



[54] METHOD OF SAMPLING, AMPLIFYING AND QUANTIFYING SEGMENT OF NUCLEIC ACID, POLYMERASE CHAIN REACTION ASSEMBLY HAVING NANOLITER-SIZED SAMPLE CHAMBERS, AND METHOD OF FILLING ASSEMBLY

[75] Inventors: James F. Brown, Clifton, Va.; Jonathan E. Silver, Bethesda, Md.; Olga V. Kalinina, Toronto, Canada

[73] Assignees: Cytonix Corporation, Beltsville, Md.; The United States of America as represented by the Department of Health and Human Services, Washington, D.C.

[21] Appl. No.: 08/838,262

[22] Filed: Apr. 17, 1997

[51] Int. Cl.<sup>7</sup> ..... G01N 33/543; G01N 33/68

[52] U.S. Cl. .... 435/6; 435/287.1; 435/287.2; 435/288.3; 435/288.7; 436/164; 436/172; 436/518; 436/524; 436/527; 436/531; 436/805; 436/809; 422/58

[58] Field of Search ..... 435/6, 287.1, 287.2, 435/288.3, 288.7; 436/164, 172, 518, 524, 527, 531, 805, 809; 422/58

[56] References Cited

U.S. PATENT DOCUMENTS

4,591,567 5/1986 Britten et al. .
4,834,946 5/1989 Levin .
4,911,782 3/1990 Brown .
5,176,203 1/1993 Larzul .
5,200,152 4/1993 Brown .
5,225,332 7/1993 Weaver et al. .
5,229,297 7/1993 Schnipelsky et al. .... 436/94
5,346,672 9/1994 Stapleton et al. .
5,380,489 1/1995 Sutton et al. .... 422/68.1
5,498,392 3/1996 Wilding et al. .
5,503,803 4/1996 Brown .
5,525,464 6/1996 Drmanac et al. .
5,585,069 12/1996 Zanzucchi et al. .

OTHER PUBLICATIONS

Wittwer et al., "The Lightcycler™: A Microvolume Multi-sample Flourimeter With Rapid Temperature Control"; Bio Techniques, vol. 22, No. 1, pp. 176-181 (Jan. 1997).
Woolley et al.; "Ultra-High-Speed DNA Fragment Separations Using Microfabricated Capillary Electrophoresis Chips", Proc. Natl. Acad. Sci. USA, vol. 91, pp. 11348-11352, Biophysics, (1994).
Wilding et al., PCR in a Silicone Microstructure; Clinical Chemistry, vol. 40, No. 9, pp. 1815-1818 (1994).
Good et al.; Generalization of Theory for Estimation of Interfacial Energies; Chemistry and Physics of Interfaces, ACS, pp. 91-96 (1971).
Burns et al.; Microfabricated Structures for Integrated DNA Analysis; Proc. Natl. Acad. Sci. USA, vol. 93, pp. 5556-5561, Genetics (1996).
Rigler, "Fluorescence Correlations, Single Molecule Detection and Large Number Screening Applications in Biotechnology"; Journal of Biotechnology, vol. 41, pp. 177-186 (1995).

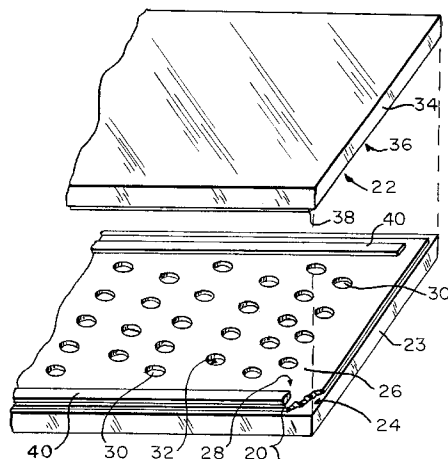
(List continued on next page.)

Primary Examiner—Christopher L. Chin
Attorney, Agent, or Firm—Kilyk & Bowersox, P.L.L.C.

[57] ABSTRACT

Methods of detecting and quantifying genomic nucleic acid molecule sequences are provided using the simultaneous amplification of a plurality of discrete nanoliter-sized samples. A miniaturized closed assembly is also provided for carrying out amplification of a nucleic acid molecule by polymerase chain reaction in multiple nanoliter-sized samples. Methods of filling miniaturized sample chambers are also provided as are methods for determining the number of template molecules in a sample by conducting replicate nucleic acid sequence amplification reactions on a set of terminally diluted samples and counting the number of positive amplification reactions. The methods can be used to detect a single starting nucleic acid target molecule.

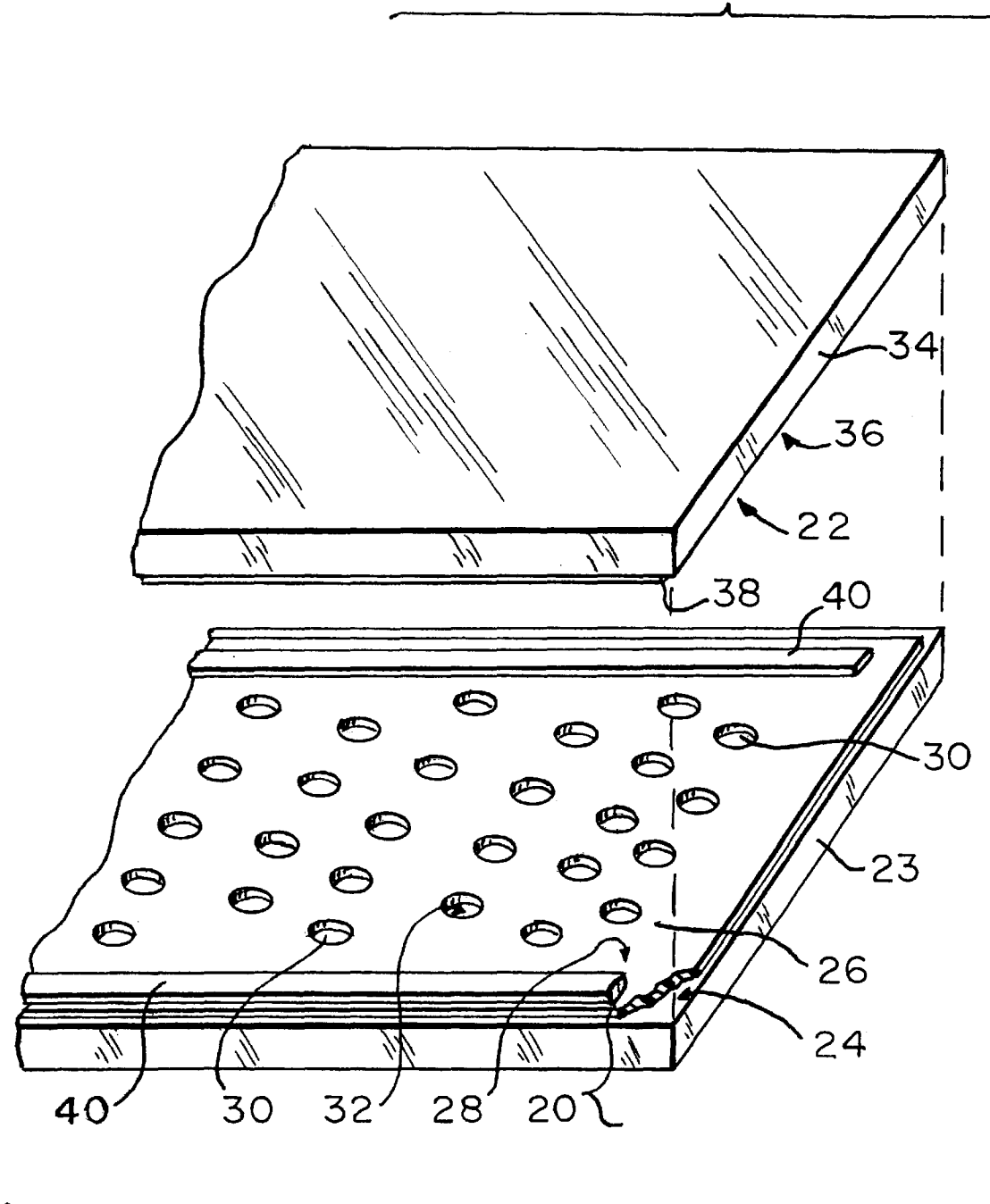
17 Claims, 7 Drawing Sheets



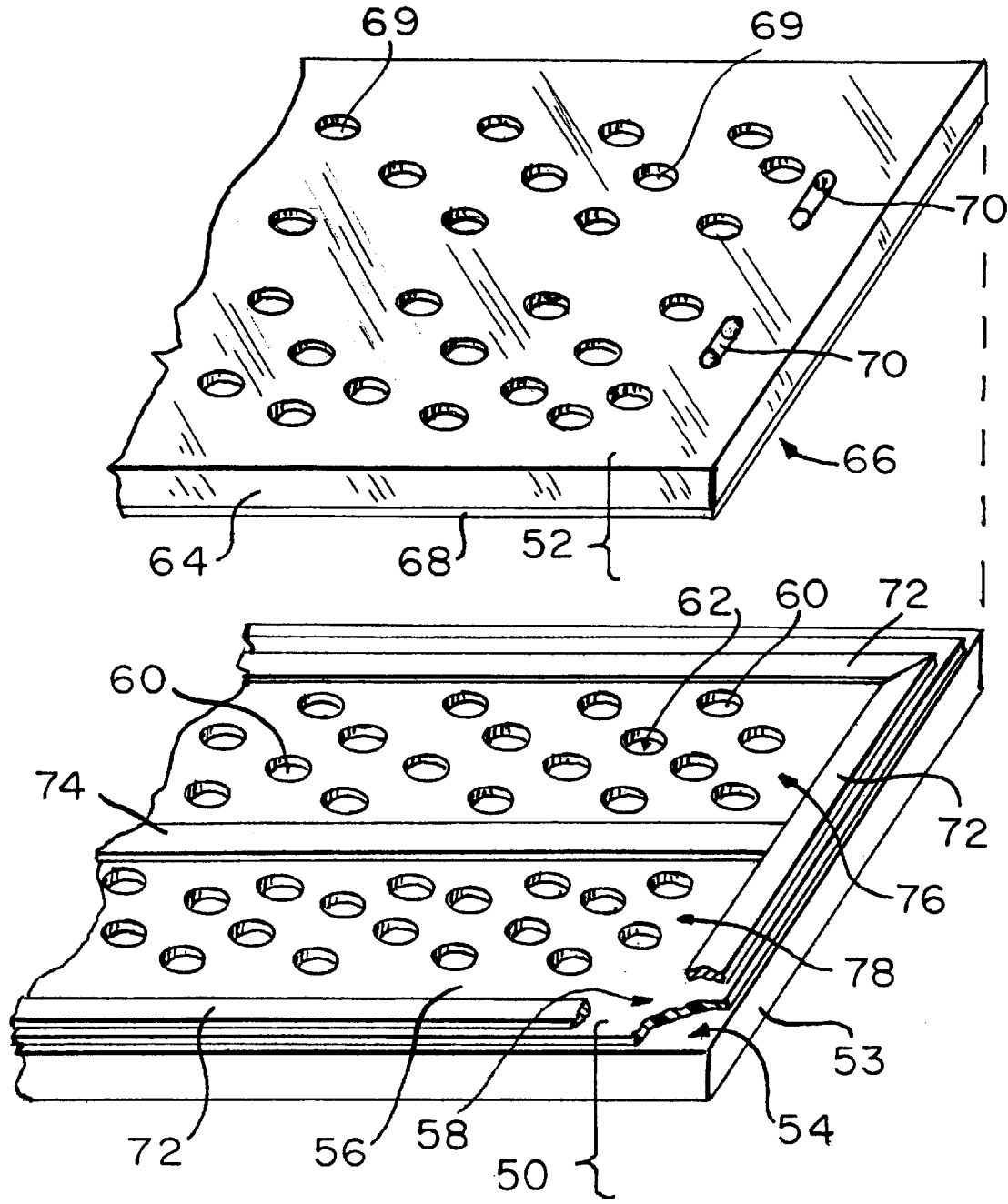
## OTHER PUBLICATIONS

- Cheng et al.; "Analysis of Ligase Chain Reaction Products Amplified in a Silicon-Glass Chip Using Capillary Electrophoresis", *Journal of Chromatography*, vol. 732, pp. 151-158 (1996).
- Kricka et al.; "Imaging of Chemiluminescent Reactions in Mesoscale Silicon-Glass Microstructures"; *J. Biolumin Chemilumin*, vol. 9, pp. 135-138 (1994).
- Woolley et al., "Ultra-High-Speed DNA Sequencing Using Capillary Electrophoresis Chips", *Anal-Chem*, vol. 67, No. 20, pp. 3676-3680 (1995).
- Hawkins et al.; "Incorporation of a Fluorescent Guanosine Analog into Oligonucleotides and its Application to a Real Time Assay for the HIV-1 Integrase 3'-Processing Reaction"; *Nucleic Acids Research*, vol. 23, No. 15, pp. 2872-2880 (1995).
- Tyagi et al.; "Molecular Beacons: Probes That Fluoresce Upon Hybridization" *Nature Biotechnology*, vol. 14, pp. 303-308 (1996).
- Holland et al.; Detection of Specific Polymerase Chain Reaction Product by Utilizing the 5'→3' Exonuclease Activity of *Thermus Aquaticus* DNA Polymerase; *Proc. Natl. Acad. Sci.*, vol. 88, *Biochemistry*, pp. 7276-7280 (1991).
- Livak et al.; "Oligonucleotides with Fluorescent Dyes at Opposite Ends Provide a Quenched Probe System Useful for Detecting PCR Product and Nucleic Acid Hybridization"; *PCR Method and Applications*, pp. 357-362 (1995).
- Sninsky et al.; "The Application of Quantitative Polymerase Chain Reaction to Therapeutic Monitoring"; *AIDS*, vol. 7 (Supp 2), pp. S29-S34 (1993).
- Becker-André et al., Absolute mRNA Quantification Using the Polymerase Chain Reaction (PCR); *Nucleic Acids Research*, vol. 17, No. 22, pp. 9437-9446 (1989).
- Gilliland et al.; "Analysis of Cytokine mRNA and DNA: Detection and Quantitation by Competitive Polymerase Chain Reaction", *Proc. Natl. Acad. Sci. USA*, vol. 87, Genetics, pp. 2725-2729 (1990).
- Higuchi et al.; "Simultaneous Amplification and Detection of Specific DNA Sequences"; *Biotechnology*, vol. 10, pp. 413-417 (1992).
- Heid et al.; "Real Time Quantitative PCR", *Genome Research*, No. 6, pp. 986-994 (1996).
- Gibson et al.; "A Novel Method for Real Time Quantitative RT-PCR"; *Genome Research*, No. 6, pp. 995-1001 (1996).
- Gerard et al.; "A Rapid and Quantitative Assay to Estimate Gene Transfer into Retrovirally Transduced Hematopoietic Stem/Progenitor Cells Using a 96-Well Format PCR and Fluorescent Detection System Universal for MMLV-Based Proviruses"; *Human Gene Therapy*, No. 7, pp. 343-354 (1996).
- Wittwer et al., "Rapid Cycle DNA Amplification", *Biotechniques*, vol. 10, No. 1, pp. 76-83 (1991).
- Chang, *Physical Chemistry With Applications to Biological Systems*, 2<sup>nd</sup> Edition, Sec. 5.4, p. 87.
- Berg, *Random Walks in Biology*, "Diffusion: Microscopic Theory", pp. 10, 49 (1983).
- Burns et al.; Microfabricated Structures for Integrated DNA Analysis, *Proc. Natl. Acad. Sci.*, vol. 93, Genetics, pp. 5556-5561 (1996).
- Cheng et al.; Chip PCR.II. Investigation of Different PCR Amplification Systems in Microfabricated Silicon-Glass Chips; *Nucleic Acids Research*, vol. 24, No. 2, pp. 380-385 (1996).
- Woolley et al.; "Functional Integration of PCR Amplification and Capillary Electrophoresis in a Microfabricated DNA Analysis Device"; *Anal. Chem.* No. 68, pp. 4081-4086 (1996).
- Wittwer et al.; Continuous Fluorescence Monitoring of Rapid Cycle DNA Amplification; *Biotechniques*, 22, pp. 130-138 (Jan. 1997).
- Hawkins et al.; Fluorescence Properties of Pteridine Nucleoside Analogs as Monomers and Incorporated into Oligonucleotides, *Analytical Biochemistry*, 244, pp. 86-95 (1997).
- Xu et al., "Direct Measurement of Single-Molecule Diffusion and Photodecomposition in Free Solution", *Science*, vol. 275, pp. 1106-1109 (Feb. 1997).
- CRC Handbook of Chemistry and Physics*, 74th Edition, Lide (Editor-In-Chief), p. 6-10 (1993-1994).

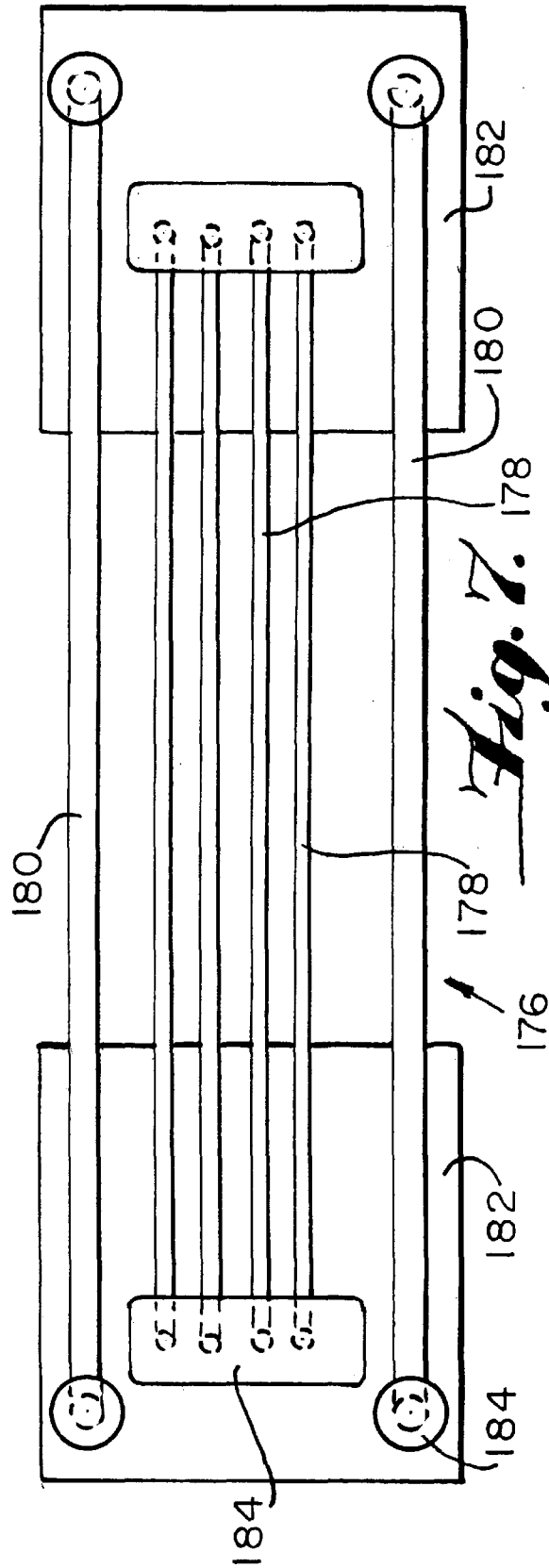
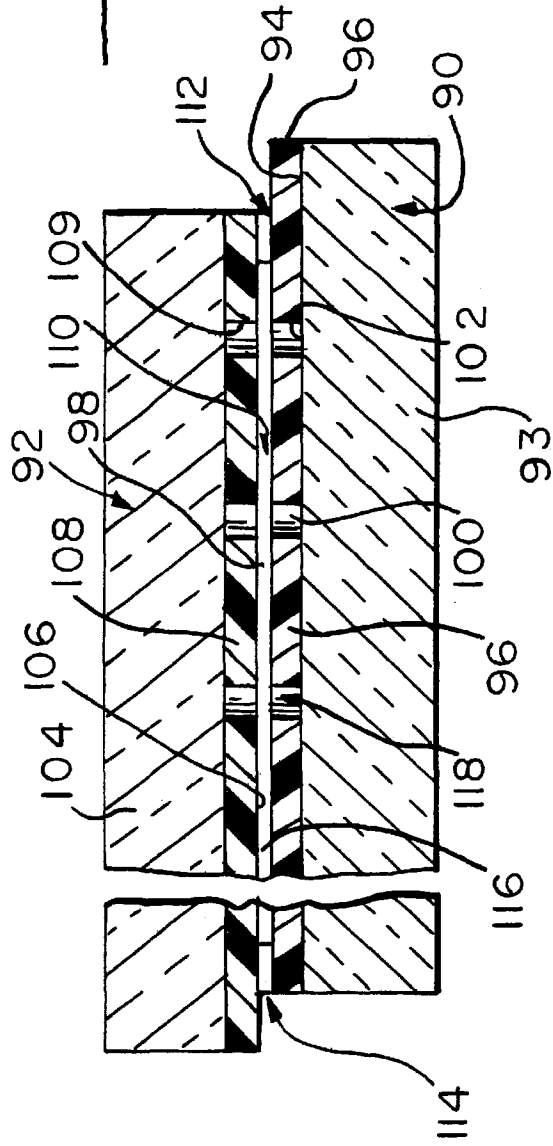
*Fig. 1.*



*Fig. 2.*



*Fig. 3.*



*Fig. 7.*

# Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

## Real-Time Litigation Alerts



Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

## Advanced Docket Research



With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

## Analytics At Your Fingertips



Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

## API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

## LAW FIRMS

Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

## FINANCIAL INSTITUTIONS

Litigation and bankruptcy checks for companies and debtors.

## E-DISCOVERY AND LEGAL VENDORS

Sync your system to PACER to automate legal marketing.