agent is selected from the group consisting of single-strand binding protein, helicase, topoisomerase, resolvase and a combination thereof.

18. The process of claim 16 wherein the accessory agent are selected from the group consisting of single stranded binding protein, RuvA, RuvB, RuvC, RecG, PriA, PriB, PriC, DnaT, DnaB, DnaC, DnaG, DnaX clamp loader, polymerase core complex, DNA ligase, a sliding clamp, and a combination thereof.

19. The process of claim 18 wherein said sliding clamp is the *E. coli* β-dimer sliding clamp, the eukaryotic PCNA sliding clamp, or the T4 sliding clamp (gp45).

20. The process of claim 1 wherein said process is performed in the presence of a RecA/ssDNA nucleoprotein filaments stabilizing agent.

21. The process of claim 20 wherein said stabilizing agent is selected from the group consisting of RecR, RecO, RecF and a combination thereof.

22. The process of claim 1 wherein said process is performed in the presences of a single strand stabilizing agent selected from the group consisting of *E. coli* SSB protein, T4 gp32 protein, and derivatives thereof.

23. The process of claim 22 wherein the derivatives of the gp32 protein is selected from the group consisting of gp32 with additional amino acids at the N terminus, gp32 with additional amino acids at the C terminus, gp32 with the substitution of lysine 3 with a non lysine amino acid, and gp32 with the substitution of arginine 4 with a non arginine amino acid and a combination thereof.

24. The process of claim 23 wherein the additional amino acids comprise a poly His tag.

25. The process of claim 22 wherein said gp32 derivative is selected from the group consisting of gp32(N), gp32(C), gp32(C)K3A, gp32(C)R4Q, gp32(C)R4T, gp32K3A, gp32R4Q, gp32R4T and a combination thereof.

26. The process of claim 22 wherein said single stranded DNA stabilizing agent is present at a concentration of between 1 μM and 20 μM.

27. The process of claims 22 wherein the T4 gp32 is at a concentration sufficient to saturate all of the primers in the reaction.

28. The process of claim 22 wherein said T4 gp32 is between 1 μM and 20 μM.

29. The process of claim 1 wherein said process is performed in the presence of a crowding agent.

30. The process of claim 29 wherein said crowding agent is selected from the group consisting of polyethylene glycols, polyvinyl alcohols, dextran, ficoll and a combination thereof.

31. The process of claim 30 wherein said polyethylene glycol selected from the group consisting of PEG1450, PEG3000, PEG8000, PEG10000, PEG compound (molecular weight 15,000 to 20,000 and also known as Carbowax 20M), and a combination thereof.

32. The process of claim 30 wherein said polyethylene glycol is between 1 to 12% of a weight or a volume of the reaction.

33. The process of claim 29 wherein the crowding agent has a concentration of between 1 and 12% weight/volume of the reaction.

34. The process of claim 1 wherein said process is performed in the presence of a recombinase loading protein.

35. The process of claim 34 wherein the recombinase loading protein is selected from the group consisting of T4 uvsY, *E. coli* recO, *E. coli* recR and a combination thereof.

36. The process of claim 35 wherein the T4 uvsY further comprise a poly-His tag at the N or C terminus.

37. The process of claims **34** wherein the recombinase loading agent is at a concentration of between 0.2 μM and 8 μM.

38. The process of claim 1 wherein said recombinase polymerase amplification process further comprises a cofactor for the recombinase agent selected from the group consisting of ATP, ATP analog, or a combination thereof.

39. The process of claim 38 wherein said ATP analog is ATP-γ-S or ddATP.

40. The process of claim 1, wherein said recombinase polymerase amplification process further comprises an ATP regeneration system to convert ADP to ATP.

41. The process of claim 40 wherein said ATP regeneration system comprises phosphocreatine and creatine kinase.

42. The process of claim 1 wherein said recombinase polymerase amplification process further comprises ADP-O—S as a recombinase agent modulator.

43. The process of claim 1, wherein said recombinase polymerase amplification process further comprises a system to regenerate ADP from AMP.

44. The process of claim 1, wherein said recombinase polymerase amplification process further comprises pyrophosphatase to convert pyrophosphate to phosphate.

45. The process of claim 1 wherein the one or more DNA polymerase is selected from the group consisting of prokaryotic polymerase, eukaryotic polymerase and phage-encoded polymerase.

46. The process of claim 45 wherein said eukaryotic polymerase is selected from the group consisting of pol-α, pol-β, pol-δ, pol-ε and a combination thereof.

47. The process of claim 45 wherein the procaryotic polymerase is selected from the group consisting of *E. coli* DNA polymerase I Klenow fragment, bacteriophage T4 gp43 DNA polymerase, *Bacillus stearothermophilus* polymerase I large fragment, Phi-29 DNA polymerase, T7 DNA polymerase, *Bacillus subtilis* Pol I large fragment (Bsu polymerase), *E. coli* DNA polymerase I, *E. coli* DNA polymerase II, *E. coli* DNA polymerase III, *E. coli* DNA polymerase IV, *E. coli* DNA polymerase V and a combination thereof.

48. The process of claim 45 wherein said one or more DNA polymerase is a fusion protein comprising a short tag sequence at the N or C terminus.

49. The process of claim 1 wherein the one or more DNA polymerase includes at least one DNA polymerase which lacks 3'-5' exonuclease activity.

50. The process of claim 1 wherein said one or more DNA polymerase comprises a DNA polymerase with strand displacing properties.

51. The process of claim 1 wherein at least one primer comprise a non-phosphate linkage between the two bases at its 3' end and is resistant to 3' to 5' nuclease activity.

52. The process of claim 1 wherein at least one primer comprise at least one locked nucleic acid at its 3' ultimate base or 3' penultimate base.

53. The process of claim 1 wherein target nucleic acid molecule is supercoiled.

54. The process of claim 1 wherein the target nucleic acid molecule is linear with two ends and wherein both ends are linked to a non-target nucleic acid molecule.

55. The process of claim 1 wherein the target nucleic acid molecule is linear with two ends and wherein one end is linked to a non-target nucleic acid molecule.

56. The process of claim 1 wherein the target nucleic acid molecule is a linear nucleic acid molecule.

57. The process of claim 1 wherein the target nucleic acid molecule is a single-stranded nucleic acid which is converted to a double stranded nucleic acid by a polymerase or a double stranded nucleic acid denatured by the action of heat or chemical treatment.

58. The process of claims **54, 55, 56** or **57** wherein the nucleic acid primers possess additional 5' bases that are not complementary to the target nucleic acid molecule.

59. The process of claim 1 wherein the first or second nucleic acid primers are at least 20 residues in length.

60. The process of claim 1 wherein the first or second nucleic acid primers are at least 30 residues in length.

61. The process of claim 1 wherein said first nucleic acid primer and said second nucleic acid primer are oriented with their 3' ends toward one another on the same double-stranded template nucleic acid molecule and are separated by between 0 and 10 bases after step (b).

62. The process of claim 1 wherein said first nucleic acid primer and said second nucleic acid primer are oriented with their 3' ends toward one another on the same double-stranded template nucleic acid molecule and are separated by between 10 and 30 bases after step (b).

63. The process of claim 1 wherein said first nucleic acid primer and said second nucleic acid primer are oriented with their 3' ends toward one another on the same double-stranded template nucleic acid molecule and are separated by between 30 and 100 bases after step (b).

64. The process of claim 1 wherein said first nucleic acid primer and said second nucleic acid primer are oriented with their 3' ends toward one another on the same double-stranded template nucleic acid molecule and are separated by more than 100 bases after step (b).

65. The process of claim 1 wherein said first nucleic acid primer and said second nucleic acid primer are oriented with their 3' ends toward one another on the same double-stranded template nucleic acid molecule and are separated by more than 1,000,000 bases after step (b).

66. The process of claim 1 wherein said recombinase polymerase amplification is performed in the presence of short, 3'-blocked, oligonucleotides with a sequence complementary to the 3' region of the nucleic acid primers.

67. The process of claim 66 wherein the short oligonucleotides are tethered to the nucleic acid primers through a covalent linker between the 5' extreme of the nucleic acid primer and the 3' or 5' end of the short oligonucleotide.

68. The process of claim 1 wherein at least one nucleic acid primer contains a short 5' region not complementary to the target nucleic acid molecule but complementary to a 3' region of the same primer.

69. The process of claim 1, wherein said process is performed between 20° C. and 50° C.

70. The process of claim 1, wherein said process is performed between 20° C. and 40° C.

71. The process of claim 1, wherein said process is performed between 20° C. and 30° C.

72. The process of claim 1 wherein said process is performed without temperature induced melting of the template nucleic acid.

73. The process of claim 1, wherein the two nucleoprotein primer are each present in a molar concentration that is at least 1000 folds greater than the concentration of the target nucleic acid molecule.

74. The process of claim 1 wherein at least one primer comprises one or more bases that is not complementary to the target nucleic acid molecule at its 5' end.

75. The process of claim 74 wherein said one or more nucleotides an oligonucleotide that comprises the sequence of a restriction endonuclease recognition site.

76. The process of claim 1 wherein at least one primer is partially single stranded and partially double stranded, wherein said primer is comprised of a longer invading-strand which possesses a specific 3' primer sequence and a shorter non-invading strand, which is complementary to the 5' end of the longer invading strand.

77. The process of claim 1 wherein said first nucleic acid primer comprise a first non invading strand and said second nucleic acid primer comprise a second non-invading strand and said first and second non-invading strand are complementary to each other.

78. The process of claim 76 wherein said invading strand, said non-invading strand, or both are labeled with a detectable moiety.

79. The process of claim 78 wherein both the invading strand and the non-invading strand are labeled and the separation of the invading strand and non-invading strand is detected.

80. The process of claim 78 wherein said detectable moiety is selected from the group consisting of a fluoro-chrome, an enzyme, a fluorescence quencher, an enzyme inhibitor, a radioactive label, and a combination thereof.

81. The process of claim 76 wherein both said invading strand and said non-invading strand are labeled and wherein the invading strand is labeled with a first fluorochrome or a first enzyme and wherein the non-invading strand is labeled with a second fluorochrome, a second enzyme, a fluorescence quencher or an enzyme inhibitor.

82. The process of claim 76 wherein both said invading strand and said non-invading strand are labeled and wherein the non-invading strand is labeled with a first fluorochrome or a first enzyme and wherein the invading strand is labeled with a second fluorochrome, a second enzyme, a fluorescence quencher or an enzyme inhibitor.

83. The process of claim 1 wherein one or both nucleic acid primers is a single stranded primer.

84. The process of claim 83, wherein said single stranded primer is labeled with a detectable moiety selected from the group consisting of a fluorochrome, an enzyme, a fluorescence quencher, an enzyme inhibitor, a radioactive label and a combination thereof.

85. The process of claim 1 wherein the recombinase agent is temperature-sensitive, wherein said temperature sensitive recombinase agent possessing strand-invasion activity at a permissive temperature and no strand-invasion activity at a non-permissive temperature.

86. The process of claim 1 wherein said process amplifies said target nucleic acid molecule is amplified in an amount selected from the group consisting of 10 fold, 100 fold, 1000 fold, or at least 1,000,000 fold.

87. The process of claim 1 wherein said steps (b) and (c) is repeated at least once.

88. The process of claim 1 wherein said steps (b) and (c) is repeated at least five times.

89. The process of claim 1 wherein the target nucleic acid molecule contains at least one specific mutation that is associated with a genetic disease.

90. The process of claim 1 wherein the target nucleic acid molecule is contained in a pathogenic organism, an onco-gene, an activated oncogene, or a repressed oncogene.

91. The process of claim 1 wherein at least one primer is complementary to a sequence associated with a genetic disease.

92. The process of claim 1 wherein at least one primer is complementary to a nucleic acid sequence in a pathogenic organism, an oncogene, an activated oncogene, or a repressed oncogene.

93. A process of nested recombinase polymerase ampli-fication comprising the steps of:

(a) amplifying a region of DNA using the recombinase polymerase amplification process of claim 1 using a first and a second primer to produce a first amplified product;

(b) amplifying said amplified product using a third and a fourth primer using the recombinase polymerase ampli-fication process of claim 1 to produce a second ampli-fied product, wherein said second amplified product is a smaller sequence contained within said first amplified product.

94. The process of claim 93 wherein said first primer, said second primer, said third primer and said fourth primer are each different.

95. The process of claim 93 wherein said one of said first and said second primer is identical to one of said third and fourth primer.

96. A recombinase polymerase amplification process of DNA amplification of a target nucleic acid molecule, said target nucleic acid molecule comprising a first and second strand of DNA, comprising the steps of

(a) contacting a recombinase agent with a nucleic acid primer to form a nucleoprotein primer which comprises a single stranded region at its 3' end;

(b) contacting the nucleoprotein primer to said target nucleic acid molecule thereby forming a double-stranded structure at a portion of said target nucleic acid molecule;

(c) extending the 3'end of said nucleic acid primer with one or more polymerases and dNTPs to generate a double-stranded nucleic acid and a displaced strand of nucleic acid; and

(d) continuing the reaction through repetition of (b) and (c) until a desired degree of amplification is reached.

97. The process of claim 96 wherein a fraction of said dNTPs comprises chain-terminating dNTPs.

98. The process of claim 96 wherein at least a fraction of said dNTPs is labeled with a detectable marker.

99. A process of detecting the presence or absence of a recombinase polymerase amplification amplified nucleic acids product, comprising the steps of:

(a) contacting a recombinase agent with a first and second nucleic acid primer to form a first and second nucle-oprotein primer, wherein said nucleic acid primer com-prises a single stranded region at its 3', wherein said first nucleic acid primer is labeled with a first member of a first binding pair, and wherein said second nucleic acid primer is labeled with a first member of a second binding pair;

(b) contacting the first and the second nucleoprotein primer to a sample suspected to contain a recombinase polymerase amplification amplified nucleic acid to form primer/recombinase polymerase amplification amplified nucleic acid complexes;

(c) contacting said primer/recombinase polymerase amplification amplified nucleic acid complexes with a first mobile solid support coated with a second member of said first binding pair and a second mobile solid support coated with a second member of said second binding pair;

(d) determining if said first mobile solid support is co-localized with said second mobile solid support to determine the presence of said recombinase polymerase amplification amplified nucleic acid product.

100. The process of claim 99 wherein the first nucleic acid primer has the same nucleic acid sequence as a primer used to produce the suspected recombinase polymerase amplification amplified nucleic acid product.

101. The process of claim 99 wherein the second nucleic acid primer has the same nucleic acid sequence as a primer used to produce the suspected recombinase polymerase amplification amplified nucleic acid product.

102. The process of claim 99 wherein the first and/or second primers are associated with the target DNA by virtue of the formation of a triple helix mediated by a recombinase, and occurring after amplification of the target by an recombinase polymerase amplification reaction.

103. A recombinase polymerase amplification process of DNA amplification of a double stranded target molecule comprising the steps of:

(a) contacting a recombinase agent selected from the group consisting of uvsX and recA, with a first and a second nucleic acid primer to form a first and a second nucleoprotein primer;

(b) contacting the first and second nucleoprotein primers to said double stranded target nucleic acid molecule thereby forming a first double stranded structure at a first portion of said target molecule and a second double stranded structure at a second portion of said target molecule such that the 3' ends of said first nucleic acid primer and said second nucleic acid primer are oriented toward each other on the same template nucleic acid molecule;

(c) contacting said target nucleic acid molecule to primosome assembly proteins and a clamp loader/polymerase III holoenzyme complex to form a replication fork at said first portion of target molecule and a second replication fork at said second portion of the target molecule;

(d) contacting said target molecule to a DNA polymerase III core, a DNA polymerase I, a primase, a helicase, and a ligase to allow coupled leading and lagging strand DNA synthesis and the migration of the replication forks towards each other; and

(e) continuing the reaction through repetition of steps (b), (c) and (d) until a desired degree of amplification is reached.

104. The process of claim 103 which is performed in the presence of dNTP, rNTP, single stranded DNA binding protein, or a combination thereof.

105. The process of claim 103 wherein said primosome assembly proteins are selected from the group consisting of PriA, PriB, PriC, DnaT, DnaC, DnaB, DnaG, Pol III holoenzyme, and a combination thereof.

106. The process of claim 103 wherein the clamp loader is a DnaX clamp loader complex.

107. The process of claim 103 wherein the DNA polymerase III holoenzyme is *E. coli* polymerase III.

108. The process of claim 103 wherein DNA polymerase I is *E. coli* polymerase I.

109. The process of claim 103 wherein the primase is *E. coli* DnaG primase.

110. The process of claim 103 wherein the helicase is *E. coli* DnaB helicase.

111. A recombinase polymerase amplification process of DNA amplification comprising the steps of:

(a) combining the following reagents in a reaction

(1) at least one recombinase;

(2) at least one single stranded DNA binding protein;

(3) at least one DNA polymerase;

(4) dNTPs or a mixture of dNTPs and ddNTPs

(5) a crowding agent;

(6) a buffer;

(7) a reducing agent;

(8) ATP or ATP analog;

(9) at least one recombinase loading protein;

(10) a first primer and optionally a second primer; and

(11) a target nucleic acid molecule;

(b) incubating said reaction until a desired degree of amplification is achieved.

112. The process of claim 111 wherein said recombinase is selected from the group consisting of uvsX, recA and derivatives and combinations thereof.

113. The process of claim 111 wherein said recombinase is a fusion protein comprising a short tag sequence at the N or C terminus.

114. The process of claim 111 wherein the recombinase comprises a C terminal deletion of acidic residues.

115. The process of claim 111 wherein the recombinase is present at a concentration of between about 0.2 μM and about 1 μM.

116. The process of claim 111 wherein the recombinase is present at a concentration of between about 1 μM and about 4 μM.

117. The process of claim 111 wherein the recombinase is present at a concentration of between about 4 μM and about 6 μM.

118. The process of claim 111 wherein the recombinase is present at a concentration of between about 6 μM and about 12 μM.

119. The process of claim 111 wherein the recombinase is present at a concentration of between about 0.2 μM and about 12 μM.

120. The process of claim 111 wherein said single stranded DNA binding protein is selected from the group consisting of *E. coli* SSB, T4 gp32, and derivatives and combinations thereof.

121. The process of claim 120 wherein said gp32 derivative is selected from the group consisting of gp32(N), gp32(C), gp32(C)K3A, gp32(C)R4Q, gp32(C)R4T, gp32K3A, gp32R4Q, gp32R4T and a combination thereof.

122. The process of claim 111 wherein said single stranded DNA binding protein is present at a concentration of between 1 μM and 30 μM.

123. The process of claim 111 wherein said DNA polymerase is a eukaryotic polymerase or a prokaryotic polymerase.

124. The process of claim 123 wherein said eukaryotic polymerase is selected from the group consisting of pol-α, pol-β, pol-δ, pol-ϵ and derivatives and combinations thereof.

125. The process of claim 123 wherein said prokaryotic polymerase is selected from the group consisting of *E. coli* DNA polymerase I Klenow fragment, bacteriophage T4 gp43 DNA polymerase, *Bacillus stearothermophilus* polymerase I large fragment, Phi-29 DNA polymerase, T7 DNA polymerase, Bsu Pol I, *E. coli* DNA polymerase I, *E. coli* DNA polymerase II, *E. coli* DNA polymerase III, *E. coli* DNA polymerase IV, *E. coli* DNA polymerase V and derivatives and combinations thereof.

126. The process of claim 123 wherein said DNA polymerase is a fusion protein comprising a short tag sequence at the N or C terminus.

127. The process of claim 111 wherein said DNA polymerase is at a concentration of between 10,000 units/ml to 16 units/ml

128. The process of claim 111 wherein said DNA polymerase is at a concentration of between 5000 units/ml to 500 units/ml.

129. The process of claim 111 wherein the one or more DNA polymerase includes at least one DNA polymerase which lacks 3'-5' exonuclease activity.

130. The process of claim 111 wherein said one or more DNA polymerase comprises a DNA polymerase with strand displacing properties.

131. The process of claim 111 wherein said dNTP is at a concentration of 1 mM to 40 μM.

132. The process of claim 111 wherein said crowding agent is polyethylene glycol, dextran and ficoll.

133. The process of claim 132 wherein said polyethylene glycol is between 1 to 12% of a weight or a volume of the reaction.

134. The process of claim 132 wherein said polyethylene glycol is selected from the group consisting of PEG1450, PEG3000, PEG8000, PEG10000, PEG compound (molecular weight 15,000 to 20,000 and also known as Carbowax 20M), and a combination thereof.

135. The process of claim 111 wherein said buffer is a Tris-HCl buffer, a Tris-Acetate buffer, or a combination thereof.

136. The process of claim 135 wherein said buffer is at a concentration of between 10 to 60 mM and at a pH of between 6.5 to 9.0.

137. The process of claim 111 wherein said reducing agent is DTT.

138. The process of claim 137 wherein said reducing agent is at a concentration of 1 to 10 mM.

139. The process of claim 111 wherein said ATP or ATP analog is selected from the group consisting of ATP, ATP-γ-S, ATP-β-S or ddATP.

140. The process of claim 139 wherein said ATP or ATP analog is at a concentration of 1 to 10 mM.

141. The process of claim 111 wherein said recombinase loading protein is selected from the group consisting of T4uvsY, *E. coli* recO, *E. coli* recR and derivatives and combinations thereof.

142. The process of claim 141 wherein said recombinase loading protein further comprise a poly-His tag at the N terminus, C terminus, or at both the N and C terminus.

143. The process of claim 111 wherein said recombinase loading protein is at a concentration of between 0.2 and 8 μM.

144. The process of claim 111 wherein the first or second primer is selected from the group consisting of DNA, RNA, PNA, LNA, morpholino backbone nucleic acid, phosphorothiorate backbone nucleic acid and a combination thereof.

145. The process of claim 111 wherein said primer is at a concentration of 100 nM to 1000 nM

146. The process of claim 111 wherein the first or second primer comprise a non-phosphate linkage between the two bases at its 3' end and is resistant to 3' to 5' nuclease activity.

147. The process of claim 111 wherein the first or second primer comprise at least one locked nucleic acid at its 3' ultimate base or 3' penultimate base.

148. The process of claim 111 wherein the first or second primer are at least 20 bases in length.

149. The process of claim 111 wherein the first or second primer are at least 30 bases in length.

150. The process of claim 111 wherein the first or second primer further comprise one or more bases at its 5' end that is not complementary to the target nucleic acid.

151. The process of claim 150 wherein said one or more bases comprise the sequence of a restriction endonuclease recognition site.

152. The process of claim 111 wherein said first or second primer is partially double stranded with a single stranded region at its 3' end.

153. The process of claim 111 wherein at least one primer is labeled with a detectable label.

154. The process of claim 153 wherein said detectable label is selected from the group consisting of a fluorochrome, an enzyme, a fluorescence quencher, an enzyme inhibitor, a radioactive label and a combination thereof.

155. The process of claim 111 wherein target nucleic acid molecule is supercoiled.

156. The process of claim 111 wherein the target nucleic acid molecule is linear with two ends and wherein both ends are linked to a non-target nucleic acid molecule.

157. The process of claim 111 wherein the target nucleic acid molecule is linear with two ends and wherein one end is linked to a non-target nucleic acid molecule.

158. The process of claim 111 wherein the target nucleic acid molecule is a linear nucleic acid molecule.

159. The process of claim 111 wherein the target nucleic acid molecule is a single-stranded nucleic acid which is converted to a double stranded nucleic acid by a polymerase or a double stranded nucleic acid denatured by the action of heat or chemical treatment.

160. The process of claim 111 wherein the target nucleic acid is at a concentration of less than 1000 copies per ml.

161. The process of claim 111 wherein the target nucleic acid is at a concentration of between 1 and 10 copies.

162. The process of claim 111 wherein said incubation is between 5 minutes and 16 hours.

163. The process of claim 111 wherein said incubation is between 15 minutes and 3 hours.

164. The process of claim 111 wherein said incubation is between 30 minutes and 2 hours.

165. The process of claim 111 wherein said incubation is performed until a desired degree of amplification is achieved.

166. The process of claim 165 wherein said desired degree of amplification is selected from the group consisting of 10 fold, 100 fold, 1000 fold, 10,000 fold, 100,000 fold and 1000000 fold.

167. The process of claim 111 wherein said incubation is performed between 20° C. and 50° C.

168. The process of claim 111 wherein said incubation is performed between 20° C. and 40° C.

169. The process of claim 111 wherein said incubation is performed between 20° C. and 30° C.

170. The process of claim 111 which is performed without temperature induced melting of the template nucleic acid.

171. The process of claim 111 wherein step (a) further comprises an accessory agent.

172. The process of claim 171 wherein said accessory agent is selected from the group consisting of helicase, topoisomerase, resolvase and a combination thereof.

173. The process of claim 171 wherein the accessory agent are selected from the group consisting of RuvA, RuvB, RuvC, RecG, PriA, PriB, PriC, DnaT, DnaB, DnaC, DnaG, DnaX clamp loader, polymerase core complex, DNA ligase and a sliding clamp.

174. The process of claim 173 wherein said sliding clamp is the *E. coli* β-dimer sliding clamp, the eukaryotic PCNA sliding clamp, or the T4 sliding clamp gp45 and a combination thereof.

175. A process of claim 171 wherein that accessory agent is a DNA Polymerase III holoenzyme complex consisting of β-Clamp, DnaX Clamp Loader, and the Polymerase Core Complex.

176. The process of claim 111 wherein step (a) further comprises a RecA/ssDNA nucleoprotein filaments stabilizing agent.

177. The process of claim 176 wherein said stabilizing agent is selected from the group consisting of RecR, RecO, RecF and a combination thereof.

178. The process of claim 176 wherein said stabilizing agent is at a concentration of between 0.01 μM to 20 μM.

179. The process of claim 111 wherein step (a) further comprises an ATP regeneration system to convert ADP to ATP.

180. The process of claim 179 wherein said ATP regeneration system comprises phosphocreatine and creatine kinase.

181. The process of claim 111 wherein step (a) further comprises a system to regenerate ADP from AMP.

182. The process of claim 111 wherein step (a) further comprises a system to convert pyrophosphate to phosphate.

183. The process of claim 111, wherein one or more reagents are freeze dried before said step (a), said reagents selected from the group consisting of the recombinase, the single stranded DNA binding protein, the DNA polymerase, the dNTPs or the mixture of dNTPs and ddNTPs, the reducing agent, the ATP or ATP analog, the recombinase loading protein, crowding agent, buffer, salts, DTT, and the first primer and optionally a second primer or a combination thereof.

184. A recombinase polymerase amplification process of DNA amplification comprising the steps of:

(a) combining the following reagents in a reaction

(1) a uvsX recombinase at a concentration of between 0.2 to 12 μM;

(2) a gp32 single stranded DNA binding protein at a concentration between 1 to 30 μM;

(3) a T4 gp43 DNA polymerase or Bsu polymerase at a concentration between 500 to 5000 units per ml;

(4) dNTPs or a mixture of dNTPs and ddNTPs at a concentration of between 1-300 μM;

(5) polyethylene glycol at a concentration of between 1% to 12% by weight or by volume;

(6) Tris-acetate buffer at a concentration of between 1 mM to 60 mM;

(7) DTT at a concentration of between 1 mM-10 mM;

(8) ATP at a concentration of between 1 mM-10 mM;

(9) uvsY at a concentration of between 0.2 μM-8 μM;

(10) a first primer and optionally a second primer, wherein said primers are at a concentration of between 50 nM to 1 μM; and

(11) a target nucleic acid molecule of at least one copy;

(b) incubating said reaction until a desired degree of amplification is achieved.

185. A recombinase polymerase amplification process of DNA amplification comprising the steps of:

(a) combining the following reagents in a reaction;

(1) 100-200 ng/μl uvsX recombinase;

(2) 600 ng/μl gp32;

(3) 20 ng/μl Bsu polymerase or T4 polymerase;

(4) 200 μM dNTPs;

(5) 1 mM DTT

(6) 3 mM ATP or an ATP analog;

(7) 16 ng/μl to 60 ng/μl uvsY

(8) 300 nM of a first primer and 300 nM of a second primer;

(9) 80 mM Potassium acetate

(10) 10 mM Magnesium acetate

(11) 20 mM Phosphocreatine

(12) 100 ng/μl Creatine kinase

(b) freeze drying the reagents from said step (a) to form freeze dried reagents;

(c) reconstituting said freeze dried reagents with

(1) Tris-acetate buffer at a concentration of between 1 mM to 60 mM;

(2) polyethylene glycol at a concentration of between 1% to 12% by weight or by volume;

(3) a target nucleic acid;

(d) incubating said reaction until a desired degree of amplification is achieved.

186. A recombinase polymerase amplification process of DNA amplification comprising the steps of:

(a) combining the following reagents in a reaction;

   (1) 100-200 ng/μl uvsX recombinase;

   (2) 300-1000 ng/μl gp32;

   (3) 10-50 ng/μl Bsu polymerase or T4 polymerase;

   (4) 50-500 μM dNTPs;

   (5) 0.1 to 10 mM DTT

   (6) 3 mM ATP or an ATP analog;

   (7) 16 ng/μl to 60 ng/μl uvsY

   (8) 50-1000 nM of a first primer and 50-1000 nM of a second primer;

   (9) 40-160 mM Potassium acetate

   (10) 5-20 mM Magnesium acetate

   (11) 10-40 mM Phosphocreatine

   (12) 50-200 ng/μl Creatine kinase

(b) freeze drying the reagents from said step (a) to form freeze dried reagents;

(c) reconstituting said freeze dried reagents with

   (1) Tris-acetate buffer at a concentration of between 1 mM to 60 mM;

   (2) polyethylene glycol at a concentration of between 1% to 12% by weight or by volume;

   (3) a target nucleic acid;

(d) incubating said reaction until a desired degree of amplification is achieved.

187. A recombinase polymerase amplification process of DNA amplification comprising the steps of:

(a) combining the following reagents in a reaction;

   (1) 100-200 ng/μl uvsX recombinase;

   (2) 300-1000 ng/μl gp32;

   (3) 10-50 ng/μl Bsu polymerase or T4 polymerase;

   (4) 50-500 μM dNTPs;

   (5) 0.1 to 10 mM DTT

   (6) 3 mM ATP or an ATP analog;

   (7) 16 ng/μl to 60 ng/μl uvsY

   (8) 50-1000 nM of a first primer and 50-1000 nM of a second primer;

   (9) 40-160 mM Potassium acetate

   (10) 5-20 mM Magnesium acetate

   (11) 10-40 mM Phosphocreatine

   (12) 50-200 ng/μl Creatine kinase

   (13) polyethylene glycol at a concentration of between 1% to 12% by weight or by volume

(b) freeze drying the reagents from said step (a) to form freeze dried reagents;

(c) reconstituting said freeze dried reagents with

   (1) Tris-acetate buffer at a concentration of between 1 mM to 60 mM;

   (2) a target nucleic acid;

(d) incubating said reaction until a desired degree of amplification is achieved.

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