(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
31 March 2005 (31.03.2005)

PCT

(10) International Publication Number
WO 2005/028110 A2

(51) International Patent Classification[7]: B01L

(21) International Application Number:
PCT/US2004/030788

(22) International Filing Date:
17 September 2004 (17.09.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/504,052	19 September 2003 (19.09.2003)	US
60/504,500	19 September 2003 (19.09.2003)	US
60/589,224	19 July 2004 (19.07.2004)	US
60/589,225	19 July 2004 (19.07.2004)	US
10/913,601	5 August 2004 (05.08.2004)	US
60/601,716	13 August 2004 (13.08.2004)	US

(71) Applicant *(for all designated States except US)*: **APPLERA CORPORATION?** [US/US]; 850 Lincoln Centre Drive, Foster City, California 94404 (US).

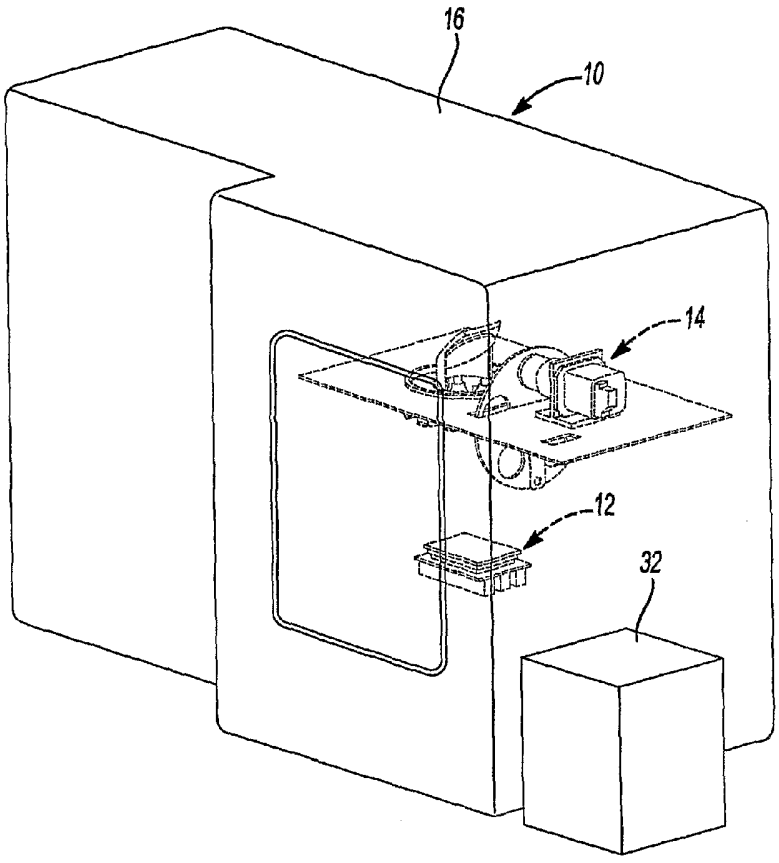
(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LI, Robin?** [US/US]; 860 Oak Street #4, San Francisco, California 94117 (US). **AMIN, Yusuf?** [US/US]; 2278 Burgundy Way, Fairfield, California 94533 (US). **CARRILLO, Albert?** [US/US]; 508 Lincoln Avenue, Redwood City, California 94061 (US). **REEVE, Vincent?** [US/US]; 730 29th Street #204, Oakland, California 94609 (US). **HARDING, Ian?** [US/US]; 147 Burbank Avenue, San Mateo, California 94403 (US). **FAWCETT, Adrian?** [GB/US]; 3606 W. Las Positas Blvd., Pleasanton, California 94588 (US). **KAO, H. Pin?** [CA/US]; 37581 Maple Street, Fremont, California 94536 (US). **LU, Mike?** [US/US]; 1057 Kelly Drive, San Jose, California 95129 (US). **LIM, Gary?** [US/US]; 2739 37th Avenue, San Francisco, California 94116 (US).

(74) Agent: **LANG, Michael?**; Harness, Dickey & Pierce, P.L.C., P.O. Box 828, Bloomfield Hills, Michigan 48303 (US).

[Continued on next page]

(54) Title: MICROPLATES USEFUL FOR CONDUCTING THERMOCYCLED NUCLEOTIDE AMPLIFICATION



(57) Abstract: A reaction plate comprises a plurality of reaction cells, wherein the plate comprises a thermal conductive material, each of the wells has an open top and a closed bottom, and a distance between the wells is less than 0.5 mm. Other embodiments include an alignment feature which may be, but not limited to, a bar code, an alignment pin, an alignment slot or a keyed corner. In various embodiments, the plate has about 6,144 wells at a pitch of about 1.125 mm. In some embodiments, each well has a volume of less than 600 nanoliters. Some embodiments include a cover for the reaction plate.

WO 2005/028110 A2

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

MICROPLATES USEFUL FOR CONDUCTING THERMOCYCLED NUCLEOTIDE AMPLIFICATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 60/504,052 filed on September 19, 2003; U.S. Provisional Application No. 60/504,500 filed on September 19, 2003; U.S. Provisional Application No. 60/589,224 filed July 19, 2004; U.S. Provisional Application No. 60/589,225 filed on July 19, 2004; U.S. Patent Application No. 10/913,601 filed on August 5, 2004 and U.S. Provisional Application No. 60/601,716 filed on August 13, 2004. The applications are incorporated herein by reference.

INTRODUCTION

[0002] The present invention relates to multi-well microtiter plate and, more specifically to high density multi-well microtiter plate.

[0003] Today, polymerase chain reaction ("PCR") processes which are associated with replicating genetic material such as DNA and RNA are carried out on a large scale in both industry and academia, so it is desirable to have an apparatus that allows the PCR process to be performed in an efficient and convenient fashion. Traditionally, screening of agents for biological activity is accomplished by placing small amounts of a sample, generally a genetic material, to be analyzed, either in liquid or solid form, in a plurality of wells formed in a microtiter plate. The sample is then exposed to the target of interest, for example, a purified protein, such as an enzyme or receptor, or a whole cell or non-biologically derived catalyst. The interaction of the sample with the target can then be measured radiochemically, spectrophotometrically, or fluorometrically. In a fluorescence measurement technique, light of a given wavelength is directed onto a sample within a well of the microtiter plate, a portion of the light is absorbed by the sample, and is reemitted at a different, typically longer, wavelength, which is then measured.

[0004] In accordance with the PCR process, the sample and a solution of reactants including the target are deposited within each well of the traditional microtiter plate. The traditional microtiter plate is then placed in a thermocycler which operates to cycle the temperature of the contents within the wells. In particular, the traditional microtiter plate is placed on a metal heating fixture in the thermocycler that is shaped to closely conform to the

underside of the traditional microtiter plate and, in particular, to the exterior portion of the wells. A heated top plate of the thermocycler then tightly clamps the traditional microtiter plate onto the metal heating fixture while the contents in the wells of the traditional microtiter plate are repeatedly heated and cooled for around 90-150 minutes. Because, the traditional microtiter plate is made from a polymeric material which is a poor thermal conductor, the walls of the wells have to be molded as thin as possible so the thermocycler can effectively heat and cool the contents in the wells. The relatively thin well walls in the traditional microtiter plate deform when they contact the metal heating fixture of the thermocycler to make good thermal contact. This requires that the traditional microtiter plate be made from a relatively non-rigid material such as polypropylene.

[0005] Unfortunately, polypropylene tends to change dimensions when heated to relieve stress in the traditional microtiter plate. As a result of the deformation of the relatively thin wells and the tendency of the traditional microtiter plate to change dimensions during the thermal cycling, it is often difficult for a scientist to remove the traditional microtiter plate from the thermocycler. More specifically, as the number of wells in the traditional microtiter plate increases from 96 wells to 384 wells to 1536 wells, the force required to remove the traditional microtiter plate from the thermocycler also increases which further deforms the relatively thin, non-rigid, traditional microtiter plate. In addition, the low thermal conductivity of the traditional plastic microtiter plates results in inconsistent heating and cooling, temperature non-uniformity between samples and limitations on the speed, or response time, at which the samples can be thermally cycled.

[0006] With the demands of increased throughput of samples and the increase in the number of probes and primers that are available, it is desirable to have microtiter plates with a greater number of wells. Since the footprint of microtiter plates are standardized, the only way to increase the number of wells is to increase the density of wells. If the density of wells is increased this will cause the well walls to be even thinner and exacerbate the problems of deformation, lack of rigidity, and thermal conductivity described above. What is needed is a high density multi-well microtiter plate that meets

the standard foot print dimensions and does not have the inherent problems of deformation, lack of rigidity, and thermal conductivity.

SUMMARY OF THE INVENTION

[0007] The present invention includes microplate manufactured from a thermal conductive material and methods for making and using the microplate. The present invention works with any system that uses thermal cycling for analysis, such as PCR or that requires heat to be transferred from a heater system through a microplate.

[0008] In embodiments of the invention, a reaction plate comprises a plurality of reaction cells, wherein the plate comprises a thermal conductive material, each of the wells has an open top and a closed bottom, and a distance between the wells is less than 0.5 mm. Other embodiments of the invention include an alignment feature which may be, but not limited to, a bar code, an alignment pin, an alignment slot or a keyed corner. In various embodiments, the plate has about 6,144 wells at a pitch of about 1.125 mm. In some embodiments, each well has a volume of less than 600 nanoliters. Some embodiments include a cover for the reaction plate.

[0009] Embodiments of the present invention include: methods for producing a thermal conductive reaction plate comprising mixing a polymer and at least one thermal conductive additive, extruding the mixed polymer and at least one thermal conductive additive to create a melt blend, cooling said extruded melt blend, pelletizing said cooled melt blend, melting said pelletized melt blend, injecting said melted blend into a mold cavity of an injection molding machine, said molding cavity includes sections shaped to form said microplate, cooling the injected melt blend to create said microplate, and removing said microplate from injection molding machine, wherein said microplate includes a plurality of wells. In some embodiments of the methods, the plate that is produced has at least 96 columns of wells and at least 64 rows of wells. In certain embodiments, the plate has at least a total of 6,000 wells and a plate footprint dimension of about 127 mm by about 85 mm.

[0010] The invention also includes methods to perform PCR using a pre-loaded multi-well microplate comprising a thermal conductive material, the methods include placing the microplate in a thermal cycling machine, cycling the machine and analyzing the results. In certain embodiments, the plate has

at least a total of 6,000 wells and a plate footprint dimension of about 127 mm by about 85 mm. In still other embodiments of this invention include a high density microplate for performing an amplification reaction on a sample comprising a plurality of polynucleotide targets, comprising a substrate comprising an array of at least 1,000 wells, wherein each well has a capacity of less than about 100 microliters and comprises a homogenous solution consisting essentially of an amplification reagents and a sample.

[0011] In another embodiment of the invention, a method for simultaneously amplifying a plurality of polynucleotide targets in a liquid sample, each polynucleotide target being present at a very low concentration within said sample, comprising applying said sample to a microplate assembly, wherein said microplate assembly comprises a high density a microplate comprising a substrate comprising a well array of at least 1,000 wells, wherein each well has a capacity of less than 100 microliters, and comprises homogenous mixture comprising essentially amplification reagents and said sample, said sample is applied to the surface of said substrate so as to contact with the sample with said reagent in said wells, and thermal cycling said microplate assembly. In some embodiments of said invention, the method further comprises at least one primer and at least one probe in the amplification reagents. In other embodiments of the invention, the method includes an array comprising at least 6,000 wells and having a volume of less than 600 nanoliters and a pitch of about 1.125 mm.

[0012] Further areas of applicability of the present invention will become apparent from the detailed description provided hereinafter. It should be understood that the detailed description and specific examples, while indicating the preferred embodiment of the invention, are intended for purposes of illustration only and are not intended to limit the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] The present invention will become more fully understood from the detailed description and the accompanying drawings, wherein:

[0014] Figure 1 is a perspective view illustrating a High-Density Sequence Detection System according to the principles of the present invention;

[0015] Figure 2 is a top perspective view illustrating an upright configuration of a thermocycler assembly and an excitation and detection assembly;

[0016] Figure 3 is a side view illustrating the thermocycler assembly and excitation and detection assembly of Figure 2;

[0017] Figure 4 is a bottom perspective view illustrating the thermocycler assembly and excitation and detection assembly of Figure 2;

[0018] Figure 5 is a bottom perspective view of Figure 2 detailing excitation assembly;

[0019] Figure 6 is a side perspective view illustrating the thermocycler assembly and excitation and detection assembly of Figure 5;

[0020] Figure 7 is a top perspective view illustrating the thermocycler assembly and excitation and detection assembly of Figure 5;

[0021] Figure 8 is a top perspective view detailing the excitation assembly of Figure 7;

[0022] Figure 9 is a top perspective view illustrating an alternate embodiment of the microplate;

[0023] Figure 10 is a top perspective view illustrating an embodiment of the microplate;

[0024] Figure 11 is a top perspective view detailing a portion of the microplate;

[0025] Figure 12 is a top perspective view detailing a portion of an alternative embodiment of the microplate;

[0026] Figure 13 is a top view of an embodiment of the microplate;

[0027] Figure 14 is a top view detail of a corner of the microplate shown in Figure 13;

[0028] Figure 15 is a cross-section of Figure 14;

[0029] Figure 16 is a top view of an alternate embodiment view detail corner shown in Figure 14;

[0030] Figure 17 is a cross-section of Figure 16;

[0031] Figure 18 is a cross-section detail illustrating a well of the microplate;

[0032] Figure 19 is a cross-section detailing an alternate embodiment of an individual well of the microplate;

[0033] Figure 20 is a cross-sectional view illustrating an embodiment employing an inflatable transparent bag;

[0034] Figure 21 is a cross-sectional view of an embodiment employing a moveable transparent window;

[0035] Figure 22 is a cross-sectional view illustrating an embodiment employing an inverted microplate;

[0036] Figure 23 is a cross-sectional view illustrating an embodiment employing a plurality of apertures;

[0037] Figure 24 is a cross-sectional view illustrating an embodiment employing a pressure chamber;

[0038] Figure 25 is a cross-sectional view of an embodiment employing a pressure chamber and an inverted microplate;

[0039] Figure 26 is a cross-sectional view of an embodiment employing a pressure chamber and a microplate having a plurality of apertures;

[0040] Figure 27 is a cross-sectional view illustrating an embodiment with an alternate pressure chamber;

[0041] Figure 27A is a cross-sectional view of an embodiment employing a vacuum assisted holder for the microplate;

[0042] Figure 28 is a cross-sectional view illustrating an embodiment with a second alternative pressure chamber;

[0043] Figure 29 is a cross-sectional view illustrating an embodiment employing a relieving port;

[0044] Figure 30 is a cross-sectional breakout illustrating an embodiment employing a heatable transparent window;

[0045] Figure 31 is a graph illustrating vignetting and shadowing;

[0046] Figure 32 is a graph illustrating vignetting and shadowing as well as illumination;

[0047] Figure 33 is a block diagram illustrating a light source and a lens;

[0048] Figure 34 is a block diagram illustrating a light source with a mirror;

[0049] Figure 35 is a block diagram illustrating a light source with both a mirror and a lens;

[0050] Figure 36 is a block diagram illustrating multiple light sources focused to a point on the object;

[0051] Figure 37 is a block diagram showing multiple light sources in a desired irradiance profile;

[0052] Figure 38 is a top view of an embodiment of the microplate employing a recessed area;

[0053] Figure 39 is a side view of Figure 38;

[0054] Figure 40 is a bottom view of Figure 38;

[0055] Figure 41 is a cross sectional view of Figure 38;

[0056] Figure 42 is a cross-sectional view of a detail of an alternate embodiment of a well employing a blind hole;

[0057] Figure 43 is a cross-sectional view of a detail of a well employing an alternate embodiment of a blind hole;

[0058] Figure 44 is a cross-sectional view of a portion of a microplate employing a through hole, a foil seal and a clear seal;

[0059] Figure 45 is a cross-sectional view of a hot roller apparatus that may be used to seal a cover to a microplate;

[0060] Figure 46 is a cross-sectional view of a cover material illustrating a different layer of the material;

[0061] Figure 47 is an alternate embodiment of the excitation assembly;

[0062] Figure 48 is an exploded view of a manual filler;

[0063] Figure 49 is a cross sectional perspective view of a manual filler;

[0064] Figure 50 is a cross sectional view of a manual filler;

[0065] Figure 51 is a top view of microfluidic channels;

[0066] Figure 52 is an enlargement of a section of microfluidic channels;

[0067] Figure 53 is an alternative top view of an alternate embodiment of microfluidic channels;

[0068] Figure 54 is a flow chart illustrating a manufacturing procedure of preloaded microplates; and

[0069] Figure 55 is a flow chart of a database of the invention.

DETAILED DESCRIPTION OF THE INVENTION

[0070] The present invention provides methods and apparatus for PCR analysis. The following definitions and non-limiting guidelines must be considered in reviewing the description of this invention set forth herein.

[0071] The headings (such as "Introduction" and "Summary,") and sub-headings used herein are intended only for general organization of topics within the disclosure of the invention, and are not intended to limit the disclosure of the invention or any aspect thereof. In particular, subject matter disclosed in the "Introduction" may include aspects of technology within the scope of the invention, and may not constitute a recitation of prior art. Subject matter disclosed in the "Summary" is not an exhaustive or complete disclosure of the entire scope of the invention or any embodiments thereof.

[0072] The citation of references herein does not constitute an admission that those references are prior art or have any relevance to the patentability of the invention disclosed herein. Any discussion of the content of references cited in the Introduction is intended merely to provide a general summary of assertions made by the authors of the references, and does not constitute an admission as to the accuracy of the content of such references. All references cited in the Description section of this specification are hereby incorporated by reference in their entirety.

[0073] The description and specific examples, while indicating embodiments of the invention, are intended for purposes of illustration only and are not intended to limit the scope of the invention. Moreover, recitation of multiple embodiments having stated features is not intended to exclude other embodiments having additional features, or other embodiments incorporating different combinations of the stated features. Specific Examples are provided for illustrative purposes of how to make, use and practice the compositions and methods of this invention and, unless explicitly stated otherwise, are not intended to be a representation that given embodiments of this invention have, or have not, been made or tested.

[0074] As used herein, the words "preferred" and "preferably" refer to embodiments of the invention that afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more

preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the invention.

[0075] As used herein, the word "include," and its variants, is intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that may also be useful in the materials, compositions, devices, and methods of this invention.

[0076] The following description of the embodiments is merely exemplary in nature and is in no way intended to limit the invention, its application, or uses. For example, the present invention may find utility in a wide variety of applications, such as in connection with Polymerase Chain Reaction (PCR) measurements; ELISA tests; DNA and RNA hybridizations; antibody titer determinations; protein, peptide, and immuno tests; recombinant DNA techniques; hormone and receptor binding tests; and the like. Additionally, the present invention is particularly well suited for use with luminescence, colorimetric, chemilumescence, or radioactivity measurement such as scintillation measurements. Although the present invention will be discussed as it relates to Polymerase Chain Reaction measurements, such enabling discussion should not be regarded as limiting the present invention to only such applications.

[0077] The analysis of the function of the estimated 30,000 human genes is a major focus of basic and applied pharmaceutical research, toward the end of developing diagnostics, medicines and therapies for wide variety of disorders. For example, through understanding of genetic differences between normal and diseased individuals, differences in the biochemical makeup and function of cells and tissues can be determined and appropriate therapeutic interventions identified. However, the complexity of the human genome and the interrelated functions of many genes make the task exceedingly difficult, and require the development of new analytical and diagnostic tools.

[0078] A variety of tools and techniques have already been developed to detect and investigate the structure and function of individual genes and the proteins they express. Such tools include polynucleotide probes, which comprise relatively short, defined sequences of nucleic acids,

typically labeled with a radioactive or fluorescent moiety to facilitate detection. Probes may be used in a variety of ways to detect the presence of a polynucleotide sequence, to which the probe binds, in a mixture of genetic material. Nucleic acid sequence analysis is also an important tool in investigating the function of individual genes. Several methods for replicating, or "amplifying," polynucleic acids are known in the art, notably including polymerase chain reaction (PCR). Indeed, PCR has become a major research tool, with applications including cloning, analysis of genetic expression, DNA sequencing, and genetic mapping.

[0079] In general, the purpose of a polymerase chain reaction is to manufacture a large volume of DNA which is identical to an initially supplied small volume of "target" or "seed" DNA. The reaction involves copying the strands of the DNA and then using the copies to generate other copies in subsequent cycles. Each cycle will double the amount of DNA present thereby resulting in a geometric progression in the volume of copies of the target DNA strands present in the reaction mixture.

[0080] A typical PCR temperature cycle requires that the reaction mixture be held accurately at each incubation temperature for a prescribed time and that the identical cycle or a similar cycle be repeated many times. For example, a PCR program may start at a sample temperature of 95° C held for 30 seconds to denature the reaction mixture. Then, the temperature of the reaction mixture is lowered to 60° C and held for one minute to permit primer hybridization. This completes one cycle. The next PCR cycle then starts by raising the temperature of the reaction mixture to 95° C again for strand separation of the extension products formed in the previous cycle (denaturation). Typically, the cycle is repeated 35 to 40 times.

[0081] A variety of devices are commercially available for the analysis of materials using PCR. In order to monitor the expression of a large number of genes, high throughput assays have been developed comprising a large number of PCR reaction chambers (or wells) on a microtiter tray, microplate, a spotted reaction plate, or similar substrate. A typical microtiter tray contains 96 or 384 wells on a plate having dimensions of about 127 by 85 mm. The present invention provides a plate which may be used in PCR

applications or the plate may be used as a microtiter plate for non-PCR assays.

[0082] In many situations it would be desirable to determine the gene expression profile test all genes in an organism. Such a test would also be useful to screen DNA or RNA from a single individual for sequence variants associated with different mutations in the same or different genes (e.g., single nucleotide polymorphisms, or "SNPs"), or for sequence variants that serve as "markers" for the inheritance of different chromosomal segments from a parent. Such tests would be also useful, for example, to predict susceptibility to disease, to determine whether an individual is a carrier of a genetic mutation, to determine whether an individual may be susceptible to adverse reactions or resistance to certain drugs, or for other diagnostic, therapeutic or research purposes.

[0083] The present invention provides methods which comprise the amplification of polynucleotides. As referred to herein, "polynucleotide" refers to naturally occurring polynucleotides (e.g., DNA or RNA), and analogs thereof, of any length. As referred to herein, the term "amplification" and variants thereof, refer to any process of replicating a "target" polynucleotide (also referred to as a "template") so as to produce multiple polynucleotides (herein, "amplicons") that are identical or essentially identical to the target in a sample, thereby effectively increasing the concentration of the target in the sample. In embodiments of this invention, amplification of either or both strands of a target polynucleotide comprises the use of one or more nucleic acid-modifying enzymes, such as a DNA polymerase, a ligase, an RNA polymerase, or an RNA-dependent reverse transcriptase. Amplification methods among those useful herein include methods of nucleic acid amplification known in the art, such as Polymerase Chain Reaction (PCR), Ligation Chain Reaction (LCR), Nucleic Acid Sequence Based Amplification (NASBA), self-sustained sequence replication (3SR), strand displacement activation (SDA), Q (3 replicase) system, and combinations thereof. The LCR is described in the literature, for example, by U. Landegren, et al., "A Ligase-mediated Gene Detection Technique", Science 241, 1077-1080 (1988). Similarly, NASBA is as described, for example, by J. Cuatelli, et al., "Isothermal in Vitro Amplification of Nucleic Acids by a Multienzyme Reaction

Modeled After Retroviral Replication", Proc. Natl. Acad. Sci. USA 87, 1874-1878 (1990).

[0084] Methods and apparatus for the use of microplates used in research and diagnostic techniques are described herein. For real time polymerase chain reaction ("PCR") measurements, wells containing assay/sample mixtures need to be tightly sealed to prevent water evaporation during thermocycling. In general, the purpose of PCR is to manufacture a large quantity of DNA which is identical to an initially supplied small quantity of target or seed DNA. The reaction involves copying the strands of the DNA and then using the copies to generate other copies in subsequent cycles. Each cycle will double the amount of DNA present thereby resulting in a geometric progression in the quantity of copies of the target DNA strands present in the reaction mixture.

[0085] In general, PCR methods comprise the use of at least two primers, a forward primer and a reverse primer, which hybridize to a double-stranded target polynucleotide sequence to be amplified. As referred to herein, a "primer" is a naturally occurring or synthetically produced polynucleotide capable of annealing to a complementary template nucleic acid and serving as a point of initiation for target-directed nucleic acid synthesis, such as PCR or other amplification reaction. Primers may be wholly composed of the standard gene-encoding nucleobases (e.g., C, A, G, T, and U) or, alternatively, they may include modified nucleobases which form base-pairs with the standard nucleobases and are extendible by polymerases. Modified nucleobases useful herein include 7-deazaguanine and 7-deazaadenine. The primers may include one or more modified interlinkages, such as one or more phosphorothioate or phosphorodithioate interlinkages. In one embodiment, all of the primers used in the amplification methods of this invention are DNA oligonucleotides

[0086] As used herein, the term "polymerase chain reaction" ("PCR") refers to the method described in U.S. Pat. Nos. 4,683,195, 4,889,818, and 4,683,202, all of which are hereby incorporated by reference. These patents describe methods for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of

introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase (e.g., Taq). The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (i.e., denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the polymerase chain reaction or PCR. Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified."

[0087] As used herein, the term "target," when used in reference to the polymerase chain reaction, refers to the region of nucleic acid of interest bounded by the primers. In PCR, this is the region amplified and/or identified. Thus, the target is sought to be isolated from other nucleic acid sequences. The terms "target sequence" and "target polynucleotide" mean a polynucleotide sequence that is the subject of hybridization with a complementary polynucleotide, e.g., a primer or probe. The sequence can be composed of DNA, RNA, an analog thereof, including combinations thereof.

[0088] The term "amplicon" means a polynucleotide sequence amplified within a target sequence, and defined by the distal ends of two primer-binding sites. A "segment" is defined as a region of nucleic acid within the target sequence. As used herein, the terms "PCR product" and "PCR fragment" and "amplicon" refer to the resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension

are complete. These terms encompass the case where there has been amplification of one or more segments of one or more target sequences.

[0089] The principles of PCR are well-known in the art, such as are described in the following references: U.S. Patent 4,683,195, Mullis et al., issued July 28, 1987; U.S. Patent 4,683,202, Mullis, issued July 28, 1987; U.S. Patent 4,800,159, Mullis et al., issued January 24, 1989; U.S. Patent 4,965,188 Mullis et al., issued October 23, 1990; U.S. Patent 5,338,671 Scalice et al., issued August 16, 1994; U.S. Patent 5,340,728 Grosz et al., issued August 23, 1994; U.S. Patent 5,405,774 Abramson et al., issued April 11, 1995; U.S. Patent 5,436,149 Barnes, issued July 25, 1995; U.S. Patent 5,512,462 Cheng, issued April 30, 1996; U.S. Patent 5,561,058, Gelfand et al., issued October 1, 1996; U.S. Patent 5,618,703 Gelfand et al., issued April 8, 1997; U.S. Patent 5,693,517, Gelfand et al., issued December 2, 1997; U.S. Patent 5,876,978, Willey et al., issued March 2, 1999; U.S. Patent 6,037,129 Cole et al., issued March 14, 2000; U.S. Patent 6,087,098, McKiernan et al., issued July 11, 2000; U.S. Patent 6,300,073 Zhao et al., issued October 9, 2001; U.S. Patent 6,406,891 , issued June 18, 2002; U.S. Patent 6,485,917, Yamamoto et al., issued November 26, 2002; U.S. Patent 6,436,677, Gu et al., issued August 20, 2002; Innis et al. In: PCR Protocols a guide to Methods and Applications, Academic Press, San Diego (1990); Schlesser et al. Applied and Environ. Microbiol, 57:553-556 (1991); PCR Technology : Principles and Applications for DNA Amplification (ed. H. A. Erlich, Freeman Press, NY, NY, 1992); Mattila et al., Nucleic Acids Res. 19: 4967 (1991); Eckert et al., PCR Methods and Applications 1,17 (1991), PCR A Practical Approach (eds. McPherson, et al., Oxford University Press, Oxford, 1991); PCR2 A Practical Approach (eds. McPherson, et al., Oxford University Press, Oxford, 1995); PCR Essential Data, J. W. Wiley & Sons, Ed. C.R. Newton, 1995; and PCR Protocols: A Guide to Methods and Applications (Innis, M, Gelfand, D., Sninsky, J. and White, T., eds.), Academic Press, San Diego (1990), McPherson et al. eds., PCR Basics from Background to Bench, Springer-Verlag Telos (2000), Innis, Gelfand and Sninsky, eds., PCR Applications: Protocols for Functional Geonomics, Academic Press, San Diego (1999), Demidor and Broude, eds., DNA Amplification: Current Technologies and Applications, Horizon Bioscience

(2004), Bustin, S.A., eds., A-Z of Quantitative PCR, International University Line (2004), Edwards et al., eds., Real Time PCR: An Essential Guide, Horizon Bioscience (2004).

[0090] In one embodiment, the amplification reaction is conducted under conditions allowing for quantitative and qualitative analysis of one or more polynucleotide targets. Accordingly, embodiments of this invention comprise the use of detection reagents, for detecting the presence of a target amplicon in an amplification reaction mixture. In a one embodiment, the detection reagent comprises a probe or system of probes having physical (e.g., fluorescent) or chemical properties that change upon hybridization of the probe to a nucleic acid target.

[0091] A primer need not reflect the exact sequence of the target but must be sufficiently complementary to hybridize with the target. The primer is substantially complementary to a strand of the specific target sequence to be amplified. As referred to herein, a "substantially complementary" primer is one that is sufficiently complementary to hybridize with its respective strand of the target to form the desired hybridized product under the temperature and other conditions employed in the amplification reaction. Noncomplementary bases may be incorporated in the primer as long as they do not interfere with hybridization and formation of extension products. In one embodiment, the primers have exact complementarity. In another embodiment, a primer comprises regions of mis-match or non-complementarity with its intended target. As a specific example, a region of noncomplementarity maybe included at the 5'-end of a primers, with the remainder of the primer sequence being completely complementary to its target polynucleotide sequence. As another example, non-complementary bases or longer regions of non-complementarity are interspersed throughout the primer, provided that the primer has sufficient complementarity to hybridize to the target polynucleotide sequence under the temperatures and other reaction conditions used for the amplification reaction.

[0092] In one embodiment, the primer comprises a double-stranded, labeled nucleic acid region adjacent to a single-stranded region. The single-stranded region comprises a nucleic acid sequence which is capable of hybridizing to the template strand. The double-stranded region, or tail, of the

primer can be labeled with a detectable moiety which is capable of producing a detectable signal or which is useful in capturing or immobilizing the amplicon product. In one embodiment, the primer is a single-stranded oligodeoxyribonucleotide. In certain embodiments, a primer will include a free hydroxyl group at the 3' end.

[0093] The primer is of sufficient length to prime the synthesis of extension products in the presence of the polymerization agent, depending on such factors as the use contemplated, the complexity of the target sequence, reaction temperature and the source of the primer. Generally, each primer used in this invention will have from about 12 to about 40 nucleotides, from about 15 to about 40, and from about 20 to about 40 nucleotides, from about 20 to about 35 nucleotides. In one embodiment, the primer comprises from about 20 to about 25 nucleotides. In embodiments for use with PNA and DNA herodynes, each primer may be in the order of 8 nucleotides. Short primer molecules generally require lower temperatures to form sufficiently stable hybrid complexes with the template.

[0094] In certain embodiments, the amplification primers are designed to have a melting temperature ("Tm") in the range of about 60-75° C. Melting temperatures in this range will tend to insure that the primers remain annealed or hybridized to the target polynucleotide at the initiation of primer extension. The actual temperature used for the primer extension reaction may depend upon, among other factors, the concentration of the primers which are used in the multiplex assays. For amplifications carried out with a thermostable polymerase such as Taq DNA polymerase, the amplification primers can be designed to have a Tm in the range of from about 60 to about 78° C. In one embodiment, the melting temperatures of different amplification primers used in the same amplification reaction are different. In an embodiment, the melting temperatures of the different amplification primers are approximately the same.

[0095] As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acid strands. Hybridization and the strength of hybridization (i.e., the strength of the association between nucleic acid strands) is impacted by many factors well known in the art including the degree of complementarity between the nucleic acids, stringency of the

conditions involved affected by such conditions as the concentration of salts, the T_m (melting temperature) of the formed hybrid, the presence of other components (e.g., the presence or absence of polyethylene glycol), the molarity of the hybridizing strands and the G:C content of the nucleic acid strands. PCR requires repetitive template denaturation and primer annealing. These hybridization transitions are temperature-dependent. The temperature cycles of PCR that drive amplification alternately denature accumulating product at a high temperature and anneal primers to the product at a lower temperature. The transition temperatures of product denaturation and primer annealing depend primarily on GC content and length. If a probe is designed to hybridize internally to the PCR product, the melting temperature of the probe also depends on GC content, length, and degree of complementarity to the target. Fluorescence probes compatible with PCR can monitor hybridization during amplification.

[0096] In some embodiments, primers are used in pairs of forward and reverse primers, referred to herein as a "primer pair." The amplification primer pairs may be sequence-specific and may be designed to hybridize to sequences that flank a sequence of interest to be amplified. In one embodiment, primer pairs comprise a set of primers including a 5' upstream primer that binds with the 5' end of the target sequence to be amplified and a 3', downstream primer that binds with the complement of the 3' end of the target sequence to be amplified. Methods useful herein for designing primer pairs suitable for amplifying specific sequences of interest include methods that are well-known in the art. Such methods include those described in e.g. http://www.ucl.ac.uk/wibr/2/services/ reldocs/taqmanpr.pdf; http://www.ukl.uni-freiburg.de/core-facility/taqman/taqindex.html; http://www.operon.com/oligos/toolkit.php; http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi; http://www.ncbi.n/m.nih.gov/BLAST/; http://bioinfo.math.rpi.edufmfold/dna/form1.cgi; and http://www.biotech.uiuc.edu/primer.htm.

[0097] In PCR, a double-stranded target DNA polynucleotide which includes the sequence to be amplified is incubated in the presence of a primer pair, a DNA polymerase and a mixture of 2'-deoxyribonucleotide triphosphates ("dNTPs") suitable for DNA synthesis. A variety of different

DNA polymerases are useful in the methods of this invention. In one embodiment, the polymerase is a thermostable polymerase. Suitable thermostable polymerases include Taq and Tth polymerases, commercially available from Applied Biosystems, Inc., Foster City, California, U.S.A.

[0098] To begin the amplification, the double-stranded target DNA polynucleotide is denatured and one primer is annealed to each strand of the denatured target. The primers anneal to the target DNA polynucleotide at sites removed from one another and in orientations such that the extension product of one primer, when separated from its complement, can hybridize to the other primer. Once a given primer binds to the target DNA polynucleotide sequence, the primer is extended by the action of the DNA polymerase. The extension product is then denatured from the target sequence, and the process is repeated.

[0099] In successive cycles of this process, the extension products produced in earlier cycles serve as templates for subsequent DNA synthesis. Beginning in the second cycle, the product of the amplification begins to accumulate at a logarithmic rate. The final amplification product, or amplicon, is a discrete double-stranded DNA molecule consisting of: (i) a first strand which includes the sequence of the first primer, which is followed by the sequence of interest, which is followed by a sequence complementary to that of the second primer and (ii) a second strand which is complementary to the first strand.

[00100] In various embodiments, a polymerase used in the methods described herein can be a DNA polymerase. A DNA polymerase used herein can be a thermostable DNA polymerase, such as, for example, a *Taq* polymerase. Furthermore, a DNA polymerase can have, in some embodiments, 5' exonuclease activity. As used herein, the term "polymerase" refers to an enzyme that synthesizes nucleic acid strands (e.g., RNA or DNA) from ribonucleoside triphosphates to deoxyribonucleoside triphosphates. The term "*Taq* polymerase" or sometimes known as just "*Taq*" refers to the native form of the *Taq* polymerase from the bacterium *Thermus aquaticus* and a cloned version that is expressed in *E. coli* or any other recombinant and/or modified forms. *Taq* Polymerase contains a polymerization dependent 5'-3' exonuclease activity. The recombinant *Taq* Polymerase expressed in *E. coli*

shows identical characteristics to native *Taq* from *Thermus aquaticus* with respect to activity, specificity, thermostability and performance in PCR. *Taq* Polymerase is available commercially from many sources including but not limited to: Applied Biosystems, Foster City CA; Invitrogen, Carlsbad, CA; Roche Molecular Systems, Inc., Pleasanton, CA; Qiagen, Inc. Valencia, CA; and Promega, Madison, WI. Some examples of suitable thermostable DNA polymerases include Taq™ (Applied Biosystems, Foster City CA), Vent™ (New England Biolabs, Beverly Mass.), Deep Vent™ (New England Biolabs, Beverly Mass.), Pyrococcus furiosus DNA polymerase (Stratagene, La Jolla Calif.), Thermotaga maritima DNA polymerase, and AmpliTaq DNA polymerase, FS™ polymerase, and Ampli DNA polymerase, Taq FS DNA polymerase (Applied Biosystems, Foster City CA).

[00101] In embodiments for amplifying an RNA target, RT-PCR a single-stranded RNA target which includes the sequence to be amplified (e.g., an mRNA) is incubated in the presence of a reverse transcriptase, two amplification primers, a DNA polymerase and a mixture of dNTPs suitable for DNA synthesis. One of the amplification primers anneals to the RNA target and is extended by the action of the reverse transcriptase, yielding an RNA/cDNA doubled-stranded hybrid. This hybrid is then denatured, and the other primer anneals to the denatured cDNA strand. Once hybridized, the primer is extended by the action of the DNA polymerase, yielding a double-stranded cDNA, which then serves as the double-stranded template or target for further amplification through conventional PCR, as described above. Following reverse transcription, the RNA can remain in the reaction mixture during subsequent PCR amplification, or it can be optionally degraded by well-known methods prior to subsequent PCR amplification. RT-PCR amplification reactions may be carried out with a variety of different reverse transcriptases, and in some embodiments, thermostable reverse-transcriptions is used. Suitable thermostable reverse transcriptases include, but are not limited to, reverse transcriptases such as AMV reverse transcriptase, MuLV, and Tth reverse transcriptase.

[00102] Temperatures suitable for carrying out the various denaturation, annealing and primer extension reactions with the polymerases and reverse transcriptases include those well-known in the art. Optional

reagents commonly employed in conventional PCR and RT-PCR amplification reactions, such as reagents designed to enhance PCR, modify Tm, or reduce primer-dimer formation, may also be employed in the multiplex amplification reactions. Such reagents are described, for example, in U.S. Patent 6,410,231, Arnold et al., issued June 25, 2002; U.S. Patent 6,482,588, Van Doorn et al., issued November 19, 2002; U.S. Patent 6,485,903, Mayrand, issued November 26, 2002; and U.S. Patent 6,485,944, Church et al., issued November 26, 2002. In various aspects, primer sequences can be selected using well known principles for predicting melting temperature (see, e.g., Owczarzy et al., *Biopolymers, 44*:217-239 (1998)). In certain embodiments, the multiplex amplifications may be carried out with commercially-available amplification reagents, such as, for example, AmpliTaq® Gold PCR Master Mix, TaqMan® Universal Master Mix and TaqMan® Universal Master Mix No AmpErase® UNG, all of which are available commercially from Applied Biosystems (Foster City, California, U.S.A.).

[00103] In one embodiment, the amplification reaction is conducted under conditions allowing for quantitative and qualitative analysis of one or more polynucleotide targets. Accordingly, embodiments of this invention comprise the use of detection reagents, for detecting the presence of a target amplicon in an amplification reaction mixture. In a one embodiment, the detection reagent comprises a probe or system of probes having physical (e.g., fluorescent) or chemical properties that change upon hybridization of the probe to a nucleic acid target. As used herein, the term "probe" refers to a polynucleotide of any suitable length which allows specific hybridization to a polynucleotide, e.g., a target or amplicon.

[00104] Oligonucleotide probes may be DNA, RNA, PNA, LNA or chimeras comprising one or more combinations thereof. The oligonucleotides may comprise standard or non-standard nucleobases or combinations thereof, and may include one or more modified interlinkages. The oligonucleotide probes may be suitable for a variety of purposes, such as, for example to monitor the amount of an amplicon produced, to detect single nucleotide polymorphisms, or other applications as are well-known in the art. Probes may be attached to a label or reporter molecule. Any suitable method

for labeling nucleic acid sequences can be used, e.g., fluorescent labeling, biotin labeling or enzyme labeling.

[00105] In one embodiment, an oligonucleotide probe is complementary to at least a region of a specified amplicon. The probe can be completely complementary to the region of the specified amplicons, or may be substantially complementary thereto. In some embodiments, the probe is at least about 65% complementary over a stretch of at least about 15 to about 75 nucleotides. In other embodiments, the probes are at least about 75%, 85%, 90%, or 95% complementary to the regions of the amplicons. Such probes are disclosed, for example, in Kanehisa, M., Nucleic Acids Res. 12: 203 (1984), The exact degree of complementarity between a specified oligonucleotide probe and amplicon will depend upon the desired application for the probe and will be apparent to those of skill in the art.

[00106] The length of a probes can vary broadly, and in some embodiments can range from a few as two as many as tens or hundreds of nucleotides, depending upon the particular application for which the probe was designed. In one embodiment, the probe ranges in length from about 15 to about 35 nucleotides. In another embodiment, the oligonucleotide probe ranges in length from about 15 to about 25 nucleotides. In another embodiment, the probe is a "tailed" oligonucleotide probe ranging in length from about 25 to about 75 nucleotides.

[00107] Accordingly, in some embodiments, detection of amplification can comprise detection of the binding of a detection probe to a detection probe sequence, as discussed below. As used herein, "detecting amplification" and "detection of amplification" can refer to detecting the quantity of an amplification product, or detecting accumulation of a product of an enzyme-catalyzed reaction which is coupled with a polymerase chain reaction. Such a product can be, for example, an unquenched fluorophore which accumulates as a result of 5'-nuclease activity of a DNA polymerase which hydrolyzes a probe comprising a fluorophore, a nucleotide sequence and a fluorescence quencher, such as, for example, a TaqMan® probe, during a polymerase chain reaction. In some configurations, detection of amplification can comprise detecting accumulation of an unquenched fluorophore, such as, for example, a fluorophore comprised by probe which is

initially quenched prior to binding, but becomes unquenched upon binding to a detection probe sequence. Such a fluorogenic probe can be, in non-limiting example, a Molecular Beacon probe.

[00108] In certain configurations, a detection probe can further comprise a label. A label can be any moiety which facilitates detection of the detection probe. In non-limiting example, a label can be a fluorophore, a hapten such as a biotin or a digoxygenin, a radioisotope, an enzyme or an electrophoretic mobility modifier.

[0100] In certain embodiments of quantitative or real-time amplification assays useful herein, total RNA from a sample is amplified by RT-PCR in the presence of amplification primers suitable for specifically amplifying a specified gene sequence of interest and an oligonucleotide probe labeled with a labeling system that permits monitoring of the quantity of amplicon that accumulates in the amplification reaction in real-time. The cycle threshold values (Ct values) obtained in such quantitative RT-PCR amplification reactions can be correlated with the number of gene copies present in the original total mRNA sample. Other quantitative methods are known such as a curve fit, standard curves and the like or any other such method known in the art may be used with the present invention. For example, U.S. Patent No. 5,766,889 to Atwood issued June 1998. Such quantitative or real-time RT-PCR reactions, as well as different types of labeled oligonucleotide probes useful for monitoring the amplification in real time, are well-known in the art. Oligonucleotide probes suitable for monitoring the amount of amplicon(s) produced as a function of time, include the 5'-exonuclease assay (TaqMan®) probes; various stem-loop molecular beacons; stemless or linear beacons; peptide nucleic acid (PNA) molecular beacons; linear PNA beacons; non-FRET probes; sunrise primers; scorpion probes; cyclicons; PNA light-up probes; self-assembled nanoparticle probes, and ferrocene-modified probes. Such probes are described, for example, in U.S. Patent 6,103,476, Tyagi et al., issued August 15, 2000; U.S. Patent 5,925,517, Tyagi et al., issued July 20, 1999; Tyagi & Kramer, 1996, Nature Biotechnology 14:303-308; PCT Publication WO 99/21881, Gildea et al., published May 6, 1999; U.S. Patent 6,355,421, Coull et al., issued March 12, 2002; Kubista et al,, 2001, SPIE 4264:53-58; U.S. Patent 6,150,097, Tyagi et

al., issued November 21, 2000; U.S. Patent 6,485,901, Gildea et al., issued November 26, 2002; Mhlanga, et al., (2001) Methods. 25:463-471; Whitcombe et al. (1999) Nat Biotechnol. 17:804-807; Isacsson et al. (2000) Mol Cell Probes. 14: 321-328: Svanvik et al (2000) Anal Biochent 281:26-35; Wolff et. al. (2001) Biotechniques 766:769-771; Tsourkas et al (2002) Nucleic Acids Res. 30:4208-4215; Riccelli, et al. (2002) Nucleic Acids Res. 30:4088-4093; Zhang et al. (2002) Shanghai 34:329-332; Maxwell et al. (2002) J. Am Chem Soc. 124:9606-9612; Eroude et al. (2002) Trends Biotechnol 20:249-56; Huang et al. (2002) Chem Res Toxicol. 15:118-126; and Yn et al. (2001) J. Am. Chem. Soc. l4: 11155-11161.

[0101] In another embodiment, the oligonucleotide probes are suitable for detecting single nucleotide polymorphisms, as is well-known in the art. A specific example of such probes includes a set of four oligonucleotide probes which are identical in sequence save for one nucleotide position. Each of the four probes includes a different nucleotide (A, G, C and T/U) at this position. The probes may be labeled with labels capable of producing different detectable signals that are distinguishable from one another, such as different fluorophores capable of emitting light at different, spectrally-resolvable wavelengths (e.g., 4-differently colored fluorophores). In some embodiments, for example SNP analysis, two colors are used for two known variants. Such labeled probes are known in the art and described, for example, in U.S. Patent 6,140,054, Wittwer et al., issued October 31, 2000; and Saiki et al., 1986, Nature 324:163-166.

[0102] One embodiment, which utilizes the 5'-exonuclease assay to monitor the amplification as a function of time is referred to as the 5'-exonuclease gene quantification assay. Such assays are disclosed, for example, in U.S. Patent 5,210,015, Gelfand et al., issued May 11, 1993; U.S. Patent 5,538,848, Livak et al., issued July 23, 1996; and Lie & Petropoulos, 1998, Curr. Opin. Biotechnol. 14:303-308).

[0103] In various embodiments, the level of amplification can be determined using a fluorescently labeled oligonucleotide, such as disclosed in Lee, L.G., et al. Nucl. Acids Res. 21:3761 (1993), and Livak, K.J., et al. PCR Methods and Applications 4:357 (1995). In such embodiments, the detection reagents include a sequence-selective primer pair as in the more general

PCR method above, and in addition, a sequence-selective oligonucleotide (FQ-oligo) containing a fluorescer-quencher pair. The primers in the primer pair are complementary to 3'-regions in opposing strands of the target segment which flank the region which is to be amplified. The FQ-oligo is selected to be capable of hybridizing selectively to the analyte segment in a region downstream of one of the primers and is located within the region to be amplified.

[0104] The fluorescer-quencher pair includes a fluorescer dye and a quencher dye that are spaced from each other on the oligonucleotide so that the quencher dye is able to significantly quench light emitted by the fluorescer at a selected wavelength, while the quencher and fluorescer are both bound to the oligonucleotide. In certain embodiments, the FQ-oligo includes a 3'-phosphate or other blocking group to prevent terminal extension of the 3'-end of the oligo. The fluorescer and quencher dyes are selected from any dye combination having the proper overlap of emission (for the fluorescer) and absorptive (for the quencher) wavelengths while also permitting enzymatic cleavage of the FQ-oligo by the polymerase when the oligo is hybridized to the target. Suitable dyes, such as rhodamine and fluorescein derivatives, and methods of attaching them, are well known and are described, for example, in, U.S. Patent 5,188,934, Menchen, et al., issued February 23, 1993, 1993; PCT Publication WO 94/05688, Menchen, et al., published March 17, 1994;). PCT Publication WO 91/05060, Bergot, et al., published April 18, 1991; and European Patent Publication 233,053, Fung, et al., published August 19, 1987. The fluorescer and quencher dyes are spaced close enough together to ensure adequate quenching of the fluorescer, while also being far enough apart to ensure that the polymerase is able to cleave the FQ-oligo at a site between the fluorescer and quencher. Generally, spacing of about 5 to about 30 bases is suitable, as described in Livak, K.J., et al. PCR Methods and Applications 4:357 (1995). In one embodiment, the fluorescer in the FQ-oligo is covalently linked to a nucleotide base which is 5' with respect to the quencher. In some embodiments, the fluorescence observed from these probes primarily depends on hydrolysis of the probe between its two fluorophores. The amount of PCR product is estimated by acquiring fluorescence once each cycle. Although hybridization of these probes appears

necessary for hydrolysis to occur, the fluorescence signal primarily results from hydrolysis of the probes, not hybridization, wherein an oligonucleotide probe with fluorescent dyes at opposite ends thereof provides a quenched probe system useful for detecting PCR product and nucleic acid hybridization, K. J. Livak et al., 4 PCR Meth. Appl. 357-362 (1995).

[0105] In various embodiments, the primer pair and FQ-oligo are reacted with a target polynucleotide (double-stranded for this example) under conditions effective to allow sequence-selective hybridization to the appropriate complementary regions in the target. The primers are effective to initiate extension of the primers via DNA polymerase activity. When the polymerase encounters the FQ-probe downstream of the corresponding primer, the polymerase cleaves the FQ-probe so that the fluorescer is no longer held in proximity to the quencher. The fluorescence signal from the released fluorescer therefore increases, indicating that the target sequence is present. In a further embodiment, the detection reagents may include two or more FQ-oligos having distinguishable fluorescer dyes attached, and which are complementary for different-sequence regions which may be present in the amplified region, e.g., due to heterozygosity. See, for example, Lee, L.G., et al. Nucl. Acids Res. 21:3761 (1993).

[0106] In various embodiments, a detection method can utilize any probe which can detect a nucleic acid sequence quantifiably. In some configurations, a detection probe can be, for example, a 5'-exonuclease assay probe such as a TaqMan® probes described herein, various stem-loop molecular beacons, a stemless or linear beacon, a PNA Molecular Beacon™, a linear PNA beacon, a non-FRET probe such as a Sunrise ®/Amplifluor ® probe, a stem-loop and duplex Scorpion ™ probe, a bulge loop probe, a pseudo knot probe, a cyclicon, an MGB Eclipse ™ probe (Epoch Biosciences), a hairpin probe, a peptide nucleic acid (PNA) light-up probe, a self-assembled nanoparticle probe, or a ferrocene-modified probe described, for example, in U.S. Patent No. 6,485,901; Mhlanga et al., *Methods 25*:463-471 (2001); Whitcombe et al., *Nature Biotechnology 17*:804-807 (1999); Isacsson et al., *Molecular Cell Probes 14*:321-328 (2000); Svanvik et al., *Anal Biochem. 281*:26-35 (2000); Wolffs et al., *Biotechniques 766*:769-771 (2001); Tsourkas et al., *Nucleic Acids Research 30*:4208-4215 (2002); Riccelli et al.,

Nucleic Acids Research 30:4088-4093 (2002); Zhang et al., Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai) *(Acta Biochimica et Biophysica Sinica) 34*:329-332 (2002); Maxwell et al., *J. Am. Chem. Soc. 124*:9606-9612 (2002); Broude et al., *Trends Biotechnol. 20*:249-56 (2002); Huang et al., *Chem Res. Toxicol. 15*:118-126 (2002); and Yu et al., *J. Am. Chem. Soc 14*:11155-11161 (2001). Labeling probes can also comprise a quencher such as a black hole quencher (Biosearch), Iowa Black (IDT), QSY quencher (Molecular Probes), and Dabsyl and Dabcel sulfonate/carboxylate Quenchers (Epoch). A detection probe can also comprise a sulfonate derivative of a fluorescent dye, a phosphoramidite form of fluorescein, or a phosphoramidite form of a carbocyanine dye such as CY5. In some embodiments, a probe can comprise an interchelating label such as ethidium bromide, SYBR® Green I, and PicoGreen®.

[0107] In certain configurations, a probe label can be a fluorophore. In a detection probe comprising a fluorophore, the fluorophore can be any fluorophore known to skilled artisans, such as FAM™, VIC®, 6-FAM™, TET, HEX, JOE, NED, LIZ, TAMRA, ROX, ALEXA Fluor, Texas Red®, a carbocyanine dye such as Cy3, Cy5, Cy7 or Cy9, or dR6G. In some configurations, the fluorophore can be FAM™ or VIC®. A fluorophore can be attached to a nucleobase moiety of a probe using chemical synthesis methods well known to skilled artisans.

[0108] In various embodiments, the amplified sequences may be detected in double-stranded form by including an intercalating or crosslinking dye, such as ethidium bromide, acridine orange, or an oxazole derivative, for example, SYBR Green®, which exhibits a fluorescence increase or decrease upon binding to double-stranded nucleic acids. Such methods are described, for example, in Sambrook, J., et al., Molecular Cloning, 2nd Ed., Cold Spring Harbor Laboratory Press, N.Y. (1989); Ausubel, F. M., et al., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., Media, Pa.; Higuchi, R., et al., Bio/Technology 10:413 (1992); Higuchi, R., et al., Bio/Technology 11:1026 (1993); and Ishiguro, T., et al., Anal. Biochem. 229:207 (1995). In a specific embodiment the dye is SYBR® Green I or II, marketed by Molecular Probes (Eugene, Oregon, U.S.A.).

[0109] In various configurations, a detection probe comprising a fluorophore can further comprise a fluorescence quencher. The detection probe, in these embodiments, can be used in a 5' nuclease assay such as a fluorogenic 5' nuclease assay, such as a Taqman® assay, in which the fluorophore or the fluorescence quencher is released from the detection probe if the detection probe is hybridized to the detection probe sequence. In these embodiments, the 5' nuclease assay can utilize 5' nucleolytic activity of a DNA polymerase that catalyzes a PCR amplification of a probe set ligation sequence. The fluorogenic 5' nuclease detection assay can be a real-time PCR assay or an end-point PCR assay. The fluorophore comprised by a detection probe in these embodiments can be any fluorophore that can be tagged to a nucleic acid, such as, for example, FAM™, VIC®, TET, HEX, JOE, NED, LIZ, TAMRA, ROX, ALEXA Fluor, Texas Red®, a carbocyanine dye such as Cy3, Cy5, Cy7 or Cy9, or dR6G.

[0110] In various configurations, a detection probe comprising a fluorophore can further comprise a fluorescence quencher. A fluorescence quencher can be any fluorescence quencher known to skilled artisans, such as a fluorescent fluorescence quencher such as TAMRA, or a non-fluorescent fluorescence quencher such as a combined non-fluorescent quencher-minor groove binder. A detection probe, in these embodiments, can be used in various fluorogenic assays in which a fluorophore comprised by a probe is initially quenched. A fluorogenic assay utilizing a fluorescence quencher, can be, for example, a 5' nuclease assay such as for example, a Taqman® assay which is a homogenous assay. In certain configurations, a fluorogenic detection assay can be a real-time PCR assay or an end-point PCR assay. Using a fluorogenic detection assay, quantitative results can be obtained, for example, with the aid of a fluorimeter, such as a fluorimeter comprised by an integrated nucleic acid analysis system.

[0111] In various configurations, at least one of the forward primer and the reverse primer can further comprise a detection probe sequence. A detection probe sequence (or its complement) can be situated within the forward primer between the first primer sequence and the sequence complementary to the target nucleic acid, or within the reverse primer between the second primer target sequence and the sequence

complementary to the target nucleic acid. A detection probe sequence can comprise at least about 10 nucleotides, at least about 15 nucleotides, at least about 20 nucleotides or at least about 30 nucleotides, up to about 50 nucleotides, up to about 60 nucleotides, or up to about 70 nucleotides. A detection probe sequence (or its complement), can be, in some configurations, a "Zip-Code" sequence (Applied Biosystems, Inc.)

[0112] In certain configurations, a detection probe can comprise an electrophoretic mobility modifier. In these embodiments, A mobility modifier can be a nucleobase polymer sequence which can increase the size of a detection probe, or in some configurations, a mobility modifier can be a non-nucleobase moiety which increases the frictional coefficient of a probe, such as a mobility modifier described in US Patents nos. 5,514,543, 5,580,732, 5,624,800, and 5,470,705 to Grossman. A detection probe comprising a mobility modifier can exhibit a relative mobility in an electrophoretic or chromatographic separation medium that allows a user to identify and distinguish the detection probe from other molecules comprised by a sample. A detection probe comprising a sequence complementary to a detection probe sequence and an electrophoretic mobility modifier can be, for example, a ZipChute™ probe supplied commercially by Applied Biosystems, Inc. In these embodiments, hybridization of a detection probe with an amplification product, followed by electrophoretic analysis, can be used to determine the identity and quantity of a target nucleic acid.

[0113] In certain configurations, a detection probe label can be a radioisotope, such as, for example, a moiety comprising H^3, C^{14}, S^{35}, P^{32}, P^{33} or I^{121}. The radioisotope can be detected using well known methods, such as autoradiography or scintillation counting.

[0114] In certain configurations, a detection probe label can be an enzyme. In some configurations, the enzyme can be any enzyme which can be detected using an enzyme activity assay. An enzyme activity assay can utilize a chromogenic substrate, a fluorogenic substrate or a chemiluminescent substrate. In non-limiting example, the enzyme can be an alkaline phosphatase, and the chemiluminescent substrate can be (4-methoxyspiro [1,2-dioxetane-3,2'(5'-chloro)-tricyclo [3.3.1.13, 7]decan]-4-yl)phenylphosphate. In some configurations, a chemiluminescent alkaline

phosphatase substrate can be CDP-Star® chemiluminescent substrate or CSPD® chemiluminescent substrate.

[0115] In some embodiments, quantitative results can be obtained using a real-time PCR analysis. Real-time PCR analysis can comprise determining a threshold cycle for detection of a fluorophore during thermal cycling of a polymerase chain reaction. Some non-limiting examples of protocols for conducting fluorogenic assays such as TaqMan® assays, including analytical methods for interpreting data, can be found in publications such as, for example, "SNPlex™ Genotyping System 48-plex", Applied Biosystems, 2004; "User Bulletin #2 ABI Prism 7700 Sequence Detection System," Applied Biosystems 2001; "User Bulletin #5 ABI Prism® 7700 Sequence Detection System," Applied Biosystems, 2001; and "Essentials of Real Time PCR," Applied Biosystems, available on the internet at http://home.appliedbiosystems.com/.

[0116] In certain aspects of these embodiments, a detection mixture can be formed which can comprise the preamplification product, a first universal primer which binds to a complement of the first PCR primer target sequence, a second universal primer which binds to a complement of the second PCR primer target sequence, a first detection probe comprising a sequence which binds to the detection probe sequence or a complement thereof, and a second detection probe comprising a sequence which binds to a sequence comprised by the target nucleic acid or a complement thereof. Any preamplification product comprised by the detection mixture can be amplified by a polymerase chain reaction. Detection of amplification of any preamplification product using the first and second detection probes can reveal the presence of the target nucleic acid. In some configurations, the first and second detection probes can comprise different labels, for example, two different fluorophores.

[0117] In some configurations of these embodiments, detection of amplification of any preamplification product can be quantitative detection, as described supra. In these configurations, detection of amplification can comprise quantifying the amplification detected by the first and second probes, and comparing the amounts of amplification revealed by each probe. In some aspects, a quantitative signal from a first detection probe can be

measured in a plurality of samples, and used, for example, as a standard for comparing samples.

[0118] In certain embodiments, the present teachings set forth herein describe methods for detecting a plurality of target nucleic acids. In various configurations, the methods can comprise forming an initial mixture comprising (a) a sample suspected of comprising the plurality of target nucleic acids, (b) a polymerase, (c) a plurality of primer sets, each primer set comprising (i) a forward primer comprising a 5' portion comprising a first primer target sequence and a 3' portion that binds to a target nucleic acid, and (ii) a reverse primer comprising a 5' portion comprising a second primer target sequence and a 3' portion that binds to a complement of the same target nucleic acid, wherein at least one of each forward primer and each reverse primer of a primer set further comprises a detection probe sequence unique for the primer set and wherein the initial mixture is formed under conditions in which a forward primer elongates if hybridized to a target nucleic acid. These methods can further comprise forming a plurality of preamplification products by subjecting the initial mixture to at least one cycle of a polymerase chain reaction, and forming a detection mixture comprising the plurality of preamplification products, a first universal primer which binds to a complement of the first PCR primer target sequence, and a second universal primer which binds to a complement of the second PCR primer target sequence; amplifying any preamplification product comprised by the detection mixture. Each of the plurality of target nucleic acids can be detected by detecting amplification of any preamplification product.

[0119] In various embodiments, the location of a fluorescent signal on a solid support such as a microplate, can be indicative of the identity of a target nucleic acid comprised by a sample. In certain configurations of these embodiments, a plurality of detection probes can be distributed to identify loci of a solid support such as wells of a microplate. In these configurations, a plurality of preamplification products can be contacted with the loci of the solid support. The preamplification products comprised by the loci can be subjected to amplification conditions, in which each locus further comprises a first universal primer and a second universal primer. A signal deriving from a detection probe, such as, for example, an increase in fluorescence intensity of

a fluorophore at a particular locus, can be detected if a preamplification product binds to a detection probe and is then amplified. The location of the locus can indicate the identity of the target nucleic acid, and the intensity of the fluorescence can indicate the quantity of the target nucleic acid. In some configurations, these embodiments can be used for multiplexing, i.e., detection in amplification in a plurality of samples.

[0120] In certain configurations of these embodiments, a detection mixture can be formed which can comprise both the first and the second preamplification products, a first universal primer which binds to a complement of the first primer target sequence, and a second universal primer which binds to a complement of the second primer target sequence. Any preamplification product comprised by the detection mixture can be amplified, and the amplification of any preamplification product can then be detected. In various aspects of these embodiments, a detection mixture can further comprise a first detection probe comprising a sequence which binds to the first detection probe sequence or a complement thereof and a second detection probe comprising a sequence which binds to the second detection probe sequence or a complement thereof. The first and second detection probes can comprise different labels, for example, different fluorophores such as, in non-limiting example, VIC and FAM. Sequences of the first and second detection probes can differ by as little as one nucleotide, two nucleotides, three nucleotides, four nucleotides, or greater, provided that hybridization occurs under conditions which allow each probe to hybridize specifically to its corresponding detection probe sequence.

[0121] In other embodiments, the invention includes preamplication methods that employ in vitro transcription (IVT). IVT is well known by artisans practicing biochemistry, molecular biology, DNA and RNA synthesis, identification, amplification, splicing, or identification, genomics, protenomics, transformation, and the like. In such embodiments, the invention includes amplifying at least one sequence in a collection of nucleic acids sequences, the processes comprising (i) synthesizing a nucleic acid by hybridizing a primer complex to the sequence and extending the primer to form a first strand complementary to the sequence and a second strand complementary to the first strand, wherein the complex comprises a primer complementary to

the sequence and a promoter region in anti-sense orientation with respect to the sequence; and (2) transcribing copies of anti-sense RNA off of the second strand. The promoter region, which may be single or double stranded, is capable of inducing transcription from an operably linked DNA sequence in the presence of ribonucleotides and a RNA polymerase under suitable conditions. Suitable promoter regions are prokaryotes, such as from T3 or T7 bacteriophage. In embodiments of the invention, the primer is a single stranded nucleotide, of sufficient length to act as a template for synthesis of extension products under suitable conditions and maybe poly (T) or a collection of degenerate sequences. In the embodiments, these methods involve the incorporation of an RNA polymerase promoter into selected cDNA molecule by priming cDNA synthesis with a primer complex comprising a synthetic oligonucleotide containing the promoter. Following synthesis of double-stranded cDNA, a polymerase generally specific for the promoter is added, and anti-sense RNA is transcribed from the cDNA template. The processive synthesis of multiple RNA molecules from a single cDNA template results in amplified, anti-sense RNA (aRNA) that serves, inter alia, as starting material for cloning procedures using random primers. The amplification, which will typically be at least about 20-40, typically to 50 to 100 or 250-fold, but may be 500 to 1000-fold or more, can be achieved from nanogram quantities or less of cDNA, and is economical, simple to perform under standard molecular biology laboratory conditions. An examples of these methods maybe found in Van Gelder et al. Proc Natl Acad Sci 87(5): 1663-1667 (1990) and US Patent 5,545,522 to Van Elder et al. issued August 13, 1996, as well as the large number of articles and patents that reference the above mentioned examples. IVT kits are available commercially for a variety of sources such as Applied Biosystems, Foster City CA; Ambion, Inc. Austin TX; Affymetrix, Santa Clara CA; and Incyte Corporation, Wilmington DE.

[0122] Still other embodiments of the invention pertain to methods, reagents, compositions, and diagnostic kits, for use in simultaneously amplifying multiple nucleic acid targets. In particular, a two-step multiplex amplification reaction wherein the first step truncates a standard multiplex amplification round to thereby "boost" the sample copy number by only a 100-1000 or more fold increase in the target. Following the first step, the resulting

product is divided into optimized secondary single amplification reactions, each containing one or more of the primer sets that were used previously in the first or multiplexed booster step. The booster step can occur using an aqueous target nucleic acid or using a solid phase archived nucleic acid. For example see U.S. Patent No. 6,605,451 to Marmaro et al. issued August 12, 2003.

[0123] In various embodiments of the invention, the present invention provides methods, reagents, and kits for carrying out a variety of assays suitable for analyzing polynucleotides or samples that include an amplification step performed in a multiplex fashion. Such embodiments also provide methods for analyzing and improving the efficiency of amplification and for carrying out gene expression analysis or other analysis such as SNP, for example. Since a plurality of different sequences are amplified simultaneously in a single reaction, the multiplex amplifications may be used in a variety of contexts to effectively increase the concentration or quantity of a sample available for downstream analysis and/or assays. In such embodiments, downstream analysis and/or assays include methods such as PCR, RT-PCR and the like. Once the sample has been multiplex amplified, it may be divided into aliquots, with or without prior dilution or concentrate, for subsequent analysis. Owing to its increased concentration or quantity, significantly more analysis or assays can be performed with multiplex amplified samples than could be performed with the original sample. In many embodiments, multiplex amplification even permits the ability to perform assays or analysis that require more sample or a higher concentration of sample than was originally available, for example, after a thousand X multiplex amplification, subsequent assays could then be performed with a thousand X less sample. In such embodiments, multiplex amplification allows the ability to perform downstream analysis for assays that may not have been possible with the original sample due to its limited quantity. For examples of such embodiments, see WO 2004/051218 to Andersen and Ruff published June 17, 2004. In some embodiments, a two stage preamplification method may be used. Such method preamplifies the sample in one vessel by IVT and, for example, this preamplification stage may be 100 X sample. In the second stage, the preamplified product is divided into aliquots and

preamplified by PCR and, for example, this preamplification stage may be 16,000 X sample or more. Although the above preamplification methods may be used in the microplate, these are only examples and are non-limiting. Any preamplification method known in the art, including isothermal methods, may be used with the present invention.

[0124] In various embodiments, the detection reagents include first and second oligonucleotides effective to bind selectively to adjacent, contiguous regions of a target sequence in the selected analyte, and which may be ligated covalently by a ligase enzyme or by chemical means. Such oligonucleotide ligation assays (OLA) are described, for example, in U.S. Patent 4,883,750, Whiteley, et al., issued November 28, 1989; and Landegren, U., et al., Science 241:1077 (1988). In this approach, the two oligonucleotides (oligos) are reacted with the target polynucleotide under conditions effective to ensure specific hybridization of the oligonucleotides to their target sequences. When the oligonucleotides have base-paired with their target sequences, such that confronting end subunits in the oligos are base paired with immediately contiguous bases in the target, the two oligos can be joined by ligation, e.g., by treatment with ligase. After the ligation step, the detection wells are heated to dissociate unligated probes, and the presence of ligated, target-bound probe is detected by reaction with an intercalating dye or by other means. The oligos for OLA may also be designed so as to bring together a fluorescer-quencher pair, as discussed above, leading to a decrease in a fluorescence signal when the analyte sequence is present.

[0125] In the above OLA ligation method, the concentration of a target region from an analyte polynucleotide can be increased, if necessary, by amplification with repeated hybridization and ligation steps. Simple additive amplification can be achieved using the analyte polynucleotide as a target and repeating denaturation, annealing, and ligation steps until a desired concentration of the ligated product is achieved.

[0126] Alternatively, the ligated product formed by hybridization and ligation can be amplified by ligase chain reaction (LCR), according to published methods. See, for example, Winn-Deen, E., et al., Clin. Chem. 37:1522 (1991). In this approach, two sets of sequence-specific oligos are

employed for each target region of a double-stranded nucleic acid. One probe set includes first and second oligonucleotides designed for sequence-specific binding to adjacent, contiguous regions of a target sequence in a first strand in the target. The second pair of oligonucleotides are effective to bind (hybridize) to adjacent, contiguous regions of the target sequence on the opposite strand in the target. With continued cycles of denaturation, reannealing and ligation in the presence of the two complementary oligo sets, the target sequence is amplified exponentially, allowing small amounts of target to be detected and/or amplified. In a further modification, the oligos for OLA or LCR assay bind to adjacent regions in a target polynucleotide which are separated by one or more intervening bases, and ligation is effected by reaction with (i) a DNA polymerase, to fill in the intervening single stranded region with complementary nucleotides, and (ii) a ligase enzyme to covalently link the resultant bound oligonucleotides. See, for example, PCT Publication WO 90/01069, to Segev, issued February 8, 1990, and Segev, D., "Amplification of Nucleic Acid Sequences by the Repair Chain Reaction" in Nonradioactive Labeling and detection of Biomolecules, C. Kessler (Ed.), Springer Laboratory, Germany (1992).

[0127] The term "end-point analysis" refers to a method where data collection occurs only when a reaction is complete. End-point analysis of PCR entails fluorescent dye signal measurement or other measurement when thermal cycling and amplification is complete. Results may be reported in terms of the change in fluorescence, i.e. fluorescence intensity units, of the fluorescent dye signal from start to finish of the PCR thermal cycling, minus any internal control signals.

[0128] The term "real-time analysis" refers to periodic monitoring during PCR. Certain systems such as the ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif.) conduct monitoring during each thermal cycle at a pre-determined, user-defined point, or continuously. Real-time analysis of the 5' nuclease assay measures fluorescent dye signal changes from cycle-to-cycle, minus the change in fluorescence from a passive internal reference. In some embodiments, the passive internal reference employs a ROX dye. In other embodiments, the passive internal reference employs a blue dye, a purple dye, a red dye or any other color of

dye. In various embodiments, the passive internal reference employs a dye that has an emission wavelength that is different than an emission wavelength of any other probe used in the analysis.

[0129] Examples of suitable dyes for use in connection with the present teachings include any of those described in, for example, Menchen, et al., U.S. Patent No. 5,188,934; Benson, et al., U.S. Patent No. 6,020,481; Lee, et al., U.S. Patent No. 5,847,162; Benson, et al., U.S. Patent No. 6,008,379; Benson, et al., U.S. Patent No. 5,936,087; Upadhya, et al., U.S. Patent No. 6,221,604; Lee, et al., U.S. Patent No. 6,191,278; Yan, et al., U.S. Patent No. 6,140,500; Mao, et al., U.S. Patent No. 6,130,101; Glazer, et al., U.S. Patent No. 5,853,992; Brush, et al., U.S. Patent No. 5,986,086; Hamilton, et al., U.S. Patent No. 6,140,494; and Hermann, et al., U.S. Patent No. 5,750,409, each of which is incorporated by reference in its entirety with regard to fluorescent dye structures, fluorescent dye synthesis, fluorescent dye conjugation to biopolymers, application of fluorescent dyes in energy transfer dyes and fluorescent dye spectral properties. Example of suitable dyes for use in connection with the present teachings include, but are not limited to, 5-carboxyfluorescein, 6-carboxyfluorescein, rhodamine green (R110), 5-carboxyrhodamine, 6-carboxyrhodamine, N,N'-diethyl-2',7'-dimethyl-5-carboxy-rhodamine (5-R6G), N,N'-diethyl-2',7'-dimethyl-6-carboxyrhodamine (6-R6G), N,N,N',N'-tetramethyl-5-carboxyrhodamine (5-TAMRA), N,N,N',N'-tetramethyl-5-carboxyrhodamine (6-TAMRA), 5-carboxy-X-rhodamine (5-ROX), 6-carboxy-X-rhodamine (6-ROX), 5-carboxy-2',4',5',7',-4,7-hexachlorofluorescein, 6-carboxy-2',4',5',7',4,7-hexachloro-fluorescein, 5-carboxy-2',7'-dicarboxy-4',5'-dichlorofluorescein, 6-carboxy-2',7'-dicarboxy-4',5'-dichlorofluorescein, 5-carboxy-2',4',5',7'-tetrachlorofluorescein, 1',2'-benzo-4'-fluoro-7',4,7-trichloro-5-carboxyfluorescein, 1',2'-benzo-4'-fluoro-7',4,7-trichloro-6-carboxy-fluorescein, 1',2',7',8'-dibenzo-4,7-dichloro-5-carboxyfluorescein, as well as other commercially available dyes as shown in Table 1.

Table 1

Fluorescent Dye	Absorbance (nm)	Emission (nm)	Extinction Coefficient
5-Fluorescein	495	520	73000
5-Carboxyfluorescein (5-FAM)	495	520	83000
6-Carboxyfluorescein (6-FAM)	495	520	83000
6-Carboxyhexachlorofluorescein (6-HEX)	535	556	73000
6-Carboxytetrachlorofluorescein (6-TET)	521	536	73000
JOE	520	548	73000
LightCycler Red 640	625	640	
LightCycler Red 705	685	705	
Oregon Green 488	496	516	76000
Oregon Green 500	499	519	84000
Oregon Green 514	506	526	85000
BODIPY FL-X	504	510	70000
BODIPY FL	504	510	70000
BODIPY-TMR-X	544	570	56000
BODIPY R6G	528	547	70000
BODIPY 650/665	650	665	101000
BODIPY 564/570	563	569	142000
BODIPY 581/591	581	591	136000
BODIPY TR-X	588	616	68000
BODIPY 630/650	625	640	101000
BODIPY 493/503	500	509	79000
5-Carboxyrhodamine 6G	524	557	102000
5(6)-Carboxytetramethylrhodamine (TAMRA)	546	576	90000
6-Carboxytetramethylrhodamine	544	576	90000

(TAMRA)			
5(6)-Carboxy-X-Rhodamine (ROX)	576	601	82000
6-Carboxy-X-Rhodamine (ROX)	575	602	82000
AMCA-X (Coumarin)	353	442	19000
Texas Red-X	583	603	116000
Rhodamine Red-X	560	580	129000
Marina Blue	362	459	19000
Pacific Blue	416	451	37000
Rhodamine Green-X	503	528	74000
7-diethylaminocoumarin-3-carboxylic acid	432	472	56000
7-methoxycoumarin-3-carboxylic acid	358	410	26000
Cy3	552	570	150000
Cy3B	558	573	130000
Cy5	643	667	250000
Cy5.5	675	694	250000
DY-505	505	530	85000
DY-550	553	578	122000
DY-555	555	580	100000
DY-610	606	636	140000
DY-630	630	655	120000
DY-633	630	659	120000
DY-636	645	671	120000
DY-650	653	674	77000
DY-675	674	699	110000
DY-676	674	699	84000
DY-681	691	708	125000
DY-700	702	723	96000
DY-701	706	731	115000

DY-730	734	750	113000
DY-750	747	776	45700
DY-751	751	779	220000
DY-782	782	800	102000
Cy3.5	581	596	150000
EDANS	336	490	5700
WellRED D2-PA	750	770	170000
WellRED D3-PA	685	706	224000
WellRED D4-PA	650	670	203000
Pyrene	341	377	43000
Cascade Blue	399	423	30000
Cascade Yellow	409	558	24000
PyMPO	415	570	26000
Lucifer Yellow	428	532	11000
NBD-X	466	535	22000
Carboxynapthofluorescein	598	668	42000
Alexa Fluor 350	346	442	19000
Alexa Fluor 405	401	421	35000
Alexa Fluor 430	434	541	16000
Alexa Fluor 488	495	519	71000
Alexa Fluor 532	532	554	81000
Alexa Fluor 546	556	573	104000
Alexa Fluor 555	555	565	150000
Alexa Fluor 568	578	603	91300
Alexa Fluor 594	590	617	73000
Alexa Fluor 633	632	647	100000
Alexa Fluor 647	650	665	239000
Alexa Fluor 660	663	690	132000

Alexa Fluor 680	679	702	184000
Alexa Fluor 700	702	723	192000
Alexa Fluor 750	749	775	240000
Oyster 556	556	570	155000
Oyster 645	645	666	250000
Oyster 656	656	674	220000
5(6)-Carboxyeosin	521	544	95000
Erythrosin	529	544	90000

[0130] Examples of energy transfer dyes suitable for use in connection with the present teachings include, but are not limited to those described in, for example, Mathies, et al. U.S. Patent No. 5,728,528, Lee, et al. U.S. Patent No. 5,863,727, Glazer, et al. U.S. Patent No. 5,853,992, Waggoner, et al., U.S. Patent No. 6,008,373, Nampalli, et al., U.S. Patent No. 6,335,440 Lee et al., U.S. Patent No. 5,800,996 Lee et al., U.S. Patent No. 5,945,526 Lee et al., U.S. Patent Application Pub. No. 2004/0126763 A1, Kumar, et al., PCT Pub. No. WO 00/13026A1, PCT Pub. No. WO 01/19841A1, and AB4303 filed in the U.S. Patent Office on September 16, 2004, each of which is incorporated herein by reference for all it discloses with regard to energy transfer dye structures, energy transfer dye synthesis, energy transfer dye linkers, alternative donor dyes, alternative acceptor dyes and energy transfer dye spectral properties.

[0131] In various configurations of these embodiments, determining the amount of target sequence, normalized to the reference sequence and relative to a calibrator, can comprise determining $-\Delta\Delta C_T$, wherein C_T is the threshold number of cycles for detection of a fluorophore in a real time PCR assay; $C_{T,q}$ is the threshold number of cycles for detection of a fluorophore for the target sample in the real time PCR assay, $C_{T,cb}$ is the threshold number of cycles for detection of a fluorophore for a calibrator sample in the real time PCR assay, $\Delta C_{T,\,q}$ is a difference in threshold cycles for the target and the endogenous reference which may be a passive internal reference, $\Delta C_{T,\,cb}$ is a difference in threshold cycles for the calibrator sample and the endogenous

reference, and $-\Delta\Delta C_T = \Delta C_{T,q} - \Delta C_{T,cb}$. In these configurations, if $-\Delta\Delta C_T$ is determined, the relative quantity of the target sequence can be determined using the relationship that relative quantity can be equal to $2^{-\Delta\Delta CT}$. In various embodiments, the above calculations are adapted for use in multiplex PCR. See for example: Livak et al. Applied Biosystems User Bulletin #2 updated October 2001.

[0132] Some embodiments preamplify the sample in order to increase the amount of target prior to distribution into the plate wells. For example, the sample to be analyzed could be collected via a needle bioposy which typically yields a small amount of sample. Distributing this sample across a large number of wells can result in variances in sample distribution which can affect the veracity of subsequent gene expression computations. In such situations, the sample can be preamplified using a pooled primer set to increase the copy number of all targets simultaneously.

[0133] Some embodiments calibrate for potential differences in preamplification efficiency that can arise from a variety of sources such as the effects of having multiple primer sets in the same reaction. Some embodiments perform calibration by computing a reference number that reflects preamplification bias. Reference number similarity for a given target across different samples is indicative that the preamplification reaction ΔCts, can be used to achieve reliable gene expression computations.

[0134] Some embodiments compute these reference numbers by collecting a sample (S_y) and processing it with two protocols. One protocol involves running individual PCR gene expression reactions for each target (T_x) of interest relative to an endogenous control (endo) such as 18s or GAPDH. These reactions will yield cycle threshold values for each target relative to the endogenous control.

$$\Delta Ct_{unamplified} T_x S_y = Ct_{unamplified} T_x S_y - Ct_{unamplified} endo$$

[0135] The other protocol involves running a single PCR preamplification step on the sample with a pooled primer set. The pooled primer set can contain primers for each target of interest. Subsequently, the amplified product can be distributed among the wells of the sample plate and PCR gene-expression assays relative to an endogenous control are run in

each well using a single primer/probe set corresponding to a single target in each well. These reactions will yield cycle threshold values for each target relative to the endogenous control.

$$\Delta CtpreamplifiedTxSy = CtpreamplifiedTxSy - Ctpreamplifiedendo$$

Now a difference between these two values can be computed,

$$\Delta\Delta CtTxSy = \Delta CtpreamplifiedTxSy - \Delta CtunamplifiedTxSy$$

[0136] A value for $\Delta\Delta CtTxSy$ that is zero or close to zero indicates that there is no bias in the preamplification of Tx.

[0137] Some embodiments use $\Delta\Delta Ct$ values computed for the same target but in different samples in order to determine the accuracy of subsequent relative expression computations. This results in the equation,

$$\Delta\Delta\Delta CtTx = \Delta\Delta CtTxSy - \Delta\Delta CtTxSz$$

[0138] If this value is zero or reasonably close to it, the preamplified ΔCt values for Tx ($\Delta CtpreamplifiedTxSy$ and $\Delta CtpreamplifiedTxSz$) can be used for relative gene expression computation between different samples via a standard relative gene expression calculation. In some embodiments, a standard relative gene expression calculation determines the amount of target sequence. In certain embodiments, a standard relative gene expression calculation employs a comparative Ct. Thus, this method can be practiced during experimental design and once the conditions have been optimized so that the $\Delta\Delta\Delta CtTx$ is reasonably close to zero, subsequent experiments will only require the computation of the ΔCt value for the preamplified reactions.

[0139] Some embodiments store the $\Delta\Delta CtTxSy$ values in a database or other storage medium. These values can then be used to convert $\Delta\Delta CtpreamplifiedTxSy$ values to $\Delta\Delta CtunamplifiedTxSy$ values. This can permit mapping $\Delta\Delta CtpreamplifiedTxSy$ values back to a common domain. One skilled in the art will appreciate that the unamplified domain need not necessarily be PCR-based and other gene expression instrument platforms can be used. This process can permit comparison of gene expression data from a variety of instrument platforms. In some cases the $\Delta\Delta CtTxSy$ values need not be stored for all different sample types (Sy) if it can be shown that the $\Delta\Delta CtpreamplifiedTx$ are reasonably consistent over different sample types.

[0140] One skilled in the art will appreciate that other types of preamplification can be used such as in vitro transcription. As well, one skilled in the art will appreciate that opportunities exist to multiplex sample in the wells and can modify the above method appropriately.

[0141] In general, diagnosis and screening for specific nucleic acids using nucleic acid amplification techniques has been limited by the necessity of amplifying a single target sequence at a time. In instances where any of multiple possible nucleic acid sequences may be present, performing multiple separate assays by this procedure is cumbersome and time consuming. For example, the same clinical symptoms generally occur due to infection from many etiological agents and therefore requires differential diagnosis among numerous possible target organisms. Cancer prognosis and genetic risk is known to be due to multiple gene alterations. Genetic polymorphism and mutations result from alterations at multiple loci and further demand determination of zygosity. In many circumstances the quantity of the targeted nucleic acid is limited so that dividing the specimen and using separate repeat analyses is often not possible. For example, see U.S. Patent No. 6,154,707 issued November 2000 and SNPlex™ Genotyping System 48-plex: Chemistry Guide: Rev. C January 2004 (Applied Biosystems, Foster City, California.

[0142] In accordance with the present invention, the wells of the microplate comprise a solution operable to perform multiplex PCR. In this embodiment, the wells are capable of having multiple PCR reactions in each individual well based on the chemistry and the probes that are included in the solution. "Multiplex PCR" is the use of more than one primer pair in the same tube. This method can be used for relative quantitation where one primer pair amplifies the target and another primer pair amplifies the endogenous reference. A multiplex reaction can be performed using either the Standard Curve Method or the Comparative C_t Method. Non limiting examples include various probes that can be used such as FAM which is a carboxy-fluorescein which has an excitation wavelength from about 485 nm and an emission wavelength from about 510-520 nm; TET which has an emission wavelength from about 517 nanometers to about 538 nanometers; the probes from the group of HEX, JOE and VIC, which have emission wavelengths from 525-535 nm to about 546-556 nm; TAMRA which is a carboxy-tetra methylrhodamine,

and has an emission wavelength from about 556 nanometers to about 580 nanometers; ROX which is a carboxy-x-rhodamine, which has an emission wavelength from about 575-585 nm to about 605-610 nm; ALEXA, which has an emission range from about 350 nanometers to about 440 nanometers; TEXAS RED, which has an emission wavelength from about 580-585 nm to about 600-610 nm; Cy3, which has an emission wavelength of about 545 nanometers to about 568 nanometers; Cy5, which has an emission wavelength of about 635-655 nm to about 665-675 nm; Cy7, which has an emission wavelength of about 715 nanometers to about 787 nanometers. Optimized interference filters precisely match the excitation and emission wavelengths for each fluorophore to block out unwanted cross-talk from spectrally adjacent fluorophores. Commercially available filters for fluorophores include FAM™/SYBR® Green I, TET, HEX™/JOE™/VIC™, TAMRA™, Texas Red®/ROX™, Cy7™, Cy5™, Cy3™,and ALEXA Fluor® 350 filter sets; (these materials and filters are well known in the art and are available through a variety of sources such as Applied Biosystems, Foster City, CA, Stratagene, San Diego, CA, Qiagen, Inc. Valencia, CA; and Promega, Madison, WI.. Filter sets for use with hybridization probes and custom filter sets are also available. Such multiplexing techniques may include deconvulsion, multicomponent analysis, spectral analysis or the like. For example, see U.S. Patent No. 6,333,501 issued December 2001_ and U.S. Patent No. 6,015,667 issued January 2000.

[0143] The invention also provides reagents and kits suitable for carrying out polynucleotide amplification methods of this invention. Such reagents and kits may be modeled after reagents and kits suitable for carrying out conventional PCR, RT-PCR, and other amplification reactions. Such kits comprise a microplate of this invention and a reagent selected from the group consisting of an amplification reagent, a detection reagent and combinations thereof. Examples of specific reagents include, but are not limited to, the reagents present in AmpliTaq® Gold PCR Master Mix, TaqMan® Universal Master Mix and TaqMan® Universal Master Mix No AmpErase® UNG, Assays-by-Design℠, Pre-Developed Assay Reagents (PDAR) for gene expression, PDAR for allelic discrimination and Assays-On-Demand®, all of which are marketed by Applied Biosystems, Inc. (Foster City, California,

U.S.A.). The kits may comprise reagents packaged for downstream or subsequent analysis of the multiplex amplification product. In one embodiment, the kit comprises a container comprising a plurality of amplification primer pairs or sets, each of which is suitable for amplifying a different sequence of interest and a plurality of reaction vessels, each of which includes a single set of amplification primers suitable for amplifying a sequence of interest. The primers included in the individual reaction vessels can, independently of one another, be the same or a different set of primers comprising the plurality of multiplex amplification primers.

[0144] In accordance with the invention, an embodiment includes a microplate comprising wells containing a solution that comprises at least one primer and at least one labeled probe. In another embodiment, the wells of the microplate contain a solution that comprises a forward PCR primer, a reverse PCR primer, and at least one FAM labeled MGB quenched PCR probe. In another embodiment, the wells of the microplate contain a solution comprising at least one probe, at least one primer and a polymerase. In various embodiments, the wells contain at least one forward PCR primer, at least one reverse PCR primer, at least one labeled MGB quenched PCR probe, at least one labeled MGB quenched PCR probe used as a endogenous control and a polymerase. In some embodiments, a ROX labeled oligio is used as a passive internal reference. Other embodiments include other dyes may be used as a passive internal reference. In various embodiments, the wells contain the necessary reagents for preamplification. In other embodiments of the invention, the wells may contain any of the above solutions in a dried form. In this embodiment, this dried form may be coated to the wells or be directed to the bottom of the well. In the application of this dried form of any of the above solutions, the mixture maybe applied as a liquid solution and the liquid phase of the mixture is dried off using methods known in the art such as heat and/or vacuum apparatus. According to this embodiment, the user needs to add an universal master mix, water and the sample to each of the wells before analysis. This embodiment is also known as a "preloaded" well or microplate.

[0145] An example of manufacturing preloaded plates is illustrated in Figure 54. A commercial spotting device 501 which may be, for example,

for example, the use of piezoelectric pumps, acoustic dispersion, liquid printers, micropiezo dispensers or the like. Commercial examples include Nanoraptor MPD, Aurora Discovery, San Diego, California; HTS, EDC Biosystems, San Jose, California; Echo 550, Labcyte Picoliter, Sunnyvale, California; Cyclone ILNIO, Caliper Life Sciences, Hopkinton, Massachusetts and the like may be employed. Such spotting device 501 is operable to deliver liquid in nanoliter increments and has the precision to spot the individual wells of a microplate. Reagents in an aqueous form are stored on source plates, for example, a 384 well plate. Such source plates are stored in a high humidity storage unit 505. The source plate 507 moves out of the storage area 505 as indicated by 506 and is placed onto spotting device 501 as indicated by 508. A microplate which is in low humidity storage unit 514 is moved out of the storage unit 501 as indicated by 514 as indicated by 515. Microplate 516 is placed on spotting device 517. The reagents in plate 507 are spotted onto selected locations of microplate 516. Once locations are spotted, the spotted microplate 510 is moved from spotting device 501 as indicated by 519. Spotted plate 510 may be moved to a quality control station 512 as indicated by 511. Quality control 512 is an optional step in this procedure. After quality control 512, spotted microplate 510 is moved back to low humidity storage unit 514 as indicated by 513. This procedure of spotting the microplates continues until all microplates in the storage unit 514 have been spotted with the reagents from the source plate 507. When a spotted microplate 510 enters storage unit 514, the spots on the microplate 510 dry in the low humidity of storage unit 514. In some embodiments, storage unit 514 may also be heated. Once all of the microplates from the storage unit 514 have been spotted with the reagents available in source plate 507, the source plate is removed and is indicated as a used source plate 503 since it has less material available and the used source plate 503 is removed from spotter 501 as indicated by 502. Used source plate 503 is returned to high humidity storage unit 505. The process continues when a next source plate 507 is moved out of the high humidity storage unit 505 containing a different set of reagents and is placed on spotter device 501. The microplates 516 that have been spotted by the first source plate are then moved, having been dried in the low humidity storage unit 514 and each microplate is spotted with the

different set of reagents from the second source plate 507. This process continues until all of the wells on the microplate 516 have been spotted or in some cases only certain wells may be spotted leaving other wells empty. In some embodiments, the reagents on source plate include at least one primer, at least one probe and a polymerase. In some embodiments of this process, a whole genome may be spotted from source plates onto the microplate. In other embodiments, a portion of the genome is spotted from the source plates onto the microplate. Once the microplate is fully spotted and has been completely dried, it is then moved, sealed and packaged for shipment to a user. In some embodiments, various primer probe sets are spotted onto the microplate. In some embodiments, reagents include buffers. In other embodiments, the reagents include additives. In such embodiments, additives may be useful for coating, stability, enhanced rehydration, preservation, and/or enhanced dispensing of reagents. In some embodiments, the dried on spots are then encapsulated with a material. In such embodiments, encapsulation may prevent moisture at standard room temperature from interacting with spotted material. In some embodiments, the microplate may be spotted in a single well several times. In such embodiments, the multiple spots may be in layers. Such embodiments may be employed for spotting multiplex PCR reagents. In some embodiments, the microplate well are first coated using the spotting device 501 in every well before the spotting of reagents is complete. Such coating may be necessary for attachment of reagents to the well. In other embodiments, reagents are placed into wells as beads. See U.S. Patent No. 6,579,367 to Bann issued June 2003 and U.S. Patent No. 6,432,719 to Bann issued August 2002.

[0146] In this embodiment, the microplate comprising the dried down reaction components may be sealed with a protective cover, stored or shipped to another location. The protective cover is releasable without leaving an adhesive residue on the microplate. The protective cover may be a different color than the cover to aid in identification and for ease of handling. The material of the protective cover is chosen to minimize static charge generation upon release from the plate. When it is time for this microplate to be used, the packaging seal is broken and the protective cover is removed and the sample, along with water and any required reaction components, is

added to the wells of this microplate. The reaction plate is then sealed with a cover. The plate is put in the thermal control system. The system is run and data is collected and analyzed. In a similar embodiment, there are additional steps of the plate with the cover and reaction mixture is then put in a centrifuge. The centrifuge is run. The spun plate is put into the thermal control system. The system is run and data is collected and analyzed.

[0147] In various embodiments of the present invention, the microplate has 6,144 wells and has dimensions similar to the SBS/ANSI standard footprint for microplates and in each of the wells there is part of a genome, the genome having, for example, 30,000 different targets. In such embodiments, in each well there may be up to five or more different probes and/or primer sets so that multiplexing PCR may be performed. In this embodiment, the genome may be from humans, from mammals, from mice, from Arabidopsis or from any other plant, bacteria, fungi or animal species. It is envisioned that this embodiment may be used for drug discovery and for diagnostics of a particular individual, animal or plant. In other embodiments, the microplate has at least 30,000 wells. In other embodiments, the genome is on a microcard and such a microcard may be analyzed with the present invention. See U.S. Patent No. 6,272,931 issued October 2001; U.S. Patent No. 6,126,899 issued October 2001; U.S. Patent No. 6,124,138 issued September 2000.

[0148] The methods of this invention are performed with equipment which aids in one or more steps of the process, including handling of the microplates, thermal cycling, and imaging. Accordingly, the present invention provides systems for simultaneously determining the genetic expression profile in a biological sample obtained from an individual member of a species relative to a standard genome for said species, comprising:

(a) a microplate assembly comprising

(i) a substrate, wherein at least about 1000 reaction chambers are formed in an array on the substrate and each chamber has a capacity of less than about 50 µL; and

(ii) a cover; wherein

(iii) each chamber comprises (1) a primer for a polynucleotide target within said standard genome, and (2) an indicator associated with said primer which emits a concentration dependent signal if the primer binds with said target;

(b) amplification reagents for performance of an amplification reaction for polynucleotide targets within said standard genome;

(c) a filling system for filling said biological sample and said reagents into said microplate assembly;

(d) a thermal cycling system for heating said microplate assembly; and

(e) a detection system for detecting said signal from said indicator.

[0149] Referring now to Figure 1, a high-density sequence detection system 10 is illustrated in accordance with the principles of the present invention. Sequence detection system 10 generally includes a thermocycler assembly 12 and an excitation and detection system 14 disposed in a housing 16.

[0150] As best seen in Figures 1-4, 6, 8,and 20-30, thermocycler assembly 12 generally includes a thermocycler block system 18, an optional thermal compliant pad 20 (Figure 23), a microplate 22, a sealing cover 24, a pressure clamp system 26, and a transparent window 28.

[0151] With reference to Figures 2,3 and 20-30, thermocycler block system 18 is illustrated having a thermocycler block 30. Thermocycler block 30 is operably coupled to a control system 32 (also see Figure 1). Control system 32 is operable to output a control signal to regulate a desired thermal output of thermocycler block 30. Thermocycler block 30 facilitates heat transfer between thermocycler block 30 and microplate 22 during analysis. This operation will be discussed in detail below; however, it should be appreciated that thermocycler block 30 may heat and/or cool microplate 22. To this end, thermocycler block 30 includes a plurality of fin members 33 disposed along a side thereof to dissipate heat. In some embodiments, thermocycler block 30 cycles temperatures or cycles and holds temperatures. In other embodiments, thermocycler block may be used isothermally, that is, holding an essentially constant temperature. In some embodiments, multiple thermocycler blocks 30 may be employed and the detection system may be

moved between the multiple blocks. In some embodiments, the thermal block 30 may comprise a single element heater and a non-active or passive cooler.

[0152] Various embodiments of apparatus useful herein comprise temperature control devices. Temperature control mechanisms are included to change the temperature of the microplate so as to change the temperature of the samples and reagents placed in the reaction chambers. In some embodiments of the invention, the temperature control devices provide thermal uniformity across the reaction substrate so as to facilitate accurate and precise quantification of the amplification reactions. In some embodiments, the temperature control device comprises: a heater; a cooler; a temperature sensor for measuring the temperature of the reaction substrate; or combinations thereof. Temperature control devices among those useful include: forced convection temperature systems that blow hot and cool air onto microplate assembly; systems for circulating heated and/or cooled gas or fluid through channels in the microplate assembly; Peltier thermoelectric devices; refrigerators, microwave, infrared, or combinations thereof. In one embodiment, the temperature control device is connected to a temperature control element of the microplate assembly (as discussed above). In one embodiment, the temperature control devices comprise a heating or cooling source in thermal connection with a heat sink. In various embodiments, the heat sink is configured so as to be in thermal connection with the microplate assembly during use of the amplification system. Temperature control devices include those generally known in the art, such as are in U.S. Patent No. 5,942,312, Smith et al., issued August 24, 1999; and U.S. Patent No. 5,928,907, Woundenberg et al., issued July 27, 1999; U.S. Patent No. 5,224,778 to Grossman issued July 6, 1993; U.S. Patent No. 5,475,610 issued December 1995 and U.S. Patent No. 5,703,342 issued December 1997.

[0153] With reference to Figure 23, thermal compliant pad 20 is disposed between thermocycler block 30 and any adjacent component, such as microplate 22 or sealing cover 24. It should be understood that thermal compliant pad 20 is optional and need not be used; however, it has been found that when used it serves to more evenly distribute heating or cooling through a contact interface between thermocycler block 30 and the adjacent

component. This arrangement minimizes localized hot spots and compensates for surface variations in thermocycler block 30, thereby providing improved thermal distribution.

[0154] The present invention provides a reaction plate comprising a plurality of reaction wells and a transparent cover. In particular, the present invention provides multiwell plates for conducting a thermocycled amplification reaction of polynucleotide in a liquid sample. Such plates comprise:

(a) a substantially planar substrate having a first and second major surface;

(b) a plurality of reaction wells located on said first major surface, wherein each of said wells has a bottom and an opening which is disposed on said first major surface;

(c) a substantially planar cover, a major surface of which is disposed adjacent to said first major surface so as to cover said openings of said wells; and

(d) a sealing member disposed between said cover and said first major surface, wherein said sealing member is operable to substantially seal said openings of said wells.

[0155] The microplates of the present invention comprise a substantially planar substrate, having a first major surface and a second major surface. As referred to herein, a "substantially planar" surface is, or is capable of being, flat having substantially two-dimensional geometry (in x- and y- dimensions) considering the surface as a whole, although it may have surface irregularities in the third (z) dimension (wherein the x-, y- and z-dimensions are mutually perpendicular axes defining the three special dimensions). A "major surface" of a substantially planar substrate refers to a surface that is defined by the x- and y-dimensions of the substrate. It is understood that a planar substrate comprises two such major surfaces – a first major surface and an opposite second major surface -- spatially separated in the z-direction by the thickness of the substrate.

[0156] The substrate has a first major surface, and second major surface. The microplate substrate may have any dimension (in the x- and y-dimensions), but is sized so as to readily handled during use, provide sufficient sample capacity (as further discussed below), and be compatible

with instrumentation used in amplification reactions. In various embodiments, the footprint dimensions of the microplate substrate are essentially the standards as specified by the Society of Biomolecular Screening (SBS) and the American National Standards Institute (ANSI) standards, published January 2004 (ANSI/SBS 1-2004), incorporated by reference herein. In such an embodiment, the footprint dimensions for the microplate 22 are about 127.76 mm (5.0299 inches) in length and about 85.48 mm (3.3654 inches) in width. The footprint is continuous and uninterrupted around the base. The four outside corners of the bottom flange should have corner radius to the outside about 3.18 mm (0.1252 inches). In an embodiment of the plate, the thickness of the plate is about 0.5 mm to about 3.0 mm. In one embodiment, the thickness of the plate is about 1.25 mm. In another embodiment, the thickness of the plate is about 2.25 mm.

[0157] The substrate comprises a plurality of wells. As referred to herein, a "well" is a feature formed in the substrate which is operable to contain a liquid during use of the microplate. Such wells have an opening, which is operable to allow the deposit of a liquid into the well. In an embodiment, the wells are formed in a major surface of the substrate.

[0158] As best seen in Figures 9-16, microplate 22 is illustrated having a substantially planar construction having a plurality of wells 34 disposed therein. Each of the plurality of wells 34 are sized to receive an aqueous solution 36 (Figures 18 and 19) having a target or seed DNA sample contained therein. Hereinafter, such combination of aqueous solution 36 and the target or seed DNA sample will be collectively referred to as assay 38. As will be described in detail below, pressure clamp system 26 is operable to apply a clamping force upon sealing cover 24, microplate 22, and thermocycler block 30 to operably seal aqueous solution 36 within the plurality of wells 34 during thermocycling.

[0159] With continued reference to Figures 9-19, microplate 22 generally includes a main body or substrate 40. Microplate 22 may include an optional skirt or flange portion 42 disposed about a periphery of main body 40 (see Figure 9). Alternatively, microplate 22 may employ a skirtless design (see Figures 10-12) depending upon existing laboratory equipment to be used. Skirt portion 42 is generally perpendicular to main body 40 and may

vary in height. Skirt portion 42 facilitates alignment of microplate 22 on thermocycler block 30. Additionally, skirt portion 42 provides additional rigidity to microplate 22 such that during handling microplate 22 remains rigid, thereby ensuring assay 38 disposed in microplate 22 does not contaminate adjacent wells.

[0160] Referring to Figures 13-17, microplate 22 may have grooves 360 or 362 around the periphery of an array of wells 34. In some embodiments, the grooves 360 have depth and width dimensions that are similar to the well 34 depth and width dimensions. In other embodiments, the grooves 362 have depth and width dimensions that are smaller than the well 34 depth and width dimensions. In certain embodiments, as shown in Figure 13, additional grooves may be at each end. It is believed that grooves 360 or 362 may improve thermal uniformity amongst the wells 34 in microplate 22. Grooves 360 or 362 may improve seal formed by sealing cover 24 and microplate 22. In addition, grooves 360 or 362 may assist in producing an essentially flat microplate 22 during the injection molding process. In some embodiments, the grooves 360 or 362 comprise a liquid solution similar to reaction assay 36 which may further improve thermal uniformity during thermal cycling.

[0161] In order to facilitate use with existing equipment, robotic implements, and instrumentation, the dimensions or footprint of main body 40 and/or skirt portion 42 of microplate 22 should conform to standards specified by the Society of Biomolecular Screening (SBS) and the American National Standards Institute (ANSI) published January 2004 (ANSI/SBS 3-2004), incorporated by reference herein. Microplate 22 may include additional alignment features 31, which may include slots, pins, cut corners, indentations, graphics, protrusions (nubs) or combinations thereof. These alignment features are placed so that they do not interfere with sealing cover 24 or wells 34; however, by locating such alignment features 31 near wells 34, improved alignment with dispensing equipment and/or thermocycler block 30 can be achieved.

[0162] Microplate 22 may include an alignment feature 31, illustrated in Figures 9 and 10 as a corner chamfer; however, it should be understood that alignment feature 31 may be a pin, slot, or other unique

feature that is capable of interfacing with a corresponding feature formed in a fixture, reagent dispensing equipment, and/or thermocycler. In other embodiments, the alignment feature may be a nub or protrusion 35 as shown in Figure 17. In such embodiments, the length dimension of microplate 22 is about 127 mm is measured from the tangent point of one nub 35 to the tangent of the opposite nub and the width of about 85 mm is calculated in the same fashion.

[0163] Additionally, with reference to Figures 38-41, microplate 22 may include a thermally isolated portion 70. Specifically, microplate 22 may be formed with thermally isolated portion 70 disposed along at least one edge of main body 40. Thermally isolated portion 70 is generally free of wells 34 and is sized to receive a marking indicia 190 (discussed in detail below) thereon. Thermally isolated portion 70 is further sized to facilitate the handling of microplate 22 by providing an area that can be easily gripped by a user or mechanical device without disrupting wells 34.

[0164] Still referring to Figures 38-41, microplate 22 may include a first groove 72 formed along a top surface 74 and a second groove 76 formed along a bottom surface 78 of microplate 22. First groove 72 and second groove 76 are positioned so as to generally extend across microplate 22 from a first side 80 to a second side 82. First groove 72 and second groove 76 are further generally aligned upon top surface 74 and bottom surface 78 to define a reduced cross section between thermally isolated portion 70 and well array portion 84 of main body 40. This reduced cross section serves as a thermal isolation barrier to generally minimize a heat sink effect of thermally isolated portion 70. This provided improved and consistent thermal cycling of well array portion 84.

[0165] Microplate 22 may be made from any one of a number of materials, such as by non-limiting example, glass, ceramic, silicon, standard plastic, plastic compounded with a thermally conductive material, polypropylene, polystyrene, polyethylene, polyethyleneterephthalate, styrene, acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer or any plastic material known to those skilled in the relative art with a melting point greater than 130°C and exhibiting a very low fluorescence when exposed to visible or non-visible light. Conductive material

such as a conductive carbon black or other conductive filler known to those skilled in the relative art may be included in the formulation of the plastic to increase thermal conductivity. "Thermal conductivity" is defined as the heat flow across a surface per unit area per unit time, divided by the negative of the rate of change of temperature with distance in a direction perpendicular to the surface. Thermal conductivity is also known as heat conductivity. Alternatively, thermal conductivity can generally be thought of as the rate at which heat is conducted through a substance. Thermal conductivity of microplate 22 improves heat distribution, thereby improving the heating and cooling of assay 38. To further increase the thermal conductivity, thermally conductive ceramic filler, such as boron nitrate filler or other ceramic filler, may be added to the formulation. It should be understood that combinations of these different materials may be used. In various embodiments, microplate 22 may be co-molded such that the bottom of the wells comprise a metal, a clear material, a glass, a quartz, tin iridium oxide, a different plastic composition or the like.

[0166] Microplate 22 may also be made of an electrically conductive material, which will improve reagent dispensing alignment. In this regard, electrically conductive material may serve to minimize static build-up on microplate 22 so that the reagent droplets will not go astray during dispensing. In some embodiments, a voltage may be applied to pull the sample into the appropriate position, particularly with a co-molded part where the flat "bottom" section is electrically conductive and the sides of the wells are not electrically conductive. In other embodiments, a high voltage field can be applied under the well or well of interest to pull the sample into the appropriate wells.

[0167] In order to increase throughput of genotyping, gene expression, and other assays, microplate 22 may include a dramatically increased density of wells 34 beyond that employed in the prior art. For example, microplate 22 has been proven using 6,144 wells, while prior art designs have been limited to no more than 1,536 wells. It should be appreciated, however, that assays used in microplate 22 are not limited to DNA assays that are homogenous, such as TAQMAN® and INVADER®, but could also include other assays such as receptor binding, enzyme, and other

high throughput screening assays. Microplate 22 may also be used for the temporary storage of reagents, samples, and other related applications.

[0168] The present invention provides microplates with any well array configuration essentially within the standard SBS footprint. In various embodiments, microplates of the present invention may be, but not limited to, any of the well array parameters described in Table 2.

Table 2

Total wells	RowsXColumns	approx. well area
96	8x12	9x9mm
384	16x24	4.5x4.5mm
1536	32x48	2.25x2.25mm
3456	48x72	1.5x1.5mm
6144	64x96	1.125x1.125mm
13824	96x144	0.75x.075mm
24576	128x192	0.5625x0.5625mm
55296	192x288	0.375x0.375mm
768	24x32	3x3mm
1024	32x32	2.25x3mm
1600	40x40	1.8x2.7mm
1280	32x40	2.25x2.7mm
1792	32x56	2.25x1.714mm
2240	40x56	1.8x1.714mm
864	24x36	3x3mm
4704	56x84	1.257x1.257mm
7776	72x108	1x1mm
9600	80x120	0.9x.09mm
11616	88x132	0.818x0.818mm
16224	104x156	0.692x0.692mm
18816	112x168	0.643x0.643mm
21600	120x180	0.6x0.6mm
27744	136x204	0.529x0.529mm
31104	144x216	0.5x0.5mm
34656	152x228	0.474x0.474mm

38400	160x240	0.45x0.45mm
42336	168x252	0.429x0.429mm
46464	176x264	0.409x0.409mm
50784	184x256	0.391x0.391mm

[0169] During analysis, each well 34 includes assay 38. As described above, assay 38 is a reaction solution, which may be a homogenous solution and may comprise a sample, two primers, at least one labeled probe, internal standard taq polymerase, and a buffer in the solution. For example, see U.S. Patent No. 5,736,333 issued April 1998. As best seen in Figure 18, assay 38 is disposed in well 34 and sealing cover 24 is disposed thereon (as will be discussed below). However, well 34 is not completely filled with assay 38, therefore a headspace 56 is defined, which may be an air or other gas gap. Care should be taken to minimize the occurrence of condensation within well 34 along sealing cover 24. Such condensation may cause light scattering and/or refraction of excitation light entering well 34 during analysis, which could adversely effect fluorescence detection.

[0170] As best seen in Figures 11 and 12, each well 34 is equivalent in size relative to each other throughout microplate 22. The plurality of wells 34 may have any cross sectional shape. For example, as seen in Figures 11 and 18, the plurality of wells 34 may define a generally circular rim portion 44 with downwardly-extending, generally continuous sidewall 46 that terminate at a bottom wall 48 in a radius. To ease manufacturing, a draft angle of sidewall 46 may be used. The particular draft angle is determined by the manufacturing method and the size of well 34. By way of non-limiting example, circular rim portion 44 is about 1.0 mm in diameter, the depth of well 34 is about 0.9 mm, the draft angle of sidewalls 46 is about 1° to more than 5° and each well 34 is spaced about 1.125 mm from center to center. In certain embodiments, the volume of each well 34 is about 500 nL.

[0171] Alternatively, as seen in Figure 12, the plurality of wells 34 may define a generally square-shaped rim portion 50 with downwardly extending sidewalls 52 that terminate at a bottom wall 54. To ease manufacturing and to minimize shadowing (which is discussed below), a draft

angle of sidewalls 52 may be used. Again, the particular draft angle is determined by the manufacturing method and the size of well 34. By way of non-limiting example, side dimension of portion 50 is about 1.0 mm in length, the depth of well 34 is about 0.9 mm, the draft angle of sidewalls 46 is about 1° to more than 5°, and each well 34 is spaced about 1.125 mm from center to center, generally indicated at A (see Figure 19). In certain embodiments, the volume of each well 34 is about 500 nL. In various embodiments, the spacing between wells as measured at the top of a wall dividing wells is less than about 0.5 m. In certain embodiments, this spacing between wells is about 0.25 mm.

[0172] However, it should be noted that advantages are associated with wells 34 having generally circular rim portion 44 and the corresponding circular sidewall 46 over wells 34 having generally square-shaped rim portion 50 and sidewalls 52. Specifically, during heating, it has been found that assay 38 may migrate through capillary action upward along edges of sidewalls 52. This may draw assay 38 from the center of well 34, thereby causing variation in depth of assay 38, which may compromise fluorescence output of assay 38 during analysis. Additionally, during manufacture of microplate 22, it is believed that cylindrically shaped mold pins, used to form wells 34 having generally circular rim portion 44 and sidewall 46, permit unencumbered flow of molten polymer thereabout, resulting in less deleterious polymer molecule orientation. Still further, the use of generally circular rim portion 44 provides more surface area along microplate 22 for improved sealing with sealing cover 24, which will be discussed below.

[0173] To improve such fluorescence detection and provide additional advantages, an alternative arrangement is provided as seen in Figures 6, 8, 19, 22, 25, and 30. As illustrated, a microplate 22' is provide having similar features as described above, however microplate 22' is inverted such that each well 34' is generally "upside-down". As best seen in Figure 19, this inverted arrangement causes assay 38 to collect adjacent sealing cover 24 and, thus, eliminates the occurrence of condensation effecting fluorescence detection and improves optical efficiency because solution is closer to well opening.

[0174] Thermocycler block 30' remains stationary and is above microplate 22. Inflatable transparent bag 62 is positioned in engaging contact with transparent window 28 and sealing cover 24. It should be appreciated that transparent window 28, inflatable bag 62, and sealing cover 24 must permit the free transmission therethrough of an excitation light 68 generated by a source 71 and the resultant fluorescence. The source 71 is positioned below microplate 22 and a detector 36' also positioned below microplate 22 is provided for receiving such fluorescence generated in response to excitation light 68.

[0175] In addition to the above embodiments, it can be seen in Figure 26 that pressure clamp system 12 may further employ a microplate 22' having a plurality of apertures 46 in place of the plurality of wells 34, 22'. The plurality of apertures 46 each extend through microplate 22'. However, according to the present embodiment, each of the plurality of apertures 46 are enclosed by a pair of cooperating sealing members 18, 18' disposed on top surface 24 and bottom surface 40, respectively.

[0176] The cover comprises a device which facilitates physical isolation of the surface of the substrate on which the reaction chambers are formed from the environment. As referred to herein, "physical isolation" refers to the creation of a barrier which substantially prevents physical transfer of reactants, amplification reaction products (e.g., amplicons), or contaminants to and from the reaction chambers. For example, such transfer includes loss of reactants or reaction products to the air or to surrounding surfaces of the microplate through, e.g., evaporation. In one embodiment, the cover is also facilitates physical isolation between reaction chambers, i.e., so reactants or amplification products are not transferred from between adjacent reaction chambers and so creating cross-contamination. Such physical isolation may be effected by the cover alone or with other elements of the microplate assembly or amplification equipment.

[0177] In various embodiments, the cover comprises a substantially planar cover substrate, having a substantially planar upper and lower surfaces, wherein the dimensions of the planar surfaces in the x- and y-dimensions are substantially greater than the thickness of the substrate in the z-direction. Cover substrate materials among those useful herein comprise

glass, silicon, quartz, nylon, polystyrene, polyethylene, polycarbonate, copolymer cyclic olefin, polycyclic olefin, cellulose acetate, polypropylene, polytetrafluoroethylene, metal, and combinations thereof. In one embodiment, the substrate comprises glass.

[0178] In various embodiments, a surface of the cover substrate is coated with a sealing material, to facilitate a uniform contact between that surface of the cover substrate, and the surface of the reaction substrate comprising the reaction chambers. Such sealing materials include compliant coatings and adhesives, such as pressure sensitive adhesives. In one embodiment, the sealing material contacts the surface of the reaction substrate surrounding each reaction chamber. In one embodiment, the amplification system (described below) comprises a clamp, a pressure chamber or a similar device operable to provide pressure onto the cover, so as to substantially seal the reaction chambers. In one embodiment, the cover having features or textures operable to interact with (e.g., by interlocking with) the opening of the reaction chambers.

[0179] For example, another embodiment contemplates real time fluorescence-based measurements of nucleic acid amplification products (such as PCR) as described, for example, in PCT Publication WO 95/30139 and U.S. patent application Ser. No. 08/235,411, each of which is expressly incorporated herein by reference. Generally, an excitation beam is directed through a sealing cover sheet into each of a plurality of fluorescent mixtures separately contained in an array of reaction wells, wherein the beam has appropriate energy to excite the fluorescent centers in each mixture. Measurement of the fluorescence intensity indicates, in real time, the progress of each reaction. For purposes of permitting such real time monitoring, each sheet in this embodiment is formed of a heat-sealable material that is transparent, or at least transparent at the excitation and measurement wavelength(s). In an embodiment, the heat-sealable sheet, in this regard, is a co-laminate of polypropylene and polyethylene. Other heat sealing materials may be employed in the present invention.

[0180] In order to seal assay 38 within each individual well 34, 34', sealing cover 24 is generally disposed across microplate 22, 22' to provide a sealing engagement between sealing cover 24 and microplate 22, 22' along a

sealing interface 58 (Figures 11, 18, and 19). Sealing cover 24 may be disposed in an optional depression 60 (Figure 20) formed in main body 40 of microplate 22, 22' to promote proper positioning of sealing cover 24 relative to the plurality of wells 34, 22'.

[0181] Sealing cover 24 is made of a generally transparent material and includes a means to seal sealing cover 24 to microplate 22. Sealing cover 24 seals wells 34, 34' and its contents (i.e. assay 38) from adjacent wells 34, 34', thus keeping sample integrity between wells and preventing cross contamination between wells. Sealing cover 24 is an optically clear film that may be abrasion resistant. Sealing cover 24 may be made such that it is able to withstand application to microplate 22 without distortion, cracking, or stretching. Sealing cover 24 may also have water impermeable-moisture vapor transmission values below 0.5 (cc-mm)/(m2-24hr-atm). In some embodiments, sealing cover 24 may also include an optic element such as a lens, lenslet, or a holographic feature. In certain embodiments, the sealing cover may be a liquid such as, for example, an oil.

[0182] Sealing cover 24 may have a high optical clarity and low fluorescence when using an excitation light, for example, 470 nm, and when detected in the UV, visible, and/or infrared spectrum. Ideally, but not necessarily, sealing cover 24 should maintain its properties in a temperature range of 4°C to 99°C and should not have inclusions (light blocking specks) greater than 50μm, scratches, or striations.

[0183] A surface of sealing cover 24 is coated with a sealing material to facilitate a uniform contact between the surface of sealing cover 24 and the surface of microplate 22. Such sealing materials may include compliant coatings and adhesives, such as pressure sensitive adhesives. Hot melt adhesive may also be used, although heat transfer to assay 38 may occur during application of sealing cover 24. Such heat transfer may cause sample cross-contamination between wells or increased evaporation. Pressure sensitive adhesives (PSA) provide an advantage due to their ease of application at low temperatures. PSA may be softened to assist in the spreading thereof during installation of sealing cover 24. If PSA is used in conjunction with PCR, PSA should be PCR compatible and processed free of nucleases, DNA and RNA, and further exhibit low florescence. Any sealing

material used may have thermal conductivity characteristics and/or electrical conductivity characteristics. Additionally, the sealing material should withstand rapid thermal cycling processes, have low water absorption properties, and have the ability to remain wetted with aqueous solutions when heated to 99°C.

[0184] For uses in PCR, any adhesive used should maintain an adhesive bond with microplate 22 when in contact with water at 99°C. The adhesive should maintain an adhesion of about 2.0 lbf per inch but should not be less than about 0.9 lbf per inch at 95°C. This requires the adhesive to have initial tack strength at room temperature to contain the sample within the well. This adhesive bond prevents, or at least minimizes, sample vapor from escaping the well by either direct evaporation or permeation of water/sample through the adhesive. In some applications it is desirable that the adhesive maintain adhesion of sealing cover 24 to microplate 22 in cold storage at 2°C to 8°C range (non-freezing conditions) for 48 hours.

[0185] An example of PSA that is commercially available that may be applicable for this invention is ARclear® DEV-8932 available from Adhesives Research, Glenrock, PA. Examples of commercially available sealing cover 24 with adhesive coatings included are GL-326™ and GL-327™ available from G and L Precision Die Cutting, Inc., San Jose, CA and ABI Prism® Optical Adhesive Sealing Cover, available from Applied Biosystems, Foster City, CA.

[0186] In some of the embodiments of the present invention, sealing cover 24 comprises multi-layers as illustrated in Figure 46. The multi-layered sealing cover comprises a friction reduction film 350, a base stock 351, a compliant layer 352, a PSA 354, and a release liner 355. The friction reduction film 350 may be Teflon or a similar friction reduction layer which is peeled off and removed after the sealing cover is applied to the microplate 22 and before the microplate 22 is placed in a PCR apparatus 10. The base stock 351 may be a scuff resistance and water impermeable layer with low to no fluorescence. The compliant layer 352 may be a soft silicone elastomer or other material known in the art that is deformable to allow the PSA to conform to irregular surfaces of the microplate. In various embodiments, the compliant

layer allows the PSA to flow around peaks of the microplate to increase bond area and resist delamination of the sealing cover. The PSA 354 is described above. In some embodiments, the PSA and the compliant layer may be one layer if the PSA has compliance. The release liner 355 is removed prior to employing the sealing cover. Once release liner 355 is removed, PSA 354 is exposed and the sealing cover may be tacky to the touch. The release liner 355 is removed in order for sealing cover to used on microplate 22.

[0187] In order to improve sealing of the wells of microplate 22, various treatments to the microplate may enhance the effectiveness of sealing cover 24 to microplate 22. In various embodiments, the microplate 22 may be made of a hydrophobic material. In other embodiments, the microplate 22 may be treated with a hydrophobic coating such as a fluorocarbon, PTFE coating or the like. In these embodiments, the hydrophobic material or coating helps eliminate water molecules that compete with the adhesive on the sealing cover 24. In other embodiments of the microplate 22, grooves are added around the edge as shown in Figures 13-17 and 38. In these embodiments, the grooves provide seal adhesion support on the outer edges of the sealing cover 24. In these embodiments, a pressure chamber gasket can be sealed against the grooved area providing an improved seal. Strain relief grooves may be added to the bottom of the microplate 22 below the grooves around the outer edges of the top of the microplate 22. In some embodiments, the grooves provide improved sealing of the gasket of the pressure chamber 104 on the sealing cover 24.

[0188] In various embodiments, microplate 22 can include well 34" which includes a blind hole 31 and contains solution 36. Blind hole 31 is of a small enough diameter that it does not fill due to the surface tension of solution 36. When solution 36 is heated during thermal cycling, the solution expands and the expanded solution fills into the blind hole 331, thus minimizing pressure on sealing cover 24. In various embodiments, well 34" may have multiple blind holes 31. In other embodiments, blind hole 331 is offset in well 34" so that well 34" may be filled with solution 36 via a micropiezo dispenser 336 as shown in Figure 43. In alternate embodiments, the blind hole 331 may be offset in well 34" so that well may be spotted using a spotting device 335 leaving a material 337 at the bottom of the well 34". In

certain embodiments, the blind hole 331 is not a hole but rather a small volume that is not vented so that the capillary forces and the surface tension of the solution 36 does not allow the small volume to fill. In certain embodiments, the top edge 333 of blind hole 331 is sharp, such sharpness helps prevent the solution 36 from entering the blind hole 331 at standard temperature and pressure.

[0189] In some embodiments of microplate 22, well 34''' may be a through hole as illustrated in Figure 44. In still other embodiments, microplate 22, well 34''' may have a molded clear top 338. With the above discussed embodiments, microplate 22 with either the through hole or the clear bottom may be filled with a solution then sealed with a foil seal 339. In these embodiments, foil seal 339 is placed against thermal cycler block 18. In such embodiments, the PSA sealing the foil 339 may be clear or may be opaque. In embodiments that a clear seal 338 is used as a separate piece from microplate 22 through hole wells 34''', a layer of mineral oil may be placed at the top of the filled well 34''' before transparent cover 338 is placed on microplate 22. In these embodiments, the clear mineral oil fills the chamber space of the well 34''' and provides the optical interface and evaporation control by overlaying the sample. In still other embodiments, foil seal 339 may be spotted using a spotting device 335 and spotting a material 337 onto foil seal 339 at the bottom of well 34'''. In such embodiments that the transparent top 338 is molded into microplate 22, sample is loaded into well 34''' when microplate22 is upside down with transparent cover 338 sitting on bench surface. Once samples have filled all wells 34''', foil seal 339 is placed on microplate 22 and microplate 22 is turned right side up so that the foil side of the microplate 22 may be placed on thermal cycler block 18. In certain embodiments that include foil seal 339, heat transferred from thermal cycler block 18 to microplate 22 is improved and is more uniform.

[0190] In certain embodiments, cover seal 24 is laminated onto microplate 22. In such embodiments, a hot roller apparatus 334, such as illustrated in Figure 45, may be used. The hot roller apparatus 334 has a top roller 342 that has a heater on it 341 and bottom roller 344 is unheated. A microplate 22 with cover seal 24 placed on top is put into hot roller apparatus 340 so that cover seal 24 is in contact with the first plate guide 345. The

sealing cover 24 on microplate 22 passes by heated roller 342 to the top roller to laminate the cover 24 to the microplate 22. The laminated microplate exits hot roller apparatus 334 as it goes by guide plates 343. In such embodiments, the heat from the roller helps reduce the viscosity of the adhesive to allow it to better adhere to the surface of the microplate 22. Standard lamination devices that are available commercially have heated bottom rollers that transfer heat to the bottom of the microplate, thus causing the sample in the microplate to heat. Hot roller apparatus 334 only has a heated roller on the top and that roller comes in contact with the sealing cover. Heated roller apparatus 334 allows better adhesive flow or softening without damaging the sample since the heat is only applied to the adhesive. In the embodiments of hot roller apparatus 334, the heating element 341 may be controlled so that the temperature of the top roller 342 may be adjusted. Hot roller apparatus 334 has variability in temperature of the top roller 342 and speed of the rollers 342, 344 as well as the clamping force between rollers 342, 344. By varying these parameters, optimal sealing of sealing cover 24 to microplate 22 may be achieved with minimal negative effects to any sample inside. In some embodiments, the bottom roller may be replaced by a moving platen.

[0191] Pressure clamp system 26 is operable to apply a force upon sealing cover 24, microplate 22, and thermocycler block 30 to operably hold microplate 22 in thermal contact with thermocycler block 30. Pressure clamp system 26 may be configured in any one of a number of orientations, such as described below. Additionally, pressure clamp system 26 may include any one of a number of components depending upon the specific orientation used. Therefore, it should be understood that variations exist that are still regarded as being within the scope of the present invention.

[0192] As best seen in Figure 20, pressure clamp system 26 may include an inflatable transparent bag 62 is positioned between and in engaging contact with transparent window 28 and sealing cover 24. In the embodiment illustrated in Figure 20, transparent window 28 and thermocycler block 30 are fixed in position against relative movement. Inflatable transparent bag 62 includes an inflation/deflation port 64 that is fluidly coupled to a pressure source 66, such as an air cylinder, which is controllable in

response to a control input from a user or control system 32. It should be understood that transparent bag 62 may include a plurality of inflation/deflation ports to facilitate inflation/deflation thereof. Upon actuation of pressure source 66, pressurized fluid, such as air, is introduced into inflatable transparent bag 62, thereby inflating transparent bag 62 in order to exert a generally uniform force upon transparent window 28 and, more importantly, upon sealing cover 24 and microplate 22. Such generally uniform force serves to provide a reliable and consistent sealing engagement between sealing cover 24 and microplate 22. This sealing engagement substantially prevents water evaporation or contamination of assay 38 during thermocycling. In certain embodiments, bag 62 is part of the transparent window 28 forming a bladder.

[0193] Still referring to Figure 20, it should be appreciated that transparent window 28, inflatable transparent bag 62, and sealing cover 24 should permit the free transmission therethrough of an excitation light 68 generated by a source 71 and the resultant fluorescence. Therefore, transparent window 28, inflatable transparent bag 62, and sealing cover 24 may be made of a material that is non-fluorescent. By way of non-limiting example, transparent window 28 may be made from vycor, fused silica, quartz, high purity glass, or combination thereof. In some embodiments, the transparent window 28 may include optical elements such as a lens, lenslets or a holographic feature.

[0194] Referring now to Figure 21, a transparent window 28' may be used that is movable to exert a generally uniform force upon a transparent bag 62' and, additionally, upon sealing cover 24 and microplate 22. That is, transparent bag 62' is provided having a fixed internal amount of fluid, such as air. Transparent window 28' is movable using any moving mechanism (not shown), such as an electric drive, mechanical drive, hydraulic drive, or the like.

[0195] Referring now to Figures 24-29, pressure clamp system 26 may further employ a pressure chamber 100 in place of transparent bag 62, 62'. More particularly, pressure chamber 100 eliminates the need for a flexible transparent bag that could potentially limit the transmission of fluorescence and/or the excitation beam.

[0196] Pressure chamber 100 is a pressurizable volume generally defined by transparent window 28', a frame 102 coupled to and descending from transparent window 28', and a circumferential chamber seal 104 disposed along an edge of frame 102. Chamber seal 104 is adapted to engage a surface to define the pressurizable, airtight, or at least low leakage, pressure chamber 100. Transparent window 28', frame 102, circumferential chamber seal 104, and the engaged surface bound the actual volume of pressure chamber 100. Chamber seal 104 may engage one of a number of surfaces that will be discussed in detail below. A port 64' provides fluid communication between pressure chamber 100 and pressure source 66.

[0197] In the interest of brevity, it should be appreciated that the particular configuration and arrangement of sealing cover 24 and microplate 22, 22' illustrated in Figures 24-29 are similar to those illustrated in Figures 20-23. Therefore, further discussion thereof is not deemed necessary.

[0198] With particular reference to Figures 24 and 26, it can be seen that chamber seal 104 may be positioned such that it engages a portion of sealing cover 24. In this regard, a downward force from transparent window 28' is exerted upon microplate 22 to maintain a proper thermal engagement between microplate 22 and thermocycler block 30. Additionally, such downward force further facilitates sealing engagement with sealing cover 24. Pressure chamber 100 is then pressurized to exert a generally uniform force upon sealing cover 24. Such generally uniform force serves to provide a reliable and consistent sealing engagement between sealing cover 24 and microplate 22. This sealing engagement substantially prevents water evaporation or contamination of assay 38 during thermocycling. It should be appreciated that this fluid pressure within pressure chamber 100 provides a consistent pressure across the sealing interface 58.

[0199] As seen in Figure 25, microplate 22 may be positioned in an inverted orientation similar to that described in connection with Figure 22. Chamber seal 104 is positioned such that it engages a portion of sealing cover 24. In this regard, a force from transparent window 28' is exerted upon microplate 22 to maintain a proper thermal engagement between microplate 22 and thermocycler block 30' and sealing engagement between sealing

cover 24 and microplate 22. Pressure chamber 100 is then pressurized to exert a generally uniform force.

[0200] With particular reference to Figures 27 and 28, it should be appreciated that chamber seal 104 of pressure chamber 100 may be positioned to engage thermocycler block 30, rather than microplate 22, 22'. In this regard, microplate 22, 22' is positioned within pressure chamber 100. As pressure chamber 100 is pressurized, force is exerted upon sealing cover 24, 24', thereby providing a sealing engagement between sealing cover 24, 24' and microplate 22, 22'. However, in some instances, the embodiment illustrated in Figure 28 may not afford sufficient thermal contact between microplate 22 or 22' and thermocycler block 30. Therefore, to improve such thermal contact between microplate 22, 22' and thermocycler block 30, optional posts 108 may be employed. Optional posts 108 are adapted to be coupled with transparent window 28' and downwardly extend therefrom. Optional posts 108 then engage at least one of microplate 22, 22' or sealing cover 24 to ensure proper contact between microplate 22, 22' and thermocycler block 30 during thermocycling.

[0201] As best seen in Figure 27a, these arrangements may be modified to include a vacuum assist system 130. In this regard, port 64' may be eliminated. Vacuum assist system 130 generally includes a pressure/vacuum source 132 fluidly coupled to a vacuum channel 134, which extends throughout thermocycler block 30. Vacuum channel 134 may be grooves or, alternatively, may include a porous or permeable section of thermocycler block 30. Vacuum channel 134 is evacuated so as to form a vacuum within a volume 136 defined by transparent window 28, O-rings 138, and thermocycler block 30. Upon actuation of pressure source 132, a vacuum is formed in vacuum channel 134. This vacuum vacates volume 138 causing outside air pressure to exert a clamping force on transparent window 28, thereby clamping sealing cover 24 against microplate 22 to ensure a proper seal and further clamping microplate 22 to thermocycler block 30 to ensure a proper thermal contact. It should be understood that vacuum assist system 130 may be formed in transparent window 28. It will be appreciated that vacuum assist systems may be employed in any of the embodiments described in Figures 20-30.

[0202] Turning now to Figure 29, another embodiment is illustrated employing a relief port 110 in fluid communication with pressure chamber 100. Relief port 110 is operable to slowly bleed gas in pressure chamber 100 and/or simultaneously remove water vapor from pressure chamber 100 to reduce condensation. Removal of water vapor is desirable as it may improve fluorescence detection. Relief port 110 may be used in connection with any of the embodiments described herein.

[0203] As best seen in Figure 30, it should be appreciated that transparent window 28, 28' may include a heating device 112. Heating device 112 is operable to heat transparent window 28, 28', which in turns serves to heat each well 34 to minimize the formation of condensation within wells 34. Condensation is believed to reduce optical performance and, thus, reduce the efficiency and/or stability of fluorescence detection.

[0204] Heating device 112 may include a layer member 114 that is laminated to transparent window 28, 28'. Layer member 114 may include a plurality of heating wires (not shown) distributed uniformly throughout layer member 114, which are each operable to heat an adjacent area. It should be understood that such heating wires may partially obstruct fluorescence transmissions. This may be acceptable in some applications. However, in applications where a more uniform fluorescence transmission is required, layer member 114 may be an indium tin oxide coating that is applied uniformly across transparent window 28, 28'. A pair of bus bars 116 are each disposed on opposing ends of transparent window 28, 28'. Electrical current may then be applied between bus bars 116 to heat the indium tin oxide coating, which provide a consistent and uniform heat across transparent window 28, 28' without interfering with fluorescence transmission. Bus bars 116 may be controlled in response to control system 32. In some embodiments, heating device 112 may be on both sides of the window. It should be appreciated that heat device 112 may be employed in any of the embodiments described in Figures 20-30.

[0205] By way of background, microplates or microtiter plates used for DNA and assays are often serialized with adhesive bar code labels that permit users to uniquely identify plates. Similarly, other analytical containers, such as individual tubes, strip tubes, and reservoirs are also labeled with a

bar code. Employing such bar codes permits the user to readily identify the contents of each container. Occasionally, such adhesive bar code labels are sufficient for biological applications. However, in many cases, such adhesive bar code labels interfere with the performance of the consumables. In these instances, it is advantageous to employ alternative methods of serializing consumables. Accordingly, alternative serialization has been found that is particularly useful in accordance with the present invention.

[0206] Microplate 22 may include marking indicia 190, such as graphics, machine readable codes (i.e. bar codes, etc.), text, logos, and the like. As referred to herein, "graphics" refers to any printing, lithograph, pictorial representation, symbol, bar code, handwriting, or any other type of writing, drawing, etching, indentations, embossments or raised marks. Marking indicia 190 is used for identification of microplates 22 to facilitate identification during processing. Such identification may be used to track preparation sequences for analysis. Furthermore, marking indicia 190 may be used for data collection such that microplates 22 can be positively identified to properly correlate acquired data with the corresponding sample. Still further, marking indicia 190 could be used for alignment where a symbol or other machine-readable graphic is put on microplate 22 such that an optical sensor or optical eye can read the location of microplate 22 and adjust the positioning of microplate 22 to permit service of wells 34 in microplate 22. The marking indicia 190 are employed as part of Good Laboratory Practices (GLP) and Good Manufacturing Practices (GMP).

[0207] Marking indicia 190 may be printed upon microplate 22 using any known printing system, such as inkjet printing, pad printing, hot stamping, and the like. Additionally, for light colored microplates 22, a dark ink may be used to create marking indicia 190. Likewise, for dark colored microplates 22, a light ink may be used to create a "reverse" indicia.

[0208] It is anticipated that microplate 22 may be made of polypropylene. Polypropylene is generally difficult to print upon, thereby a surface treatment is desired, such as flame treatment or corona treatment, treating with a surface primer and acid washing. Alternatively, a UV-curable ink may be used for printing on polypropylene microplates.

[0209] Laser activated pigment may be added to microplate material to obtain improved contrast between the graphic and the substrate. For example, an antimony-doped tin oxide pigment that is easily dispersed in polymers with marking speeds as high as 190 inches per second may be achieved. This pigment absorbs laser light and provides the necessary conversion of laser energy to thermal energy for most YAG laser marking applications, for example, Englehard Mark-It Laser Marking Pigment.

[0210] Furthermore, marking indicia 190 may be printed upon microplate 22 using a CO_2 laser marking system. To this end, laser marking systems are operable to evaporate material from a surface of microplate 22. CO_2 laser etching typically minimizes color changes of marking indicia 190 relative to the remaining portions of microplate 22. Therefore, if desired, a YAG laser system may be used, which provide improved contrast and reduced material deformation.

[0211] Still further, radio frequency identification (RFID) tags may be used to electronically identify microplate 22. To this end, RFID tags 192 may be attached or molded within microplate 22. An RFID reader (not shown) may be integrated into high-density sequence detection system 10 to automatically read the unique identification and/or data handling parameters of microplate 22. Also see U.S. Patent Application Serial No. 10/85,093 filed March 19, 2004.

[0212] As can be appreciated, marking indicia 190 provides a number of advantageous. Specifically, marking indicia 190 provides high-quality identification that becomes part of microplate 22 or other consumable that can not be easily removed or modified. Furthermore, marking indicia 190, especially as a bar code, minimizes any thermal interference during thermal cycling. Still further, the use of marking indicia 190 reduces labor associated with applying prior art labels and further eliminates the potential incompatibility between reagents and label adhesives. Lastly, RFID tags 192 provide an additional advantage in that they do not require line-of-sight for readability.

[0213] As best seen in Figures 2-8, excitation and detection system 14 generally includes an excitation assembly 200 for generating excitation light 68 and a detection assembly 300 for detecting and measuring a resultant

fluorescence. Excitation assembly 200 generally comprises a plurality of excitation lamps 210 generating excitation light 68 in response to control system 32. Excitation assembly 200 may direct excitation light 68 to a single well 34 or across a plurality of wells 34. Excitation light 68 may be a radiant energy having a wavelength that permits detection of photo emitting probes in assay 38 disposed in wells 34 of microplate 22.

[0214] By way of background, it should be understood that the accuracy of the analysis of assay 38 is often dependent upon how accurately the resultant fluorescence intensity can be measured. The fluorescence from wells 34 on microplate 22 are often measured simultaneously using a CCD camera. In a perfect optical system, if all of the wells had the same concentration of dye, each of the wells would produce the identical fluorescence signal. However, in prior art designs, wells near the center of the microplate may appear significantly brighter (i.e. output more signal) than wells near the edge of the microplate despite the wells outputting the same amount of fluorescence. There are several reasons for this condition in current designs—vignetting, illumination/irradiance profile, and shadowing.

[0215] Briefly, with respect to vignetting, camera lens often collect more light from the center of the frame relative to the edges. This may reduce the efficiency of the detection system. Additionally, the irradiance profile is often not uniform. Most commercially available irradiance sources have a greater irradiance (watts/meter^2) value near the center compare to the edges of the irradiance zone. For a given dye, until the dye saturates or bleaches, the amount of fluorescence is proportional to the irradiance of the illumination source. Therefore, if the excitation light is brighter at the center, then the fluorescence signal from a well near the edge would be dimmer than an identical well near the center. Lastly, shadowing can occur due to the depth of the wells. That is, unless the excitation light is perpendicular to the plate, some part of the well will not be properly illuminated. In other words, the geometry of the well will block some of the light from reaching the bottom of the well. In addition, the amount of light emitted which can be collected will vary from center to edge. As should be appreciated by one skilled in the art, noise sources are often constant across the field of view of the camera. Therefore, for wells near the edges that output a smaller amount of

fluorescence, the signal to noise ratio is reduced, thereby reducing the efficiency of the system. As best seen in Figure 31, a graph illustrates the relative intensity or light transmission versus well location. As can be seen from the graph, the effects of vignetting and shadowing causes the light intensity to drop off along the edges of field.

[0216] The present invention overcomes, or at least minimizes, these effects such that identical wells output generally identical fluorescence irrespective of their location. By using the profile from Figure 31, the optimum irradiance profile can be calculated. With reference to Figure 32, a corresponding irradiance profile, represented by dashed line, can be used having a higher irradiance along the edges. This irradiance profile, when coupled with the effects of vignetting and shadowing, creates generally uniform signal strength across all of wells 34 of microplate 22.

[0217] As seen in Figures 2-8 and 47, the plurality of excitation lamps 210 of excitation assembly 200 are fixedly mounted to a support structure 212. Support structure 212 is generally planar in construction and is adapted to be mounted within housing 16. The plurality of excitation lamps 210 may be arranged in a generally circular configuration and directed toward microplate 22, 22' to promote uniform excitation of assay 38 in wells 34. Unlike prior art designs that illuminate an assay from a single position or from a single side, the present invention permits a generally uniform excitation that is free of shadowing. The plurality of excitation lamps 210 are arranged in a generally circular configuration about an aperture 214 formed in support structure 212. Aperture 214 permits the free transmission of fluorescence therethrough for detection by detection assembly 300, as will be described below. Other methods can also be used to create the illumination profile holographic diffuser. See U.S. Patent No. 6,744,502 to Hoff issued June 1, 2004.

[0218] As best seen in Figures 33-37, each of the plurality of excitation lamps 210 may be configured to achieve the desired irradiance profile. Specifically, as seen in Figure 33, each excitation lamp 210 may include a lens 216. Lens 216 is shaped to provide a desired irradiance profile (see Figure 32). The exact shape of lens 216 is dependent upon the desired irradiance profile at microplate 22, the illumination/irradiance profile at

excitation lamps 210, and the size and position of microplate 22 relative to excitation lamps 210. The shape of lens 216 may be calculated in response to the particular application using commercially available software, such as ZEMAX and/or ASAP.

[0219] As seen in Figure 34, each excitation lamp 210 may include a mirror 218. Mirror 218 is shaped to provide a desired irradiance profile (see Figure 32). The exact shape of mirror 218 is dependent upon the desired irradiance profile at microplate 22, the illumination/irradiance profile at excitation lamps 210, and the size and position of microplate 22 relative to excitation lamps 210. The shape of mirror 218 may be calculated in response to the particular application using commercially available software, such as ZEMAX and/or ASAP.

[0220] Turning now to Figure 35, each excitation lamp 210 may include a combination of lens 216 and mirror 218 to achieve the desired irradiance profile. Again, lens 216 and mirror 218 may be calculated in response to the particular application using commercially available software, such as ZEMAX and/or ASAP.

[0221] As described above, excitation assembly 200 may include a plurality of excitation lamps 210. It is anticipated that each excitation lamp 210 may be of conventional intensity distribution wherein a higher irradiance is produced along the center of the beam relative to the edges. Moreover, as seen in Figure 36, each of the plurality of excitation lamps 210 may be aligned such that their optical centers converge on a single point 220. Although this configuration is regarded as being within the scope of the present invention, a desired irradiance profile (see Figure 32) may be achieved by directing each of the plurality of excitation lamps 210 at a predetermined location 222a-222*n*. Accordingly, excitation lamps 210 having a conventional intensity distribution may be aligned to provide the desired irradiance profile (see Figure 32). Still further, excitation lamps 210 may include lens 216 and/or mirror 218 and further be aligned as illustrated in Figure 37 to achieve more complex irradiance profiles. As can be appreciated, employing any of the above techniques described herein provides improved irradiance across microplate 22, thereby improving the result signal to noise ratio of wells 34 along the edge of microplate 22.

[0222] It is anticipated that excitation lamps 210 may be any one of a number of sources. By way of non-limiting example, excitation lamp 210 may include a laser transmitting light of a wavelength of about 488 nm, an Argon ion laser, an LED, a halogen bulb, or any other known source. In some embodiments, the LED is a MR16 design from Opto Technologies (Wheeling IL; http://www.optotech.com/MR16.htm). In other embodiments, the LED is provided by LumiLEDS (San Jose CA; http://www.lumileds.com/) In still other embodiments, the halogen is essentially similar to the 75 W, 21 V dc lamps that are used by the 7000 and 50 W, 12 V D/C lamps and to those used for 7300 and 7500 instruments (Applied Biosystems, Foster City CA). The LEDs may be controlled in several ways, including temperature to prevent intensity and spectral shift. The intensity can be controlled via a photodiode feedback system, utilizing PWM (pulse width modulation) control to modulate the power of the LED. In some embodiments, the PWM is digital. In various embodiments, the sources may include a means for temperature control. In such embodiments, the temperature control may be a cooling device. In such embodiments, the temperature control may hold lamp at an essentially constant temperature. It should be appreciated that any of the excitation sub-assemblies shown in Figures 2-8 and 47 described above may be interchanged with each other and for instance, the inverted optical bench may also employ any of the excitation sub-assemblies illustrated in Figures 2-5 and 47. In certain embodiments, shutters are included for each of the sources.

[0223] As best seen in Figures 1-4 and 8, detection system 300 of excitation and detection system 14 is used to detect and/or gather fluorescence emitted from assay 38 during analysis. To this end, detection system 300 include a collection mirror 310, a filter assembly 312, and a collection camera 314. After the excitation light passes through the lenses into the sample well of the microplate 22, the sample in the well is illuminated, thereby exciting a probe generating an emission of light a different wavelength which is detected by the detector 300 of the optical system. In accordance with various embodiments of the present invention, a detection system is provided for analyzing emission light from the reaction chambers. In accordance with various embodiments, the optical system includes a light

separating element such as a light dispersing element. Light dispersing elements include elements that separate light into its spectral components, such as transmission gratings, reflective gratings, prisms, and combinations thereof. Other light separating elements include beam splitters, dichroic filters, and combinations thereof that are used to analyze a single bandpass wavelength without spectrally dispersing the incoming light. In embodiments with a single bandpass wavelength light processing element, the optical detection device is limited to analyzing a single bandpass wavelength, thereby one or more light detectors each having a single detection element may be provided. In various embodiments, the optical detection system may further include a light detection device for analyzing light from a sample for its spectral components. In various embodiments, the detector 300 is a light detection device comprising a multi-element photodetector 314. Examples of multi-element photo detectors include, but are not limited to, charge coupled devices (CCDs), diode arrays, photomultiplier tube arrays, charge injection devices (CIDs), CMOS detectors and avalanche photodiodes. In certain embodiments, the photodetector is a CCD camera. In some embodiments, the emission light may be focused on the multi-element photo detector 314 by a lens. In some embodiments, the detector 300 comprises an ORCA-ER cooled CCD 314 with a lens (f = 50 mm, f# = 2.0) (available from Hamamatsu, Japan). In such embodiments, the fold mirror 310 is an Aluminum 120 mm diameter mirror with 1/4 or 1/2 wave flatness and 40/20 scratch dig first surface (available from JML Optics, Rochester NY or Edmund Optics, Barrington NJ). In various embodiments, mirrors may be used to collect the emission light or to direct the emission light towards the multi-element photo detector 314. In an embodiment of the invention, a detector 300 is used in combination with a filter wheel 312. With the filter wheel 312, the microplate 22 is scanned numerous times, each time with a different filter. In an embodiment, the contents of the entire plate are imaged simultaneously. In some embodiments, the CCD is mounted to the lens and prealigned.

[0224] In one embodiment, such a suitable apparatus comprises a platform for supporting a microplate of this invention; a focusing element selectively alignable with an area (e.g., reaction chambers) on a microplate; an excitation (light) source to produce an excitation beam that is focused by

the focusing element into a selected reaction chambers when the focusing element is in the aligned position; and a detection system to detect a selected emitted energy from a sample placed in the reaction chambers. In embodiments of this invention, the focusing element is selectable in an aligned position or an unaligned position relative to at least one of said reaction chambers. Also some embodiments include at least one of said the platform and the focusing element that rotates about a selected axis of rotation to move the focusing element between the aligned position and the unaligned position. Apparatus among those useful herein are described, for example, in U.S. Patent 6,015,674, Woudenberg et al., issued January 18, 2000; U.S. Patent 6,563,581, Oldham et al., issued May 13, 2003; and U.S. Patent Application Publication 2003/0160957, Oldham et al., published August 28, 2003.

[0225] In embodiments of this PCR system, the system additionally comprises a microprocessor 32 operable to control the system and to collect data. In such embodiments, the microprocessor 32 also comprises software and devices operable for data collection; for coordination of electronic, mechanical and optical elements of the system; and for thermal cycling. In such embodiments, data analysis includes organization, manipulation and reporting of measurements and derived quantities necessary to determine relative gene expression within the sample, between samples, and across multiple runs, and the ability for data archiving, data retrieval, database analysis and bioinformatics functionality from the data collection data analysis. Analysis of raw data can include compensating for (PSF), background, intensity profile, optical crosstalk, detector and optical path variability and noise, misalignment, movement during operation. This may be accomplished utilizing internal controls in several wells, as well as calibrating the system. The data analysis may include different imaging or image subtracting. Such data analysis may include difference imaging which is comparing an image from one point in time to the image at a different time. Still other data analysis may include curve fitting based on a specific gene or a gene set. Data analysis may include using NTC (no template control) background or baseline correction. Other data analysis may include error estimation which includes confidence values in terms of Ct, for example. See U.S. Patent Application

No. 60/517,506 filed November 4, 2003 and AB Docket 5043 USP2 filed November 10, 2003.

[0226] In various embodiments, the present teachings can provide a system for detecting one or more identifiable signals associated with one or more biological samples, the system including a segmented detector including a plurality of pixels that are capable of forming an optical image of fluorescent light emitted from the biological samples, a readout component that is capable of reading an output signal from each pixel, wherein the output signal includes a charge collected and transferred from the pixel and wherein the readout component includes an output register that receives transferred charges from the plurality of pixels for readout, a controller that is capable of correcting signal noise from the output signal, wherein signal noise includes a dark current contribution and a readout offset contribution, a processor capable of determining the dark current contribution and the readout offset contribution.

[0227] In various embodiments, the present teachings can provide a method for reducing signal noise from an array of pixels of a segmented detector for biological samples, wherein the signal noise includes a dark current contribution and readout offset contribution, the method including providing a substantially dark condition for the array of pixels, wherein the dark condition includes being substantially free of fluorescent light emitted from the biological samples, providing a first output signal from a binned portion of the array of pixels by collecting charge for a first exposure duration, transferring the collected charge to an output register and reading out the register, wherein transferring of the collected charge from the binned pixels includes providing a gate voltage to a region near the binned pixels to move collected charge from the binned pixels, and wherein the collected charge is transferred in a manner that causes the collected charge to be shifted to the output register, providing a second output signal from each pixel by collecting charge for a second exposure duration, transferring the collected charge to the output register, and reading out the register, providing a third output signal by resetting and reading out the output register, determining the dark current contribution and the readout offset contribution from the first output signal, the second output signal, and the third output signal.

[0228] In various embodiments, the present teachings can provide a method of characterizing signal noise associated with operation of a charge-coupled device (CCD) adapted for analysis of biological samples, wherein the signal noise includes a dark current contribution, readout offset contribution, and spurious change contribution, the method including providing a plurality of first data points associated with first outputs provided from the CCD under a substantially dark condition during a first exposure duration, providing a plurality of second data points associated with second outputs provided from the CCD under the substantially dark condition during a second exposure duration wherein the second duration is different from the first duration, providing a plurality of third data points associated with third outputs provided from a cleared output register of the CCD without having charge transferred thereto, determining the dark current contribution per unit exposure time by comparing the first data points and the second data points, determining the readout offset contribution from the third data points, and determining the spurious charge contribution based on the dark current contribution and the readout offset contribution. See U.S. Patent Application No. 10/913,601 filed August 5, 2004; U.S. Patent Application 10/660,460 filed September 11, 2003 and U.S. Patent Application No. 10/660,110 filed September 11, 2003.

[0229] Referring to Figure 55, plated reactions 531 are analyzed on instrument 532 and data is collected and manipulated. The processed data is sent to database 533 and software is used to generate information 534.

[0230] A gene expression analysis system may be built using computer software that organizes analysis sessions into studies and stores them in a database. An analysis session can be though of as the results of running a plate on the instrument. To analyze session data, one can load an existing study that contains analysis session data or create a new study and attach analysis session data to it. Studies may be opened and reexamined an unlimited number of times to reanalyze the analysis session data or to add other analysis sessions to the analysis. Figure 55 illustrates the data flow for a typical study.

[0226] The gene expression analysis system database stores the analyzed data for each plate run on the instrument as an analysis session on the database. The software can identify each analysis session

by the bar code of the associated plate and the date on which it was created. Once analysis sessions have been assigned to a study, various functions can be performed. These include but are not limited to designating replicates, removing outliers, filtering data out of a particular view or report, correction of preamplification values via stored values and computation of gene expression values.

[0227] Real time PCR assays typically measure relative quantities of DNA template using the Ct value from the PCR growth curve. The measured Ct value for a sample for a given assay typically varies depending on the instrument it is measured on and the microplate in which the PCR assay is run. Such variation can arise from manufacturing differences in the instrument detection system and thermal nonuniformity produced by variations in the production of the microplate.

[0228] A common method to reduce nonuniformity is to allocate a percentage of the wells in the microplate for use as controls. Such controls usually assay a DNA template that is present in every sample that would be measured in the consumable, such as GAPDH or some other "housekeeping" gene for human samples. Samples are measured as the difference between the Ct measured for sample wells and the Ct measured in these control wells. Such a measure is commonly known as the ΔCt. Differences in the delta Ct for samples run on different microplates are commonly referred to as $\Delta\Delta$Ct. Such calculations are described above.

[0229] The use of such controls reduces the variations and non-sample related artifacts from data, resulting in a higher quality of data. However, a drawback of using naturally occurring DNA templates such as housekeeping genes is that they can also vary from sample to sample, making sample to sample comparisons qualitative.

[0230] For the high density microplate, several wells may be allocated for controls. Some of these wells may be spotted with assays that detect an artificial template. The templates would not have any sequence in common with the samples that would be run in them. The artificial template may be introduced to the wells either when the assay is

spotted and dried, mixed into the sample mix filled into the wells, or filled into the wells in an operation separate from the sample mix. The difference in these two approaches is explained below.

[0231] If the artificial template is spotted and dried into the wells at a known and well defined concentration, the Ct value measured from these wells would serve as a well known control value that would correct for instrument, plate and sample filling/pipetting variations. For this type of control, the sample is used to fill the well but the sample would not contain any template that would be amplified by the assay.

[0232] If the artificial template is filled into the wells at a known and well-defined concentration, the Ct value measured from these wells may be used to correct for variations from sample filling and pipetting.

[0233] In some embodiments, artificial templates may also be detected in sample wells as an internal control for those wells. In this case, the assay for the artificial template would produce a different signal from the sample assay for detection.

[0234] In embodiments that a preamplification method is used to amplify the sample DNA prior to the RT-PCR assay, the artificial template may also be designed such that it is preamplified. Thus, if the artificial template is introduced to the sample prior to preamplification and subsequently measured on the plate, its Ct value could be used to correct for variations in the efficiency of sample preamplification as well as pipetting errors.

[0235] In various embodiments, control wells on the plate may be allocated to contain fluorescent dye. The dye is dried down into the plates and hydrolyzed at the time of sample filling. Such wells may be used to improve calibration of the detection system for optical aberrations. In embodiments that a dye is at known concentration, the signals from these wells may be used to optimize the detection sensitivity of the system (such as the exposure time of the CCD in an imaging system). In some embodiments, a series dilution of control wells can be used as well.

[0236] Embodiments may be used as controls for identification of well position. When the sample wells on the plate are filled, there may be a passive internal reference dye (PIR) in each well such as ROX. The

signal from the PIR may be used to locate the wells in an imaging system. However, for imaging systems, there is frequently a small shift in magnification for different colors. Thus, if only a single dye is imaged, dye locations are known only for that dye. To measure the locations of wells for the assay dyes, the background fluorescence from the Taqman assays may be used. The current Taqman assays use probes that contain a quenched dye. The quenching of the dye is not 100 % efficient and as such, there is always a background signal emitted from the unquenched probes. Thus, prior to beginning the PCR assay, the background signals from these assays may be used to determine the locations of wells in an imaging system for the assay dyes.

[0237] Embodiments used as controls for filling errors. Signals from the PIR may be used to determine if sample filling errors have occurred by looking for an absent or an abnormally high or low signal in the PIR detection image or channel. These signals may indicate an empty well, or an overfilled or underfilled well, respectively. Embodiments used as controls for spotting errors. In a similar fashion to the PIR, background signals from the Taqman assays may be used to determine if spotting errors occurred by looking for an absent or an abnormally high or low signal in the assay dye detection image or channel.

[0238] In some embodiments, assays for naturally occurring templates may also be used as controls on the platform. Embodiments may be used as quality control for spotting assay. During manufacturing of the plate, it may be desirable to have a nondestructive method to determine if spotting of the wells is successful, and that the orientation of the master plate with respect to the Mustang consumable is correct. One embodiment is to measure (by imaging or scanning) the weak background fluorescence of the dried down assays to determine if the wells were spotted correctly and in the correct orientation. Another embodiment is to introduce one or more fluorescent dyes into the assay mix prior to spotting. When dried down, the fluorescent dyes may be measured to determine if spotting was performed correctly. In some embodiments, the addition of extra dyes to the spot assay mix may be useful for spotting assays that do not have an inherent fluorescent signal, as would be the case for SYBR

assays. In such embodiments, these dyes could further be used as internal controls for identifying filling and spotting errors.

[0239] In various embodiments, control wells may be placed onto a plate to test for detection of sealing failures. In one embodiment, a dye filled well may be surrounded by eight buffer wells. In the event of a seal failure, dye from central well leaks into the buffer wells. The leak is detected by an increase in signal in the buffer wells. In addition to an artificial template, any naturally occurring DNA sequence may be used for the purposes described. The template may be a synthetic oligonucleotide or plasmid, genomic DNA, or other natural DNA or RNA. The template may contain analogs of naturally occurring nucleotides with modifications to the base, sugar, or phosphate backbone, including PNAs. As such the term "exogenous template" will be used here in place of "artificial template."

[0240] In some embodiments, probes and primers designed for use as internal controls may also consist of oligonucleotides containing modifications or additions to the base, sugar, or phosphate backbone, including PNAs. If the exogenous template is introduced to a sample prior to reverse transcription and subsequently measured on the plate, its Ct value may be used to correct Ct values resulting from variations in efficiency of reverse transcription or variations in sample quantity.

[0241] In certain embodiments, multiple exogenous templates at varying relative concentrations may be added at the reverse transcription, preamplification or to the assay mixture to produce a standard curve for absolute quantitation of samples on the plate. The resulting signals may also be used to normalize data obtained from different plates or different samples on the same plate.

[0242] In various embodiments, wells without probes or primers or wells that are completely empty may be used for the purpose of background correction. Control wells without template may also be used for this purpose and for the purpose of confirming lack of well contamination by other samples. Control wells without assays may be used to confirm lack of contamination during spotting.

[0243] In still other embodiments, wells containing varying amounts of a single or multiple dye may be used to determine if the instrument is capable of detecting signal within the expected dynamic range independent of assay performance. Wells containing varying amounts of a single or multiple dyes may be used to correct for optical crosstalk or other means of signal correction or normalization. In certain embodiments, pin hole arrays are used for optical calibration.

[0244] In certain embodiments, all the controls described above may be incorporated into a single plate to be used to verify instrument performance in the field at the time of installation or during manufacture. Also see U.S. Patent No. 6,358,679 issued 2002 and U.S. Application No. 2003/0027179 published February 2003.

[0245] In various embodiments, the microplate is manufacturing using a composition comprising plastic compound such as a resin or polymer and a thermally conductive material. In such embodiments, the composition is either low fluorescing or no fluorescing. According to the present invention, the microplate is molded from a plastic formulated for increased conductivity. The higher thermally-conductive, such as a carbon filler, material included in the composition can be in any form. The material may be in the form of powder particles, fibers, or flakes, or any combination thereof. Powder particles are ground to a desired size and are characterized by a particle size which represents an average particle size for the lot. The powder particle size may be between one tenth of a micron and 50 microns. When mixed homogeneously with a resin, powders generally produce an equal (i.e. isotropic) thermal conductivity in all directions throughout the composition.

[0246] The particles included in the composition also, or alternatively, can be flakes. Flakes are irregularly shaped particles typically produced by rough grinding or shaving and can be characterized by a mesh size through which the flakes will pass. The size of flakes for use in the present invention maybe between one and 200 microns Like powders, homogenous compositions containing flakes generally have thermal conductivities which are equal in all directions.

[0247] Fibers or rods ("fibers") are another or an additional form of carbonaceous particle that can be used. Fibers are long thin particles. Fibers are usually described by their lengths and diameters. The length of the fibers maybe in the ranges between 2 mm and 15 mm. The diameter of the fibers maybe in the ranges between 1 mm and 5 mm. The inclusion of fibers in the composition has the added benefit of reinforcing the resin and adding material strength.

[0248] The invention includes any combination of particles and resin. Mixtures of particles of different types of shapes can be included in one composition. The particles maybe made of any material having a greater thermal conductivity than the resin. Particles having greater thermal conductivity will increase the thermal conductivity of the compositions relative to compositions having an equal weight percentage of particles having lower thermal conductivities. For certain thermal conductive materials, the particles may be a metal. Examples of suitable metals are copper, nickel, steel, silver, platinum gold, copper, steel, iron, and titanium The choice of metals is dictated at least by the cost of the materials. Other factors to be considered are the amount of thermal conductivity required and the type of environment in which the composition is to be used.

[0249] Carbon fillers such as carbon, graphite, or impervious graphite are relatively inexpensive forms of suitable carbonaceous material. Graphite has the advantage of being readily and cheaply available in a variety of shapes and sizes. Graphite is a refined form of carbon having a more structured state. Impervious graphite is a non-porous, solvent-resistant form of graphite. By using more refined grades, a more consistent thermal conductivity can be achieved.

[0250] While many resins are suitable, liquid crystal polymers ("LCPs") may be a type of resin to be used in the composition. Representative classes of polymers from which the thermotropic liquid crystal polymers suitable for use in the present invention may be selected include wholly aromatic polyesters, aromatic-aliphatic polyesters, wholly aromatic poly (ester-amides), aromatic-aliphatic poly (ester-amides),

aromatic polyazomethines, aromatic polyester-carbonates, and mixtures of the same.

[0251] In the present invention, non-metallic, thermally-conductive materials are added and dispersed within the polymer matrix. These materials impart thermal conductivity to the non-conductive polymeric matrix. It is important that nonmetallic materials be used, because metal contaminants can react and bind with the reactants in the wells causing analytical problems. Further, the thermally-conductive materials should have low fluorescence so that background fluorescence levels are kept to a minimum. In some embodiments, the plate comprising a thermal conductive material is coated with an inert coating, thus allowing a broader range of thermal conductive additives including metals.

[0252] Suitable non-metallic, thermally-conductive materials include, metal oxides such as alumina, magnesium oxide, zinc oxide, and titanium oxide; ceramics such as silicon nitride, aluminum nitride, boron nitride, boron carbide, and carbon materials such as carbon black or graphite. Mixtures of such fillers are also suitable. Generally, the thermally-conductive fillers comprise about 30 to about 60% by weight of the total composition and more particularly about 38 to about 48% by weight of the composition.

[0253] The thermally conductive material can be in the form of particles, granular powder, whiskers, fibers, nanotubes or any other suitable form. The particles or granules can have a variety of structures and a broad particle size distribution. For example, the particles or granules can have flake, plate, rice, strand, hexagonal, or spherical-like shapes with a particle size in the range of 0.5 to 300 microns or smaller in the case of nanotube. In some instances, the thermally conductive material can have a relatively high aspect (length to thickness) ratio of about 10:1 or greater. Alternatively, the thermally conductive material can have a relatively low aspect ratio of about 5:1 or less. For example, boron nitride grains having an aspect ratio of about 4:1 can be used. Both low aspect and high aspect ratio materials can be added to the polymer matrix as described in McCullough, U.S. Pat. No. 6,048,919.

[0254] The thermally-conductive material and optional reinforcing material are intimately mixed with the non-conductive polymer matrix to form the polymer composition. If desired, the mixture may contain additives such as, for example, flame retardants, antioxidants, plasticizers, dispersing aids, marking additives, and mold-releasing agents. In certain embodiments, such additives are biologically inert. The mixture can be prepared using techniques known in the art.

[0255] The thermally conductive additives can be any material with a thermal conductivity greater than the base polymer. Carbon fibers and other graphitic materials some of which have thermal conductivities that are reportedly as high as 3000-6000 W/mk. Non-electrically conductive materials can also be used including, for example, crystalline silica (3.0 W/mk), aluminum oxide (42 W/mk), diamond (2000 W/mk), aluminum nitride (150-220 W/mk), crystalline boron nitride (1300 W/mk) and silicon carbide (85 W/mk).

[0256] Specifically, the plastic material may be a polypropylene, polystyrene, polyethylene, polyethyleneterephthalate, styrene, acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer or any plastic material known to those skilled in the relative art with a melting point greater than 130°C and exhibiting a very low fluorescence when exposed to visible or non-visible light. Conductive material such as a conductive carbon black or other conductive filler known to those skilled in the relative art is included in the formulation of the plastic to increase thermal conductivity. To further increase the thermal conductivity, thermally conductive ceramic filler such as boron nitrate filler or other ceramic filler known to those skilled in the relative art may be added to the formulation. In various embodiments, the composition comprises RTP199X 104849. In other embodiments, the composition comprises CoolPoly E1201. In other embodiments, the filler may be electrically conductive filler. In various embodiments, combinations of the different types of formulations in various proportions maybe used. In some embodiments, the composition comprises about 80% of the RTP199X104849 and about 20% polypropylene.

[0257] As indicated above, there may be more than one thermally conductive additive added to the polymer to make the thermally conductive material. In fact, thermally conductive additives that have different shapes can be mixed together to contribute to an overall thermal conductivity that is higher than anyone of the individual additives alone would give. Moreover, an expensive thermally conductive additive (e.g., carbon fiber) can be mixed with a less expensive thermally conductive additive to reduce costs.

[0258] The resulting composition may be shaped into a microplate using any suitable molding process such as melt-extrusion, casting, or injection molding. In general, injection-molding involves the steps of: a) feeding the composition into the heating chamber of a molding machine and heating the composition to form a molten composition (liquid plastic); b) injecting the molten composition into a mold cavity; c) maintaining the composition in the mold under high pressure until it cools; and d) removing the molded article

[0259] The present invention includes the steps of a method for making microplate using the thermally conductive material that is a polymer mixed with one or more thermally conductive additives. The microplate maybe manufactured by mixing a polymer or a resin and one or more thermally conductive additives to form a thermally conductive material. In an embodiment, the microplate is made from polymer such as polypropylene and a thermally conductive additive such as a carbon filler.

[0260] In some embodiments the steps in manufacturing the microplate includes extruding the polymer that is mixed with one or more thermally conductive additives to create a melt blend. In particular, the polymer and thermally conductive additive(s) can be fed into a twin-screw extruder with the help of a gravimetric feeder to create a well dispersed melt blend. The extruded melt blend is then run through a water bath and cooled before being pelletized and dried. The pelletized melt blend is heated and melted by an injection molding machine which then injects the melt blend into a mold cavity of the injection molding machine. The mold cavity includes sections shaped to form the microplate The injection molding machine then cools the injected melt blend to create the

microplate. Finally, the microplate is removed from the injection molding machine.

[0261] A multiport mold used during injection molding has at least one opening and at least one exit. Microplates made through the methods of injection molding, extrusion, and lost-core molding are included in the invention. In various embodiments the mold has a opening, through which the composition flows, essentially in the center of the mold face that forms the bottom of the microplate. In certain embodiments, the mold has one or more exit ports around the perimeter of the microplate.

[0262] In an alternate embodiment, a method of producing a thermally conductive reaction plate, the method comprising:

a. mixing a polymer and at least one thermally conductive additive;

b. extruding the mixed polymer and the at least one thermally conductive additive to create a melt blend;

c. cooling said extruded melt blend; pelletizing said cooled melt blend;

d. melting said pelletized melt blend;

e. injecting said melted blend into a mold cavity of an injection molding machine, said mold cavity includes sections shaped to form said microplate;

f. cooling the injected melt blend to create said microplate; and

g. removing said microplate from the injection molding machine, wherein said microplate includes a plurality of wells. Such embodiments may include a one-step method where two or more resin pellet types are stirred together and the combination is placed in the injection molding machine, to be melt-blended during the injection molding process.

[0263] In certain embodiments, the manufacturing steps for making the plates may include: receiving pelleted material from resin supplier; drying pelleted material in Conair resin dryer; transferring dried material with vacuum system into hopper on 120 ton Nissei press; molding parts on 120 ton Nissei press using mold designed for microplate of

desired quantity of wells and any alignment features that may be included; trimming gates; and packaging the microplate.

[0264] In embodiments of the present invention, a chemical modality comprises chemical treatment or modification of the surface of the well so as to anchor an amplification reagent to the surface. As referred to herein, "anchor" refers to an attachment of the reagent to the surface, directly or indirectly, so that the reagent is available for reaction during an amplification method of this invention, but is not removed or otherwise displaced from the surface prior to amplification during routine handling of the substrate and sample preparation prior to amplification. In certain embodiments, the amplification reagent is anchored by covalent or non-covalent bonding directly to the surface of the substrate. In certain embodiments, an amplification reagent is bonded, anchored or tethered to a second moiety ("immobilization moiety") which, in turn, is anchored to the surface of the substrate. In certain embodiments of the present invention, an amplification reagent may be anchored to the surface through a chemically releasable or cleavable site, for example by bonding to an immobilization moiety with a releasable site. The reagent may be released from an array upon reacting with cleaving reagents prior to, during or after the array assembly. Such release methods include a variety of enzymatic, or non-enzymatic means, such as chemical, thermal, or photolytic treatment.

[0265] In one embodiment, the amplification reagent comprises a primer, and at least one probe which is released from the surface during a method of this invention. In one embodiment, a primer is initially hybridized to a polynucleotide immobilization moiety, and subsequently released by strand separation from the immobilized polynucleotides upon the surface. In another example of primer release, a primers is covalently immobilized on the surface via a cleavable site and released. For example, an immobilization moiety may contain a cleavable site and a primer sequence. The primer sequence may be released via selective cleavage of the cleavable sites. In certain embodiments, the immobilization moiety is a polynucleotide which contains one or more cleavable sites and one or more primer polynucleotides. A cleavable site

may be introduced in an immobilized moiety during in situ synthesis. Alternatively, the immobilized moieties containing releasable sites may be prepared before they are covalently or noncovalently immobilized on the solid support. For example, U.S. Patent Application Serial No. 60/504,500 to Woudenberg et al. filed September 19, 2003.

[0266] Chemical moieties for immobilization attachment to solid support include those comprising carbamate, ester, amide, thiolester, (N)-functionalized thiourea, functionalized maleimide, amino, disulfide, amide, hydrazone, streptavidin, avidin/biotin, and gold-sulfide groups. Methods of forming immobilized reagent arrays useful herein include methods well known in the art. Such methods are described, for example, in U.S. Patent 5,445,934, Fodor et al., issued August 29, 1995; U.S. Patent 5,700,637, Southern issued December 23, 1997; U. S. Patent 5,700,642, Monforte et al., issued December 23, 1997; U.S. Patent 5,744,305, Fodor et al., issued April 28, 1998; U.S. Patent 5,830,655, Monforte et al., issued November 3, 1998; U.S. Patent 5,837,832, Chee et al., issued November 17, 1998; U.S. Patent 5,858,653, Duran et al., issued January 12, 1999; U.S. Patent 5,919,626, Shi et al., issued July 6, 1999; U.S. Patent 6,030,782, Anderson et al., issued February 29, 2000; U.S. Patent 6,054,270, Southern, issued April 25, 2000; U.S. Patent 6,083,763, Balch, issued July 4, 2000; U. S. Patent 6,090,995, Reich et al., issued July 18, 2000; PCT Patent Publication W099/58708, Friend et al., published November 18, 1999; Protocols for oligonucleotides and analogs; synthesis and properties, Methods Mol. Biol. Vol. 20 (1993); Beier et al., Nucleic Acids Res. 27: 1970-1977 (1999); Joos et al., Anal. Chem. 247: 96-101 (1997); Guschin et al., Anal. Biochem. 250: 203-211 (1997); Czarnik et al., Accounts Chem. Rev. 29: 112-170 (1996); Combinatorial Chemistry and Molecular Diversity in Drug Discovery, Ed. Kerwin J. F. and Gordon, E. M., John Wiley & Son, New York (1997); Kahn et al., Modern Methods in Carbohydrate Synthesis, Harwood Academic, Amsterdam (1996); Green et al., Curr. Opin. in Chem. Biol. 2: 404-410 (1998); Gerhold et al., TIBS, 24: 168-173 (1999); DeRisi, J., et al., Science 278: 680-686 (1997); Lockhart et al., Nature 405: 827-836 (2000); Roberts et al., Science 287: 873-880 (2000); Hughes et al., Nature Genetics 25: 333-

337 (2000); Hughes et al., Cell 102 : 109-126 (2000); Duggan, et al., Nature Genetics Supplement 21: 10-14 (1999); and Singh-Gasson et al., Nature Biotechnology 17 : 974-978 (1999).

[0267] In embodiments of the present invention, the chemical modality comprises chemical treatment or modification of the surface of the array so as to anchor an amplification reagent to the surface. In some embodiments, the amplification reagent is affixed to the surface so as form a patterned array (herein, "immobilized reagent array") of reaction spots. As referred to herein, "anchor" refers to an attachment of the reagent to the surface, directly or indirectly, so that the reagent is available for reaction during an amplification method of this invention, but is not removed or otherwise displaced from the surface prior to amplification during routine handling of the substrate and sample preparation prior to amplification. In certain embodiments, the amplification reagent is anchored by covalent or non-covalent bonding directly to the surface of the substrate. In certain embodiments, an amplification reagent is bonded, anchored or tethered to a second moiety ("immobilization moiety") which, in turn, is anchored to the surface of the substrate. In certain embodiments of the present invention, an amplification reagent may be anchored to the surface through a chemically releasable or cleavable site, for example by bonding to an immobilization moiety with a releasable site. The reagent may be released from an array upon reacting with cleaving reagents prior to, during or after the array assembly. Such release methods include a variety of enzymatic, or non-enzymatic means, such as chemical, thermal, or photolytic treatment.

[0268] In one embodiment, the amplification reagent comprises at least one primer, which is released from the surface during a method of this invention. In one embodiment, at least one primer is initially hybridized to a polynucleotide immobilization moiety, and subsequently released by strand separation from the array-immobilized polynucleotides upon array assembly. In another example of primer release, at least one primer is covalently immobilized on an array via a cleavable site and released before, during, or after array assembly. For example, an immobilization moiety may contain a cleavable site and a primer

sequence. The primer sequence may be released via selective cleavage of the cleavable sites before, during, or after assembly. In certain embodiments, the immobilization moiety is a polynucleotide which contains one or more cleavable sites and one or more primer polynucleotides. A cleavable site may be introduced in an immobilized moiety during in situ synthesis. Alternatively, the immobilized moieties containing releasable sites may be prepared before they are covalently or noncovalently immobilized on the solid support. In some embodiments, multiple areas of the well have cleavage sites with each of the sites having at least one different primer and in certain embodiments, each of the sites comprise a different primer and/or probe set.

[0269] Chemical moieties for immobilization attachment to solid support include those comprising carbamate, ester, amide, thiolester, (N)-functionalized thiourea, functionalized maleimide, amino, disulfide, amide, hydrazone, streptavidin, avidin/biotin, and gold-sulfide groups. Methods of forming immobilized reagent arrays useful herein include methods well known in the art. Such methods are described, for example, in U.S. Patent 5,445,934, Fodor et al., issued August 29, 1995; U.S. Patent 5,700,637, Southern issued December 23, 1997; U. S. Patent 5,700,642, Monforte et al., issued December 23, 1997; U.S. Patent 5,744,305, Fodor et al., issued April 28, 1998; U.S. Patent 5,830,655, Monforte et al., issued November 3, 1998; U.S. Patent 5,837,832, Chee et al., issued November 17, 1998; U.S. Patent 5,858,653, Duran et al., issued January 12, 1999; U.S. Patent 5,919,626, Shi et al., issued July 6, 1999; U.S. Patent 6,030,782, Anderson et al., issued February 29, 2000; U.S. Patent 6,054,270, Southern, issued April 25, 2000; U.S. Patent 6,083,763, Balch, issued July 4, 2000; U. S. Patent 6,090,995, Reich et al., issued July 18, 2000; PCT Patent Publication W099/58708, Friend et al., published November 18, 1999; Protocols for oligonucleotides and analogs; synthesis and properties, Methods Mol. Biol. Vol. 20 (1993); Beier et al., Nucleic Acids Res. 27: 1970-1977 (1999); Joos et al., Anal. Chem. 247: 96-101 (1997); Guschin et al., Anal. Biochem. 250: 203-211 (1997); Czarnik et al., Accounts Chem. Rev. 29: 112-170 (1996); Combinatorial Chemistry and Molecular Diversity in Drug Discovery, Ed. Kerwin J. F. and

Gordon, E. M., John Wiley & Son, New York (1997); Kahn et al., Modern Methods in Carbohydrate Synthesis, Harwood Academic, Amsterdam (1996); Green et al., Curr. Opin. in Chem. Biol. 2: 404-410 (1998); Gerhold et al., TIBS, 24: 168-173 (1999); DeRisi, J., et al., Science 278: 680-686 (1997); Lockhart et al., Nature 405: 827-836 (2000); Roberts et al., Science 287: 873-880 (2000); Hughes et al., Nature Genetics 25: 333-337 (2000); Hughes et al., Cell 102 : 109-126 (2000); Duggan, et al., Nature Genetics Supplement 21: 10-14 (1999); and Singh-Gasson et al., Nature Biotechnology 17 : 974-978 (1999).

[0270] A filling system comprises any apparatus which facilitates the placement of amplification reagents or sample on the surface of the substrate, effecting placement of such reagents or sample in reaction chambers. Such apparatus among those useful herein include devices for pouring of reagents or samples onto the surface so as to substantially cover the entire surface. In one embodiment the filling system comprises a device for spotting or spraying of reactants to specific wells. For example, by use of piezoelectric pumps, acoustic dispersion, liquid printers or the like. Commercial examples include Scout MPD, Aurora Discovery, San Diego, California; HTS, EDC Biosystems, San Jose, California; Echo 550, Labcyte Picoliter, Sunnyvale, California; Cyclone ILNIO Caliper Life Sciences, Hopkinton, Massachusetts and the like. In one embodiment, the filling apparatus comprises a vacuum pump operable to fill of wells the microplate assembly. Filling systems may also include devices for applying centrifugal force to the microplate assembly, operable to disperse reagents or sample across the surface of the substrate into reaction chambers. In one embodiment, the filling system is in fluid communication with a filling device in the microplate assembly. In order to realize such filling, various devices and methods are known at present; the most common ones of these devices are air-cushion pipettes, valve-controlled piston-displacement pipettes, piezoelectric pipettes and needle pipettes. Known micro filling devices are described in DE-A-19706513 and in DE-A-19802368. These known devices are based on a functional principle according to which a liquid to be dosed has applied thereto an acceleration by a displacer within a pressure chamber. DE-A-19913076

discloses a micro filling device by means of which a plurality of microdroplets can be applied to a substrate; in this micro filling device the whole dosing head is acted upon by an acceleration.

[0271] WO 00/62932 discloses methods and devices for discharging extremely small, dosed amounts of liquids, the discharge amounts mentioned being in the range of from 0.1 nl to 100 microliter According to this publication a capillary is used, which is provided with a discharge opening and which has connected thereto at least one gas line via a junction point. Via the capillary, a gas blast is introduced in the gas line so that an amount of liquid contained in the capillary section between the junction point and the discharge opening will be discharged from the discharge opening in a dosed amount. This publication also mentions the possibility of producing a pipetting array making use of a plurality of filling devices of the type described hereinbefore.

[0272] DE 19648694 C1 discloses a bidirectional dynamic micropump comprising a pump chamber as well as an inlet and an outlet for the pump chamber with different flow resistances. A diaphragm borders on the pump chamber, whereby the delivery direction of the micropump can be controlled by suitably shaping the control pulse for the diaphragm. WO 97/15394 discloses a plate having a plurality of apertures which extend therethrough. The apertures have a large opening towards one surface of the plate and a small nozzle opening towards the opposite surface of said plate. By applying a pressure on the large opening, a jet of liquid can be ejected through the small nozzle opening. Another micro filling device is known from WO 99/36176, said filling device comprising a liquid reservoir and a channel which is in fluid communication with the liquid reservoir. Openings are formed in opposed walls of the channel so that the liquid present between the openings can be discharged, in a dosed amount, by applying a pressure to one of the openings.

[0273] U.S. Patent Application No. 20040074557 to Zengerle et al. published April 22, 2004 discloses a micro filling device comprises a media reservoir used for accommodating a liquid to be dosed, a nozzle connected via a connecting channel to the media reservoir and adapted to be filled via said connecting channel with the liquid to be dosed, and a

drive unit for applying, when actuated, to a liquid contained in the media reservoir and in the nozzle a force of such a nature that a substantially identical pressure will be exerted on said liquid contained in the media reservoir and in the nozzle. Flow resistances of the connecting channel and of the nozzle are dimensioned such that, in response to an actuation of the drive unit, a volumetric flow in the connecting channel will be small in comparison with a volumetric flow in the nozzle, said volumetric flow in the nozzle causing an ejection of the liquid to be dosed from an ejection opening of the nozzle.

[0274] Figures 48-53 illustrate a high density multiport manual filling card. This manual filling card 400 has many possible configurations. This is due to many possible variations of the small microfluidic channels 416. In one embodiment, manual filler 400 has 96 sample input ports 401. Each of these sample input ports 401 fluidly communicates with microfluidic channels 416. Microfluidic channels 416 comprise channels 406 which connect the through hole wells 405. Around these through hole wells is a lip 404 and in some embodiments, a small ramp 412 connects path 403 with through hole 405. Once the sample is put into a sample input port 401, it heads toward the microfluidic channel 416, first passing an elevated member 410 which helps evenly spread the sample throughout the microfluidic channel 416. The sample moves through the microfluidic channels 416 and collects in the through holes 405. The fluid is then held in through holes 405 by capillary action. The sample loads all 64 through holes 405 and is split evenly among through holes 405. A microplate 22 is mated with manual filler so that each through hole 405 is above a well 34. The combined unit of the manual filler and the microplate is placed in a centrifuge which is spun. The force of the centrifuge moves the sample from the through hole plates 405 into well 34 of microplate 22. The manual filler is then removed from the microplate. The microplate is then ready for additional reagents to be added or to be sealed with sealing cover and then placed into a thermal cycling machine. Microfluidic channels may have various patterns as illustrated in Figures 51 and 53. In some embodiments, the manual filler may have a sample input layer 402 and a microfluidic channel layer 406. In some embodiments, microfluidic

channel layer 406 has microfluidic channel 416 and through holes 405 etched into the layers. In other embodiments, the sample input layer and microfluidic layer are one unit.

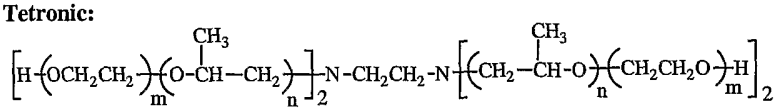
[0275] In some embodiments, microfluidic channels 416 are coated with polymeric materials. Embodiments of the invention include the hydrophobic surface of a microfluidic device to a hydrophilic one by surface adsorption of a surfactant in the presence, or absence, of a co-agent, resulting in high-speed transport of aqueous samples through capillary effect.

[0276] In some embodiments, the microfluidic device may be made of polyolefins; poly(cyclic olefins); polyethylene terephthalate; poly(alkyl (meth)acrylates); polystyrene; poly(dimethyl siloxane); polycarbonate; structural polymers, for example, poly(ether sulfone), poly(ether ketone), poly(ether ether ketone), and liquid crystalline polymers; polyacetal; polyamides; polyimides; poly(phenylene sulfide); polysulfones; poly(vinyl chloride); poly(vinyl fluoride); poly(vinylidene fluoride); or their copolymers. Injection molding, stamping embossing, machining, or any methods known to those who are skilled in the art, may be used to prepare micro-fluidic devices.

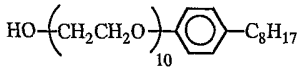
Table 3 – Surfactants for coating microfluidic plates

NO.	Name	MW	HLB
1	Tetronic 901	4700	3
2	Tetronic 1107	1500	24
3	Tetronic 1301	6800	2
4	Poly(styrene-b- ethylene oxide)	Mn: 3600-67000	
5	Poly(styrene-b-sodium acrylate)	Mn: 1800-42500	
6	Triton X-100		13.5
7	Triton X-100 reduced		
8	Tween 20	1228	16.7
9	Tween 85	1839	11
10	Span 83	1109.56	3.7
11	Span 80	428.62	4.3
12	Span 40	402.58	6.7

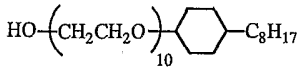
Tetronic:



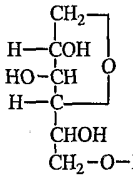
Triton X-100:



Triton X-100 reduced:

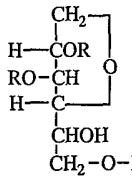


Span 80:



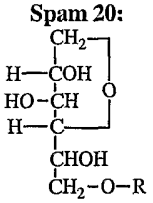
(R = -$COC_{17}H_{33}$ oleate)

Spam 83:



(R = -$COC_{17}H_{33}$ oleate)

Spam 20:



(R = -$COC_{11}H_{23}$ laurate)

Tween:

Poly(oxyethylene) sorbitan monolauate

5

Table 4 – Surfactants for Wetting Polyproplylene

Acids:	
Dodecyl sulfate, Na salt	$CH_2(CH_2)_{11}OSO_3^- Na^+$
Octadecyl sulfate, Na salt	$CH_3(CH_2)_{17}OSO_3^- Na^+$
Quaternary ammonium compounds:	
Cetyltrimethylammonium bromide	$CH_3(CH_2)_{15}\overset{+}{N}(CH_3)_3\ Br^-$
Octadecyltrimethylammonium bromide	$CH_3(CH_2)_{17}\overset{+}{N}(CH_3)_3Br^-$
Ethers:	
Brij-52	$CH_3(CH_2)_{15}(OCH_2CH_2)_2OH$
Brij 56	$CH_3(CH_2)_{15}(OCH_2CH_2)_{10}O$
Brij 58	$CH_3(CH_2)_{15}(OCH_2CH_2)_{20}O$
Brij 72	$CH_3(CH_2)_{17}(OCH_2CH_2)_2OH$
Brij 76	$CH_3(CH_2)_{17}(OCH_2CH_2)_{10}O$
Brij 78	$CH_3(CH_2)_{17}(OCH_2CH_2)_{20}O$
Esters:	
Poly(ethylene glycol) monolaurate	$CH_3(CH_2)_{10}CO(OCH_2CH_2)$
Poly(ethyleneglycol) distearate	$CH_3(CH_2)_{16}$-CO-$(OCH_2)_9$-C
Poly(ethyleneglycol)dioleate	$CH_3(CH_2)_7CH=CH(CH_2)_7$-(-O-CO-$(CH_2)_7CH=CH$

[0277]

[0278] The use of a co-agent may enhance the hydrophilicity and/or improve shelf life of the treated surface. Co-agents may be a water-soluble or slightly water-soluble homopolymer or copolymers prepared by monomers including, for example, (meth)acrylamide; N-methyl (methyl)acrylamide, N,N-dimethyl (methyl)acrylamide, N-ethyl (meth)acrylamide, N-*n*-propyl (meth)acrylamide, N-*iso*-propyl (meth)acrylamide, N-ethyl-N-methyl (meth)acrylamide, N,N-diethyl (meth)acrylamide, N-hydroxymethyl (meth)acrylamide, N-(3-hydroxypropyl) (meth)acrylamide, N-vinylformamide, N-vinylacetamide, N-methyl-N-vinylacetamide, vinyl acetate that can be hydrolyzed to give vinylalcohol after polymerization, 2-hydroxyethyl (meth)acrylate, 3-

hydroxypropyl (meth)acrylate, N-vinypyrrolidone, poly(ethylene oxide) (meth)acrylate, N-(meth)acryloxysuccinimide, N-(meth)acryloylmorpholine, N-2,2,2-trifluoroethyl (meth)acrylamide, N-acetyl (meth)acrylamide, N-amido(meth)acrylamide, N-acetamido (meth)acrylamide, N-tris(hydroxymethyl)methyl (meth)acrylamide, N-(methyl)acryloyltris(hydroxymethyl)methylamine, (methyl)acryloylurea, vinyloxazolidone, vinylmethyloxazolidone, or a combination thereof. In various embodiments, the co-agent can be poly(acrylic acid-co-N,N-dimethylacrylamide) or poly(N,N-dimethyl acrylamide-co-styrene sulfonic acid).

[0279] In various embodiments, the filling system may comprise a device to remove excess reagents or sample from the surface of the substrate. In embodiments of this invention, such a device is operable by centrifugal force, vacuum, and combinations thereof. The filling system may comprise a wiping device, such as a squeegee, which is drawn across the surface of the substrate so as to remove excess reactant.

[0280] In accordance with the invention, an embodiment includes a microplate comprising wells containing a solution that comprises a PCR, primer and a labeled probe. In another embodiment, the wells of the microplate contain a solution that comprises a forward PCR primer, a reverse PCR primer, a FAM labeled MGB quenched PCR probe and buffers. In another embodiment, the wells of the solution contain the contents of a TaqMan reagent kit. In another embodiment, the wells of the microplate contain a solution comprising a probe, a primer and a polymerase. In other embodiments of the invention, the wells contain any of the above solutions in a dried form. In this embodiment, this dried form may be coated to the wells or be directed to the bottom of the well. According to this embodiment, the user only needs to add water and the sample to each of the wells. This embodiment is also known as a "preloaded" well or microplate.

[0281] In this embodiment, the microplate comprising the dried down reaction components may be sealed with a protective cover, stored or shipped to another location. The protective cover is releasable without leaving the adhesive residue on the microplate. The protective cover is

different than the cover to aid in identification and for ease of handling. The material of the protective cover is chosen to minimize static charge generation upon release from the plate. When it is time for this microplate to be used, the packaging seal is broken and the protective cover is removed and the sample, along with water and any required reaction components, is added to the wells of this microplate. The reaction plate is then sealed with a cover. The plate is put in the PCR system with the plate being put down on its cover. The system is run and data is collected and analyzed. In a similar embodiment, there are additional steps of the plate with the cover and reaction mixture is then put on a centrifuge with the plate being put down on its cover. The centrifuge is run, forcing all of the solution against the cover. The spun plate is put into the PCR system. The system is run and data is collected and analyzed.

[0282] The present invention provides a method for PCR using microtiter plate comprising a plurality of sample wells in a thermal cycling system, the method comprising:

a. placing a sample and a reaction mixture into a well;
b. sealing the plate with a cover;
c. placing the plate into the thermal cycling system ; and
d. starting the system to complete the PCR process.

[0283] In various embodiments of the invention, the plate is put in the PCR system. The system is run and data is collected and analyzed. In a similar embodiment, there are additional steps of the plate with the cover and reaction mixture is then put on a centrifuge. The centrifuge is run, forcing all of the solution to the bottom of the wells. The spun plate is put into the PCR system. The system is run and data is collected and analyzed.

[0284] The reaction plate may be used for genotyping, gene expression, or other DNA assays preformed by PCR. Assays performed in the plate are not limited to DNA assays such as Taqman, Invader, Taqman Gold but also include other assays such as receptor binding, enzyme, and other high throughput screening assays in general. The plate may also be used for the temporary storage of reagents and other related applications.

[0285] The present invention provides a method for PCR using microtiter plate comprising a plurality of sample wells in a thermal cycling system, the method comprising;

a. placing a sample and a reaction mixture into a well;

b. sealing the plate with a cover;

c. placing the plate into the thermal cycling system such that the sample and the reaction mixture are in contact with the cover; and

d. starting the system to complete the PCR process.

[0286] In various embodiments of the invention, the plate is put in the PCR system with the plate being put down on its cover. The system is run and data is collected and analyzed. In a similar embodiment, there are additional steps of the plate with the cover and reaction mixture is then put on a centrifuge with the plate being put down on its cover. The centrifuge is run, forcing all of the solution against the cover. The spun plate is put into the PCR system. The system is run and data is collected and analyzed.

[0287] In accordance with the invention, various embodiments include a microplate comprising wells containing a solution that comprises a PCR, primer and a label probe. In another embodiment, the wells of the microplate contain a solution that comprises a forward PCR primer, a reverse PCR primer, a FAM labeled MGB quenched PCR probe and buffers. In another embodiment, the wells of the solution contain a TaqMan reagent kit. In another embodiment, the wells of the microplate contain a solution comprising a probe, a primer and a polymerase. In other embodiments of the invention, the wells contain any of the above solutions in a dried form. In this embodiment, this dried form may be coated to the wells or be directed to the bottom of the well. According to this embodiment, the user only needs to add water and the sample to each of the wells. This embodiment is also known as a "preloaded" well or microplate.

[0288] In this embodiment, the microplate comprising the dried down reaction mixture may be sealed with a protective cover, stored or shipped to another location. The protective cover is releasable in one piece without leaving adhesive residue on the microplate. The protective

cover is visibly different than the cover to aid in identification and for ease of handling. The material of the protective cover is chosen to minimize static charge generation upon release from the plate. When it is time for this microplate to be used, the package seal is broken and the protective cover is removed and the sample, along with universal master mix and water, is added to the wells of this microplate. The reaction plate is then sealed with a cover. The plate is put in the PCR system with the plate being put down on its cover. The system is run and data is collected and analyzed. In a similar embodiment, there are additional steps of the plate with the cover and reaction mixture is then put in a swing bucket type centrifuge with the plate being put down on its cover. The centrifuge is run, forcing all of the solution against the cover. The spun plate is put into the PCR system. The system is run and data is collected and analyzed.

[0289] The invention provides a method for performing a PCR analysis using a reaction plate comprising a plurality of preloaded wells, the method comprising:

a. placing a sample and a solution into the wells to create a reaction mixture;

b. sealing a cover to the plate;

c. placing the plate into a thermal cycling system;

d. cycling the system; and

e. analyzing results.

[0290] In this embodiment, the microplate comprising the dried down reaction mixture may be sealed with a protective cover, stored or shipped to another location. The protective cover is releasable in one piece without leaving residue on the microplate. The protective cover is visibly different than the cover to aid in identification and for ease of handling. The material of the protective cover is chosen to minimize static charge generation upon release from the plate. When it is time for this microplate to be used, the packaging seal is broken and the protective cover is removed and the sample, along with water, is added to the wells of this microplate. The reaction plate is then sealed with a cover. The plate is put in the PCR system. The system is run and data is collected and analyzed. In a similar embodiment, there are additional steps of the plate with the cover and

reaction mixture is then put in a centrifuge. The centrifuge is run, forcing all of the solution to the bottom of the well. The spun plate is put into the PCR system. The system is run and data is collected and analyzed.

[0291] The invention also provides reagents and kits suitable for carrying out polynucleotide amplification methods of this invention. Such reagents and kits may be modeled after reagents and kits suitable for carrying out conventional PCR, RT-PCR, and other amplification reactions. Such kits comprise a microplate of this invention and a reagent selected from the group consisting of an amplification reagent, a detection reagent and combinations thereof. Examples of specific reagents include, but are not limited to, the reagents present in AmpliTaq® Gold PCR Master Mix, TaqMan® Universal Master Mix and TaqMan® Universal Master Mix No AmpErase® UNG, Assays-by-Design^{SM}, Pre-Developed Assay Reagents (PDAR) for gene expression, PDAR for allelic discrimination and Assays-On-Demand®, all of which are marketed by Applied Biosystems, Inc. (Foster City, California, U.S.A.). The kits may comprise reagents packaged for downstream or subsequent analysis of the multiplex amplification product. In one embodiment, the kit comprises a container comprising a plurality of amplification primer pairs or sets, each of which is suitable for amplifying a different sequence of interest and a plurality of reaction vessels, each of which includes a single set of amplification primers suitable for amplifying a sequence of interest. The primers included in the individual reaction vessels can, independently of one another, be the same or a different set of primers comprising the plurality of multiplex amplification primers.

[0292] In certain embodiments of the present invention, the microplate has 6,144 wells and has the dimensions of the SBS/ANSI standard footprint for microplates and in each of the wells there is part of a genome, the genome having 30,000 different targets. In each well there are five different assays each with a different dye so that multiplexing PCR maybe performed. In this embodiment, the genome may be from humans, from mammals, from mice, from Arabidopsis or from any other plant, bacteria, fungi or animal species. It is envisioned that this embodiment may be used for drug discovery and for diagnostics of a particular individual, animal or plant.

[0293] In some embodiments of the invention, the methods of this invention are performed with equipment which aids in one or more steps of the process, including handling of the microplates, thermal cycling, and imaging. Accordingly, the present invention provides systems for simultaneously determining the genetic expression profile in a biological sample obtained from an individual member of a species relative to a standard genome for said species, comprising:

(a) a microplate assembly comprising:

(i) a substrate, wherein at least about 1000 reaction chamber are formed in an array on the substrate and each chamber has a capacity of less than about 50 µL;

(ii) a cover; wherein

(iii) each chamber comprises (1) a primer for a polynucleotide target within said standard genome, and (2) an indicator associated with said primer which emits a concentration dependent signal if the primer binds with said target;

(b) amplification reagents for performance of an amplification reaction for polynucleotide targets within said standard genome;

(c) a filling system for filling said biological sample and said reagents into said microplate assembly;

(d) a thermal cycling system for heating said microplate assembly; and

(e) a detection system for detecting said signal.

[0294] In various configurations of the present invention, there can be provided a method for supplying to a consumer assays useful in obtaining structural genomic information, such as the presence or absence of one or more single nucleotide polymorphisms (SNPs), and functional genomic information, such as the expression or amount of expression of one or more genes. As such, the assays can be configured to detect the presence or expression of genetic material in a biological sample. The method includes providing a web-based user interface configured for receiving orders for stock assays, providing a web-based user interface configured for receiving requests for design of custom assays and for ordering said assays, and delivering to the consumer at least one custom or stock assay in response to

an order for the one custom or stock assay placed by the consumer. In certain other aspects, the present invention can also be directed; to a system and to methods for constructing a system for providing to a consumer assays configured to detect presence or expression of genetic material. Stock gene expression assays provided by the web-based user interface can include, in some configurations assays for at least about 10,000 or more expressed genes. In certain configurations, gene expression assays for multi-exon genes can be made up of probes and primers designed to lie on exon-exon boundaries to preclude amplification of genomic DNA.

[0295] In various configurations of the invention as described above, the method can further include providing a web-based gene exploration platform configured to provide information to assist a consumer in selecting one or both of a stock assay and a custom assay. The present invention, in various configurations, can also include a search resource provided to identify genetic material. The search resource may provide one or more parameters identifying gene structure or function for selection by the consumer. Assays that detect the presence or expression of genetic material may include assays for detecting SNPs or for detecting expressed genes. In various configurations, the ordering interface can be configured to receive criteria related to the SNP or to the expressed transcript for which an assay is ordered. Such methods, kits, assays, web interfaces, etc are disclosed in US Patent Publication 20040018506 to Koehler et al. published January 29, 2004.

[0296] According to various embodiments, an assay kit is provided that includes: a container containing assay reagents; and a separate data storage medium that contains data about the assay reagents. The assay reagents can be adapted to perform an allelic discrimination or expression analysis reaction when admixed with at least one target polynucleotide. The other reagents can be, for example, components conventionally used for polymerase chain reactions (PCR), and can include non-reactive components. The container can be sealed and can be packaged with the separate data storage medium in a package, for example, in a box. The container can have a machine-readable label that provides information about the contents of the container.

[0297] According to various embodiments, the data stored on the data storage medium can include computer-readable code that can be used to adjust, calibrate, direct, set, run, or otherwise control an apparatus, for example, a scientific or laboratory instrument. According to various embodiments, methods are provided wherein the data is used to cause an apparatus to automatically perform a polymerase chain reaction of a target analyte that is mixed with the assay reagents. Methods are also provided whereby the kit is shipped to a customer. See US Patent Publication 20040072195 to Hunkapiller et al. published April 15, 2004.

[0298] According to various embodiments, a method of compiling a library of polynucleotide data sets is provided. The data sets can correspond to polynucleotides that each can function as (A) a primer for producing a nucleic acid sequence that is complementary to at least one target nucleic acid sequence including a target SNP, (B) a probe for rendering detectable the at least one target nucleic acid sequence including a target SNP, or (C) both (A) and (B). The method can include the step of selecting for the library polynucleotide data sets that each correspond to a respective polynucleotide that contains a sequence that is complementary to a respective first allele included in each of the at least one target nucleic acid sequences, if, under a set of reaction conditions a number of parameters are met by each polynucleotide corresponding to the data sets included in the library. The parameters can include: (1) the respective polynucleotide has a background signal value less than or equal to a first defined value, where the background signal value is a first normalized ratio of a fluorescence intensity of the respective polynucleotide reacted with first assay reactants in the absence of the target nucleic acid sequence, and under first conditions of fluorescence excitation, to a dye fluorescence intensity of a passive-reference dye under the first conditions; (2) the respective polynucleotide has a signal generation value of greater than or equal to a second defined value, wherein the signal generation value is the difference between (i) a second normalized ratio of the fluorescence intensity of the respective polynucleotide reacted with the first assay reactants in the presence of the target nucleic acid sequence, to the dye fluorescence intensity and (ii) the background signal value; (3) the respective polynucleotide has a specificity value of less than or equal to a

third defined value, wherein the specificity value is the difference between (i) a third normalized ratio of the fluorescence intensity of the respective polynucleotide reacted with second assay reactants that contain a second allele included in the at least one target nucleic acid sequence to the dye fluorescence intensity, wherein the second allele differs from the first allele, and (ii) the background signal value; (4) at least one individual from a population of individuals has a genotype identifiable under the first conditions, that results from reacting the respective polynucleotide with the first assay reactants and in the presence of the target nucleic acid sequence, wherein the population includes at least one individual that has the identifiable genotype and at least one individual that does not have the identifiable genotype; and (5) at least one individual from the population has an identifiable minor allele of the identifiable genotype, under the first conditions that results from reacting the respective polynucleotide with the first assay reactants in the presence of the target nucleic acid sequence, wherein the population includes at least one individual that has the identifiable minor allele, and at least one individual that does not have the identifiable minor allele. See US Patent Publication 20030190652 to De La Vega et al published October 9, 2003.

[0299] The present invention provides devices and methods for containing and handling small quantities of liquids, including methods and devices for performing amplification reactions on liquid samples containing polynucleotides. Embodiments of the present invention include multiwell plates for conducting a thermocycled amplification reaction of polynucleotide in a liquid sample, comprising:

(a) a substantially planar substrate having a first and second major surface;

(b) a plurality of reaction wells located on said first major surface, wherein each of said wells has a bottom and an opening which is disposed on said first major surface;

(c) a substantially planar cover, a major surface of which is disposed adjacent to said first major surface so as to cover said openings of said wells; and

(d) a sealing member disposed between said cover and said first major surface, wherein said sealing member is operable to substantially seal said openings of said wells when said wells contain said liquid and said plate is inverted so that said liquid is in contact with said cover.

[0300] In one embodiment, the seal comprises a pressure sensitive adhesive coated on to the first major surface of the substrate, the major surface of the cover, or both of the surfaces. In one embodiment, the plate comprises at least about 6144 wells. In one embodiment, the wells comprise a dried primer and probe for conducting the amplification reaction.

[0301] The present invention also provides methods, including methods for performing thermocycling amplification of a liquid polynucleotide sample, comprising:

(a) loading a quantity of said sample into multiwell plate comprising

(i) a substantially planar substrate having a first and second major surfaces; and

(ii) a plurality of reaction wells located on said first major surface, wherein each of said wells has a bottom and an opening which is disposed on said first major surface;

(b) covering said plate with a substantially planar cover, a major surface of which is disposed adjacent to said first major surface so as to cover said openings of said wells;

(c) inverting said plate, so said quantity of sample contacts said cover;

(d) thermocycling said plate so as to effect amplification of the polynucleotides in said quantity of sample.

[0302] The present invention provides devices and methods for containing and handling small quantities of liquids, including methods and devices for performing amplification reactions on liquid samples containing polynucleotides. Embodiments of the present invention include multiwell plates for conducting a thermocycled amplification reaction of polynucleotide in a liquid sample, comprising:

(a) a substantially planar substrate having a first and second major surface;

(b) a plurality of reaction wells located on said first major surface, wherein each of said wells has a bottom and an opening which is disposed on said first major surface;

(c) a substantially planar cover, a major surface of which is disposed adjacent to said first major surface so as to cover said openings of said wells; and

(d) a sealing member disposed between said cover and said first major surface, wherein said sealing member is operable to substantially seal said openings of said wells when said wells contain said liquid and said plate is inverted so that said liquid is in contact with said cover.

[0303] In one embodiment, the seal comprises a pressure sensitive adhesive coated on to the first major surface of the substrate, the major surface of the cover, or both of the surfaces. In one embodiment, the plate comprises at least about 6144 wells. In one embodiment, the wells comprise a dried primer and probe for conducting the amplification reaction.

[0304] The present invention also provides methods, including methods for performing thermocycling amplification of a liquid polynucleotide sample, comprising:

(a) loading a quantity of said sample into multiwell plate comprising

(i) a substantially planar substrate having a first and second major surfaces; and

(ii) a plurality of reaction wells located on said first major surface, wherein each of said wells has a bottom and an opening which is disposed on said first major surface;

(b) covering said plate with a substantially planar cover, a major surface of which is disposed adjacent to said first major surface so as to cover said openings of said wells;

(c) inverting said plate, so said quantity of sample contacts said cover;

(d) thermocycling said plate so as to effect amplification of the polynucleotides in said quantity of sample.

[0305] According to the principles of the present invention, a clamping apparatus for a high density microplate is provided having an advantageous construction. More particularly, the clamping apparatus of the

present invention employs a microplate having a first surface and an opposing second surface. A plurality of wells is formed in the first surface of the microplate. Each of the plurality of wells is sized to receive an assay therein for testing. A first sealing member is placed adjacent to the first surface of the microplate. This first sealing member cooperates with the microplate to define a first sealing interface between the first sealing member and each of the plurality of wells in the microplate. A pressure device is selectively actuated to exert a pressure upon the first sealing interface in response to a control input. The microplate can be oriented in an upright or inverted position. Additionally, the pressure device can include an inflatable transparent bag, a movable member in conjunction with an already inflated transparent bag, or a pressurizable pressure chamber. This arrangement permits consistent and reliable sealing of each of the plurality of wells to minimize contamination and/or evaporation.

[0306] The present invention provides a multiwell plate for conducting a thermocycled amplification reaction of polynucleotide in a liquid assay. In such embodiments, the plate comprises a substantially planar substrate having a first and second major surfaces with a plurality of reaction wells located on said first major surface, wherein each of said wells has a bottom and an opening which is disposed on said first major surface; and a substantially planar sealing cover, a major surface of which is disposed adjacent to said first major surface so as to cover said openings of said wells. In some embodiments the substrate comprises a thermal conductive material.

[0307] According to the principles of the present invention, a vacuum assist apparatus is provided having an advantageous construction. More particularly, the vacuum assist apparatus includes a microplate. The microplate includes a first surface and an opposing second surface. A plurality of wells are formed in the first surface of the microplate. Each of the plurality of wells being sized to receive an assay therein. A support base having a fluid passage. The microplate is positioned adjacent and in contact with the support base. A pressure device, in fluid communication with the fluid passage, exerts a vacuum within the fluid passage to actively retain the microplate in the contact with the support base.

[0308] According to the principles of the present invention, a reaction plate is provided according to the principles of the present invention having an advantageous construction. The reaction plate includes a main body portion, having a first surface and an opposing second surface, and a groove disposed about the first surface of the main body portion. The groove separates the main body portion into an inboard section and an outboard section. A plurality of wells are formed in the inboard section of the first surface and each of the plurality of wells being sized to receive an assay therein.

[0309] The present invention provides an apparatus for monitoring a formation of a nucleic acid amplification product in real time. The apparatus includes a plurality of light sources arranged in an essentially circular pattern uniformly illuminating a sample holder and illuminating a volume of a sample with an excitation beam with the sample holder holding the sample of nucleic acid to be amplified. Such embodiments include a detector detecting a first fluorescent emission signal from the sample and a second fluorescent emission signal from a standard and an analysis system for receiving the first and second emission signals from said detector at a plurality of times during the amplification and producing a plurality of corrected intensity signals, each corrected intensity corresponding to a relationship between the intensities of the first and second emission signals at any given time.

[0310] The present invention provides methods and apparatus for the identification of thermal conductive microplates. In some embodiments the identification includes radio frequency identification devices. Other embodiments include graphics that are printed on the plate and such graphics may be used for identification and/or as an alignment feature. In certain embodiments, the graphics are two dimensional. The present invention also provides method for incorporating the identification with the plate.

[0311] According to the principles of the present invention, a vacuum assist apparatus is provided having an advantageous construction. More particularly, the vacuum assist apparatus includes a microplate. The microplate includes a first surface and an opposing second surface. A plurality of wells are formed in the first surface of the microplate. Each of the

plurality of wells being sized to receive an assay therein. A support base having a fluid passage. The microplate is positioned adjacent and in contact with the support base. A pressure device, in fluid communication with the fluid passage, exerts a vacuum within the fluid passage to actively retain the microplate in the contact with the support base.

[0312] The examples and other embodiments described herein are exemplary and are not intended to be limiting in describing the full scope of apparatus, systems, compositions, materials, and methods of this invention. Equivalent changes, modifications, variations in specific embodiments, apparatus, systems, compositions, materials and methods may be made within the scope of the present invention with substantially similar results. Such changes, modifications or variations are not to be regarded as a departure from the spirit and scope of the invention. All patents cited herein, as well as, all publications, applications, websites, articles, brochures and product information discussed herein, are incorporated in their entirety herein by reference.

CLAIMS

What is claimed is:

1. A reaction plate comprising a plurality of reaction wells, wherein

a) a substantially planar plate having substantially rectangular first and second opposing planar surfaces, wherein said rectangular surfaces are from about 120 to about 140 mm in one dimension and from about 80 to about 90 mm in a perpendicular dimension, and wherein said plate comprises a thermal conductive material;

b) a plurality of wells located on a major surface of said plate, wherein each of said wells has an open top and closed bottom; and

c) the distance between adjacent wells is less than about 0.5 mm.

2. A plate according to Claim 1, wherein the thermally conductive material further comprises a thermal conductive filler and a polymer from the group consisting of polypropylene, polystyrene, polyethylene, polyethylene terephthalate, styrene acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer.

3. A plate according to Claim 2, wherein the material comprises a polypropylene and a carbon filler.

4. A plate according to Claim 1, wherein the plate comprises a material having electrical conductivity.

5. A plate according to Claim 1, further comprising a barcode.

6. A plate according to Claim 1, further comprising an alignment feature.

7. A plate according to Claim 6, wherein the alignment feature is selected from the group consisting of alignment pins, alignment slots, keyed alignment corners, and combinations thereof.

8. A plate according to Claim 1, wherein the rectangular surfaces are from about 125 to about 130 mm in one dimension, and from about 83 to about 87 mm in said perpendicular dimension.

9. A plate according to Claim 8, wherein said rectangular surfaces are about 127 mm in one dimension and about 85 mm in said perpendicular dimension.

10. A plate according to Claim 1, wherein the top of the well is essentially circular in the plane of said major surface.

11. A plate according to Claim 10, wherein the diameter of said top is from about 0.20 mm to about 2.0 mm.

12. A plate according to Claim 11, wherein a diameter of the top of the well is about 0.9 mm.

13. A plate according to Claim 10, wherein a pitch of the wells is from about 0.25 mm to about 2.50 mm.

14. A plate according to Claim 13, wherein the pitch of the wells is about 1.125 mm.

15. A plate according to Claim 1, wherein the top of the well is essentially square in the plane of said major surface.

16. A plate according to Claim 15, wherein a side dimension of the top of the well is about 0.7 mm to about 1.2 mm.

17. A plate according to Claim 16, wherein a side dimension of the top of the well is about 0.9 mm.

18. A plate according to Claim 15, wherein a pitch is about 0.75 mm to about 1.50 mm.

19. A plate according to Claim 15, wherein the pitch is about 1.125 mm.

20. A plate according to Claim 1, wherein a thickness of the wall between said wells is from about 0.05 mm to about 0.25 mm.

21. A plate according to Claim 20, wherein the thickness of the wall at the top of the well is about 0.15 mm.

22. A plate according to Claim 1, wherein a draft angle from the top of a well to the bottom is at least 1 degree.

23. A plate according to Claim 22, wherein the draft angle from the top of a well to the bottom is about 5 degrees.

24. A plate according to Claim 1, further comprising at least 6000 wells.

25. A plate according to Claim 24, further comprising about 6144 wells.

26. A plate according to Claim 1, further comprising at least 96 columns of wells and at least 64 rows of wells.

27. A plate according to Claim 1, further comprising a cover.

28. A plate according to Claim 27, further comprising a seal operable to seal the cover to the plate.

29. A plate according to Claim 27, wherein a clamping mechanism is operable to seal the cover to the plate.

30. A plate according to Claim 27, wherein said seal comprises a pressure sensitive adhesive.

31. A plate according to Claim 27, wherein the cover is transparent.

32. A plate according to Claim 27, wherein the cover is made of a thermal conductive material.

33. A method of producing a thermal conductive microplate, the method comprising:

a) mixing a polymer and at least one thermal conductive additive into a pelletized blend;

b) melting said pelletized blend;

c) injecting said melted blend into a mold cavity in an injection molding machine, said mold cavity shaped to form said microplate, wherein the injecting is through a mold cavity face that creates a bottom surface of the microplate;

d) cooling the injected melt blend to create said microplate; and

e) removing said microplate from the injection molding machine, wherein said microplate includes a plurality of wells.

34. A method according to Claim 33, wherein the plate has at least 6000 wells.

35. A method according to Claim 33, wherein the plate has at least 96 columns of wells and at least 34 rows of wells.

36. A method according to Claim 33, wherein the plate has dimensions of about 127 mm by about 85 mm.

37. A method according to Claim 33, wherein the polymer is from the group consisting of polypropylene, polystyrene, polyethylene, polyethylene terephthalate, styrene acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer.

38. A method according to Claim 33, wherein the polymer is polypropylene and the thermally conductive material is a carbon filler.

39. A method according to Claim 34, wherein the plate has about 6144 wells.

40. An apparatus to perform PCR analysis with a thermo cycling machine, the apparatus comprising:

a) a plate comprising a thermal conductive material and a plurality of wells;

b) a homogenous mixture comprising at least one primer, a polymerase, and at least one probe in at least one of the wells; and

c) a cover.

41. An apparatus according to Claim 40 wherein said mixture further comprises a sample.

42. An apparatus according to Claim 40, wherein the mixture further comprises a buffer.

43. An apparatus according to Claim 40, wherein the thermally conductive material further comprises a thermal conductive filler and a polymer from the group consisting of polypropylene, polystyrene, polyethylene, polyethylene terephthalate, styrene acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer.

44. An apparatus according to Claim 43, wherein the material comprises polypropylene and a carbon filler.

45. An apparatus according to Claim 40, wherein the material has electrical conductivity.

46. An apparatus according to Claim 40, further comprising a means of sealing the cover to the plate.

47. An apparatus according to Claim 40, wherein pressure sensitive adhesive operably seals the cover to the plate.

48. An apparatus according to Claim 40, wherein the cover is transparent.

49. An apparatus according to Claim 40, wherein the plate has at least 6000 wells.

50. An apparatus according to Claim 40, wherein the plate has at least 96 rows of wells and at least 64 columns of wells.

51. An apparatus according to Claim 40, wherein the plate has dimensions of about 127 mm by about 85 mm.

52. A method of performing PCR analysis using a preloaded multi-well microplate comprising thermal conductive material and a plurality of wells, the method comprising:

a) loading the microplate with a sample and reagents

b) placing the microplate in thermo cycling machine;

c) cycling the plate;

d) detecting results; and

e) analyzing the results.

53. A method according to Claim 52 wherein the material further comprises a thermal conductive filler and a polymer from the group consisting of polypropylene, polystyrene, polyethylene, polyethylene terephthalate, styrene acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer.

54. A method according to Claim 53, wherein the material further comprises polypropylene and a carbon filler.

55. A method according to Claim 52, further comprising preloading at least on of the wells with at least one primer.

56. A method according to Claim 52, further comprising preloading at least one of the wells with at least one probe.

57. A method according to Claim 52, further comprising preloading at least one of the wells with a polymerase.

58. A method according to Claim 52, wherein the plate has at least 6,000 wells.

59. A method according to Claim 52, further comprising preloading with a mixture designed to analyze a genome.

60. A method according to Claim 52, wherein the plate has at least 64 rows of wells and at least 96 columns of wells.

61. A method according to Claim 52, wherein the plate has dimensions of about 127 mm by about 85 mm.

62. A high density microplate, for performing an amplification reaction on a sample comprising a plurality of polynucleotide targets, comprising a substrate comprising an array of at least about 1000 wells, wherein each well each has a capacity of less than about 100 µL and

comprises a homogenous mixture consisting essentially of amplification reagents.

63. A high density microplate according to Claim 62, wherein said wells have a depth:width aspect ratio of from about 2:1 to about 3:2.

63. A high density microplate according to Claim 62, wherein said array comprises at least about 6,000 wells.

64. A high density microplate according to Claim 62, wherein said array comprises at least about 30,000 wells.

65. A high density microplate according to Claim 62, wherein said amplification reaction is PCR.

66. A high density microplate according to Claim 62, wherein the well pitch from about 0.5 to about 2.0 mm.

67. A high density microplate according to Claim 66, wherein the well pitch is about 1.125 mm.

68. A high density microplate according to Claim 66, wherein said substrate additionally comprises an alignment fixture.

69. A high density microplate of Claim 62, wherein said amplification reagents are operable to analyze a genome.

70. A method according to Claim 62, wherein the amplification reagents comprise at least one primer, at least one probe, and a polymerase.

71. A method for simultaneously amplifying a plurality of polynucleotide targets in a liquid sample, each polynucleotide target being present at very low concentration within said sample, comprising:

(a) applying said sample to a microplate assembly, wherein said microplate assembly comprises a high density microplate comprising a substrate comprising a well array of at least about 1000 wells; wherein each well has a capacity of less than about 100 µL and comprises a homogenous solution comprising amplification reagents and said sample ; and

(b) thermal cycling said microplate assembly.

72. A method according to Claim 71, wherein said amplification reagents comprise at least one primer.

73. A method according to Claim 71, said amplification reagents comprise at least one probe.

74. A method according to Claim 71, said amplification reagents comprise a polymerase.

75. A method according to Claim 71, wherein said wells have a depth:width aspect ratio of from about 2:1 to about 3:2.

76. A method according to Claim 71, wherein said array comprises at least about 6,000 wells.

77. A method according to Claim 71, wherein said wells have a volume less than about 100 microliters.

78. A method according to Claim 77, wherein said wells have a volume of from about 10 nl to about 600 nl.

79. A method according to Claim 71, wherein said amplification reaction is PCR.

80. A method according to Claim 71, wherein said microplate assembly additionally comprising an alignment device.

81. A method according to Claim 71, wherein said microplate assembly comprises a thermally conductive material.

82 A method according to Claim 81, wherein the thermally conductive material further a thermal conductive filler and a polymer from the group consisting of polypropylene, polystyrene, polyethylene, polyethylene terephthalate, styrene acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer.

83. A method according to Claim 82, wherein the material comprises polypropylene and a carbon filler.

84. A microplate comprising:

a main body portion having a first surface and an opposing second surface;

a plurality of wells formed in said first surface, each of said plurality of wells being sized to receive an assay therein; and

an integrated identification feature coupled with said main body portion.

85. The microplate according to Claim 84 wherein said integrated identification feature comprises a radio frequency identification device coupled with said main body portion.

86. The microplate according to Claim 85 wherein said radio frequency identification device is embedded in said main body portion.

87. The microplate according to Claim 84 wherein said integrated identification feature comprises a marking indicia disposed on said main body portion.

88. The microplate according to Claim 87 wherein said marking indicia is chosen from the group consisting essentially of printing, lithograph, pictorial representation, symbol, bar code, writing, drawing, etching, indentations, embossments, and raised marks.

89. The microplate according to Claim 87 wherein said marking indicia is an alignment feature.

90. The microplate according to Claim 84 wherein said main body portion is made of a thermal conductive filler and a polymer chosen from the group consisting essentially of polypropylene, polystyrene, polyethylene, polyethylene terephthalate, styrene acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer.

91. The microplate according to Claim 7 wherein said main body portion is further made of a polypropylene and a carbon filler.

92. The microplate according to Claim 84 wherein said plurality of wells is at least 6,000 wells.

93. The microplate according to Claim 84 wherein said main body portion defines a footprint of about 127 mm by about 85 mm.

94. A microplate comprising:

a main body portion having a first surface and an opposing second surface;

a plurality of wells formed in said first surface, each of said plurality of wells being sized to receive an assay therein; and

a radio frequency identification device coupled with said main body portion.

95. The microplate according to Claim 94 wherein said radio frequency identification device is embedded in said main body portion.

96. The microplate according to Claim 94 wherein said main body portion is made of a thermal conductive filler and a polymer chosen from the group consisting essentially of polypropylene, polystyrene, polyethylene, polyethylene terephthalate, styrene acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer.

97. The microplate according to Claim 96 wherein said main body portion is further made of a polypropylene and a carbon filler.

98. The microplate according to Claim 94 wherein said plurality of wells is at least 6,000 wells.

99. The microplate according to Claim 94 wherein said main body portion defines a footprint of about 127 mm by about 85 mm.

100. A microplate comprising:

a main body portion having a first surface and an opposing second surface;

a plurality of wells formed in said first surface, each of said plurality of wells being sized to receive an assay therein; and

a marking indicia disposed on said main body portion.

101. The microplate according to Claim 100 wherein said marking indicia is chosen from the group consisting essentially of printing, lithograph, pictorial representation, symbol, bar code, writing, drawing, etching, indentations, and embossments.

102. The microplate according to Claim 100 wherein said marking indicia is an alignment feature.

103. The microplate according to Claim 100 wherein said main body portion is made of a thermal conductive filler and a polymer chosen from the group consisting essentially of polypropylene, polystyrene, polyethylene, polyethylene terephthalate, styrene acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer.

104. The microplate according to Claim 103 wherein said main body portion is further made of a polypropylene and a carbon filler.

105. The microplate according to Claim 100 wherein said plurality of wells is at least 6,000 wells.

106. The microplate according to Claim 100 wherein said main body portion defines a footprint of about 127 mm by about 85 mm.

107. A method for printing a marking indicia on a microplate, said method comprising:

providing a thermally conductive microplate having a plurality of wells;

treating a surface of said thermally conductive microplate in preparation for printing; and

printing a marking indicia onto said thermally conductive microplate.

108. The method according to Claim 107 wherein said printing a marking indicia further comprises:

printing said marking indicia with curable ink; and

curing said ink.

109. The method according to Claim 108 wherein said curing said ink comprises curing said ink using ultraviolet light.

110. The method according to Claim 107 wherein said treating a surface is a treatment chosen from the group consisting essentially of flame treatment, corona treatment, acid wash treatment, photolithography treatment, or coating.

111. The method according to Claim 107 wherein said providing a thermally conductive microplate comprises providing a thermally conductive microplate being made of a thermally conductive filler and a polymer from the group consisting of polypropylene, polystyrene, polyethylene, polyethylene terephthalate, styrene acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer.

112. The method according to Claim 111 wherein said providing a thermally conductive microplate further includes providing a thermally conductive microplate further being made of a polypropylene and a carbon filler.

113. The method according to Claim 107 wherein said printing a marking indicia comprises printing a marking indicia chosen from the group consisting essentially of a printing, lithograph, pictorial representation, symbol, bar code, writing, drawing, etching, indentations, embossments, and alignment feature.

114. A method for marking a microplate, said method comprising:

providing a thermally conductive microplate having a plurality of wells; and

laser etching a marking indicia onto said thermally conductive microplate.

115. The method according to Claim 114 wherein said providing a thermally conductive microplate comprises providing a thermally conductive microplate being made of a thermally conductive filler and a polymer from the

group consisting of polypropylene, polystyrene, polyethylene, polyethylene terephthalate, styrene acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer.

116. The method according to Claim 115 wherein said providing a thermally conductive microplate further includes providing a thermally conductive microplate further being made of a polypropylene and a carbon filler.

117. The method according to Claim 114 wherein said laser etching a marking indicia comprises laser etching a marking indicia chosen from the group consisting essentially of a printing, lithograph, pictorial representation, symbol, bar code, writing, drawing, etching, indentations, embossments, and alignment feature.

118. A multiwell plate for conducting a thermocycled amplification reaction of polynucleotide in a liquid assay, said plate comprising:

(a) a substantially planar substrate having first and second major surfaces;

(b) a plurality of reaction wells located on said first major surface, wherein each of said wells has a bottom and an opening which is disposed on said first major surface;

(c) a substantially planar sealing cover, a major surface of which is disposed adjacent to said first major surface so as to cover said openings of said wells;

wherein the substrate comprises a thermal conductive material.

119. A plate according to Claim 118 further comprising at least one blind hole in the bottom of the wells.

120. A plate according to Claim 118, wherein the plurality of reaction wells is at least 6000 wells.

121. A plate according to Claim 118, wherein the sealing cover is essentially transparent.

122. A plate according to Claim 118, further comprising a pressure sensitive adhesive.

123. A plate according to Claim 118, wherein the sealing cover comprises a friction reduction film, a face stock, and a pressure sensitive adhesive.

124. A plate according to Claim 123, wherein the sealing cover further comprises a compliant layer.

125. A plate according to Claim 123, wherein in the sealing cover further comprises a release liner.

126. A plate according to Claim 118, further comprising at least about 24,000 wells.

127. A plate according to Claim 119, wherein the pressure sensitive adhesive is operable to seal the cover to the plate.

128. A plate according to Claim 118, wherein the cover is a flexible membrane.

129. A plate according to Claim 118, wherein the cover further comprises a heater.

130. A plate according to Claim 118, wherein the cover comprises indium tin oxide.

131. A plate according to Claim 118, wherein the cover comprises a thermally conductive material.

132. A plate according to Claim 118, wherein the material further comprises a thermal conductive filler and a polymer from the group consisting of polypropylene, polystyrene, polyethylene, polyethylene terephthalate, styrene acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer.

133. A plate according to Claim 132, wherein the material further comprises a polypropylene and a carbon filler.

134. A plate according to Claim 118, wherein the material has electrical conductivity.

135. A plate according to Claim 118, further comprising a graphic.

13. An apparatus for sealing a microplate assembly comprising a cover and a microplate, the apparatus comprising:

a heated roller; and

an unheated roller;

wherein said heated roller and said unheated roller have axes of rotation that are essentially parallel and are spaced apart sufficiently to receive said assembly between said heated roller and said unheated roller while applying pressure between said cover and said microplate.

137. An apparatus according to Claim 136, further comprising a temperature controller controlling the temperature of the heated roller.

138. An apparatus according to Claim 136, further comprising at least one pressure controller controlling the pressure exerted by at least one of said rollers on said microplate.

139. An apparatus according to Claim 136, wherein the microplate comprises a thermal conductive material.

140. An apparatus according to Claim 136, further comprising a motor operable to rotate at least one of said rollers.

141. An apparatus according to Claim 140, further comprising a speed controller controlling the speed of said roller.

142. An apparatus according to Claim 136, wherein said apparatus further comprises a pressure sensitive adhesive.

143. An apparatus according to Claim 136, wherein said cover comprises a friction reduction film, a face stock, and a pressure sensitive adhesive.

144. An apparatus according to Claim 133, wherein said sealing cover further comprises a compliant layer.

145. An apparatus according to Claim 143, wherein in said sealing cover further comprises a release liner.

146. An apparatus according to Claim 142, wherein the pressure sensitive adhesive operable to seal the cover to the plate.

147. An apparatus according to Claim 146, wherein the cover is a flexible membrane.

147. An apparatus according to Claim 146, wherein the cover further comprises a heater.

148. An apparatus according to Claim 139, wherein the material comprises a thermal conductive filler and a polymer from the group consisting of polypropylene, polystyrene, polyethylene, polyethylene terephthalate, styrene acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer.

149. An apparatus according to Claim 148, wherein the material comprises a polypropylene and a carbon filler.

150. An apparatus according to Claim 149, wherein the material that has electrical conductivity.

151. An apparatus according to Claim 146, wherein the assembly further comprises a graphic.

152. A method of sealing a cover to a microplate, the method comprising:

removing a release liner from the cover;

placing the cover on the microplate; and

laminating the cover to the microplate.

153. A method according to Claim 152, further comprising activating a pressure sensitive adhesive.

154. A method according to Claim 152, further comprising removing a protective liner from the face of the cover.

155. A method according to Claim 152, wherein the microplate comprises a thermal conductive material.

156. A method according to Claim 152, further comprising maximizing laminating conditions.

157. A reaction plate comprising:

a main body portion having a first surface and an opposing second surface;

a groove disposed about said first surface of said main body portion, said groove separating said main body portion into an inboard section and an outboard section; and

a plurality of wells formed in said inboard section of said first surface, each of said plurality of wells being sized to receive an assay therein.

158. The reaction plate according to Claim 157 wherein said main body portion is made of a thermally conductive material.

159. The reaction plate according to Claim 158 wherein said thermally conductive material further comprises a thermal conductive filler and a polymer chosen from the group consisting essentially of polypropylene, polystyrene, polyethylene, polyethylene terephthalate, styrene acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer.

160. The reaction plate according to Claim 157 wherein said thermally conductive material further comprises a polypropylene and a carbon filler.

161. The reaction plate according to Claim 157, further comprising:

a sealing cover engagable at least in part with said groove to cover said plurality of wells.

162. The reaction plate according to Claim 161 wherein said sealing cover includes an adhesive at least partially disposed in said groove.

163. The reaction plate according to Claim 157, further comprising:

marking indicia disposed on said outboard portion of said outboard section of said main body portion.

164. The reaction plate according to Claim 163 wherein said marking indicia is chosen from the group consisting essentially of printing, lithograph, pictorial representation, symbol, bar code, writing, drawing, etching, indentations, embossments, and raised marks.

165. The reaction plate according to Claim 157 wherein said groove is operable to generally thermally isolate said outboard section from said inboard section.

166. The reaction plate according to Claim 157 wherein said groove is operable to maintain said main body portion in a generally flat position during manufacture.

167. The reaction plate according to Claim 157 wherein said main body portion defines a footprint of about 127mm by about 85mm.

168. The reaction plate according to Claim 157 wherein a depth of one of said plurality of wells is generally equal to a depth of said groove.

169. The reaction plate according to Claim 157 wherein said groove is operable to receive a liquid therein.

170. A reaction plate for thermocycled amplification reaction of polynucleotide in an assay, said reaction plate comprising:

a thermally conductive main body portion having a first surface and an opposing second surface;

a groove disposed about said first surface of said main body portion, said groove separating said main body portion into an inboard section and an outboard section; and

a plurality of wells formed in said inboard section of said first surface, each of said plurality of wells being sized to receive the assay therein.

171. The reaction plate according to Claim 170 wherein said thermally conductive main body portion further comprises a thermal conductive filler and a polymer chosen from the group consisting essentially of polypropylene, polystyrene, polyethylene, polyethylene terephthalate, styrene acrylonitrile, cyclic polyolefin, syndiotactic polystyrene, polycarbonate and liquid crystal polymer.

172. The reaction plate according to Claim 171 wherein said thermally conductive main body portion further comprises a polypropylene and a carbon filler.

173. The reaction plate according to Claim 170, further comprising:

a sealing cover engagable at least in part with said groove to cover said plurality of wells.

174. The reaction plate according to Claim 170 wherein said sealing cover includes an adhesive at least partially disposed in said groove.

175. The reaction plate according to Claim 170, further comprising:

marking indicia disposed on said outboard portion of said outboard section of said main body portion.

176. The reaction plate according to Claim 170 wherein said marking indicia is chosen from the group consisting essentially of printing, lithograph, pictorial representation, symbol, bar code, writing, drawing, etching, indentations, embossments, and raised marks.

177. The reaction plate according to Claim 170 wherein said groove is operable to generally thermally isolate said outboard section from said inboard section.

178. The reaction plate according to Claim 170 wherein said groove is operable to maintain said main body portion in a generally flat position during manufacture.

179. The reaction plate according to Claim 170 wherein said main body portion defines a footprint of about 127mm by about 85mm.

180. The reaction plate according to Claim 170 wherein a depth of one of said plurality of wells is generally equal to a depth of said groove.

181. The reaction plate according to Claim 170 wherein said assay further comprises at least one primer, at least one probe and a polymerase.

182. The reaction plate according to Claim 170 wherein said assay is a homogenous mixture.

183. The reaction plate according to Claim 170 wherein said assay includes reagents operable to perform multiplex PCR.

184. The reaction plate according to Claim 170 wherein said groove is operable to receive a liquid therein.

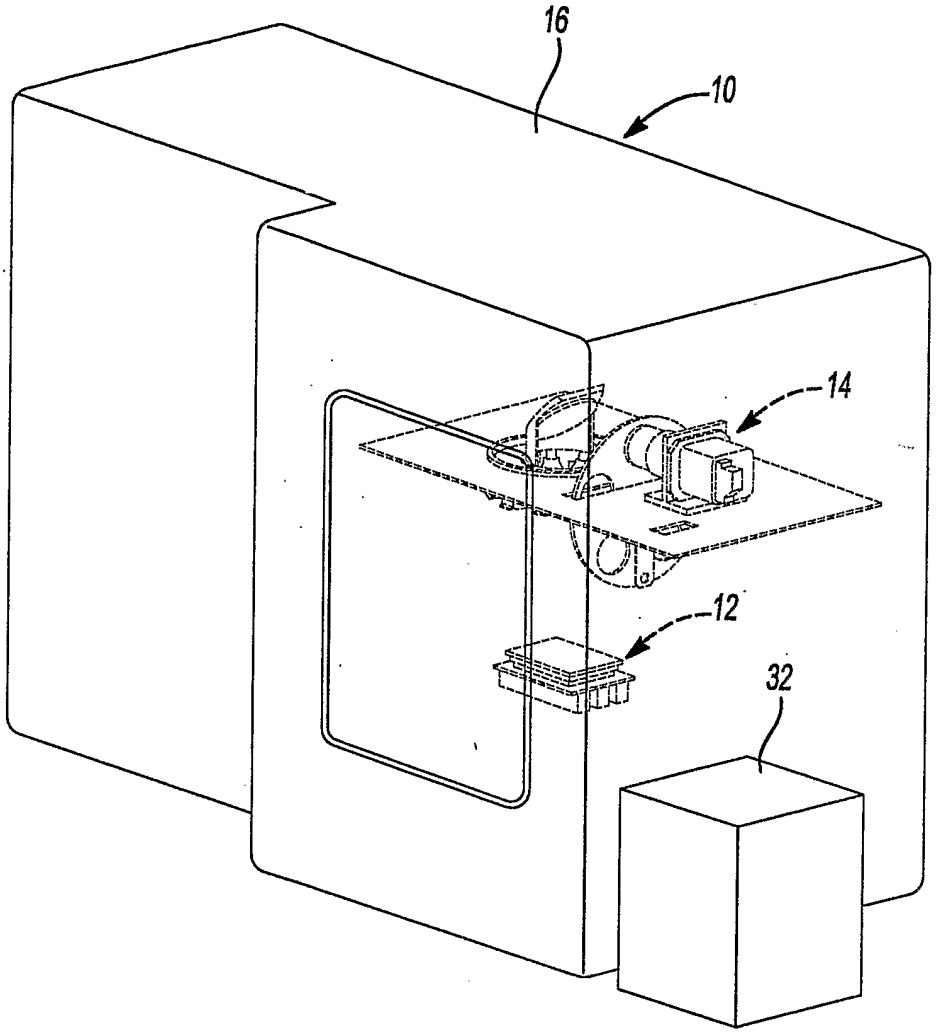


Fig-1

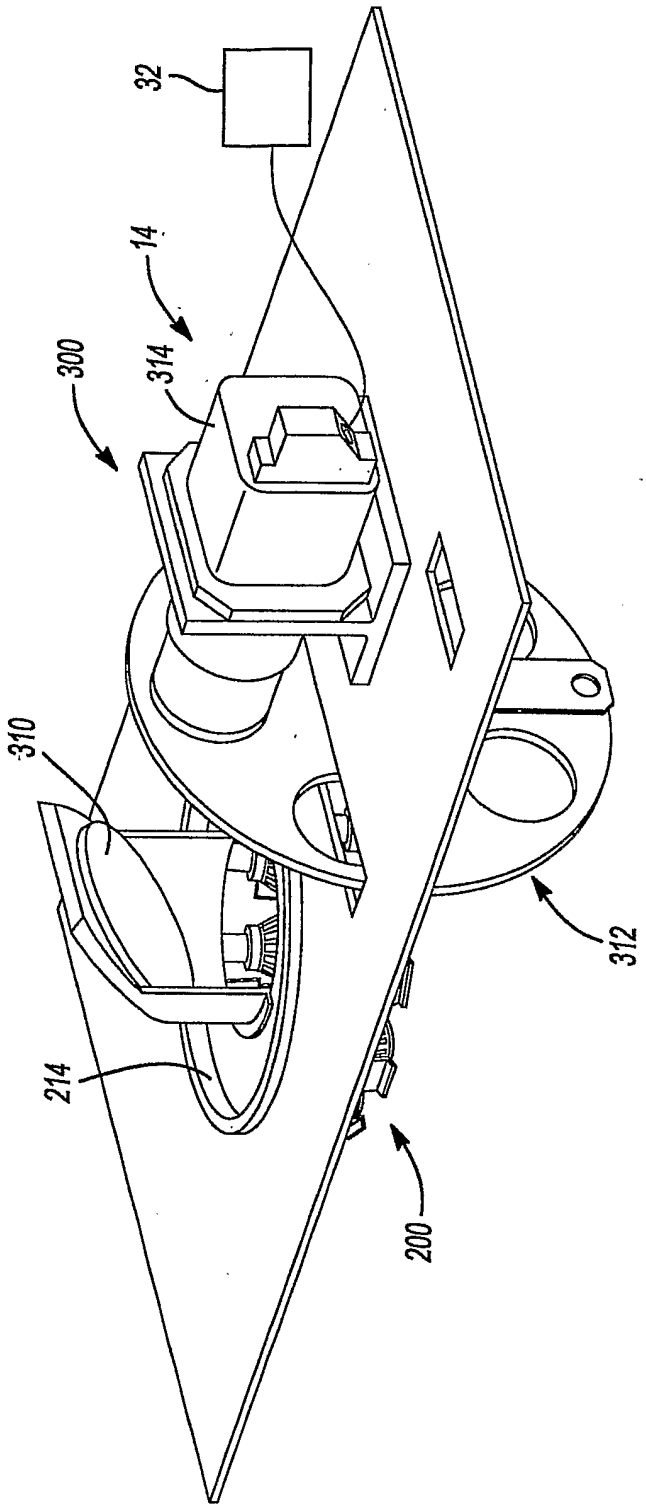
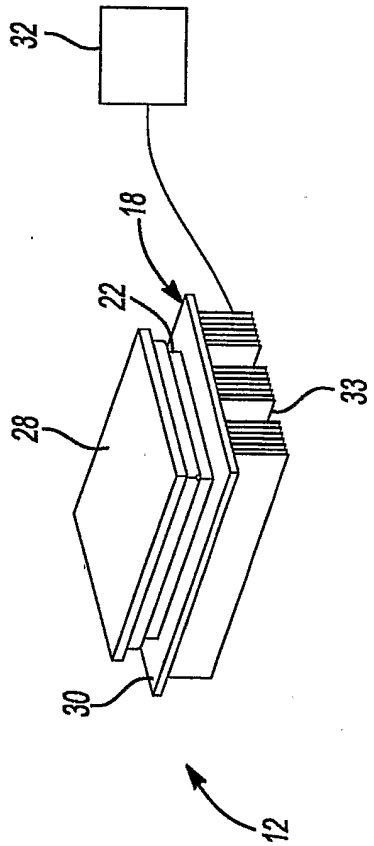


Fig-2



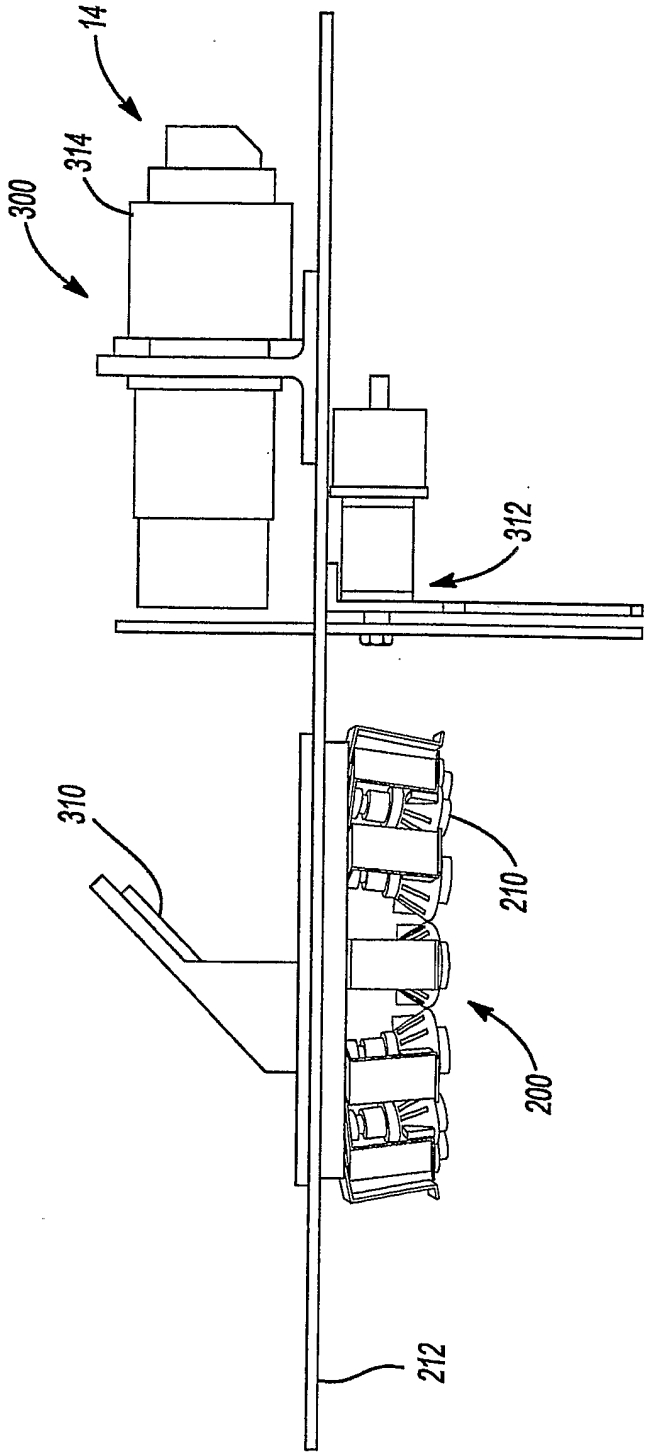
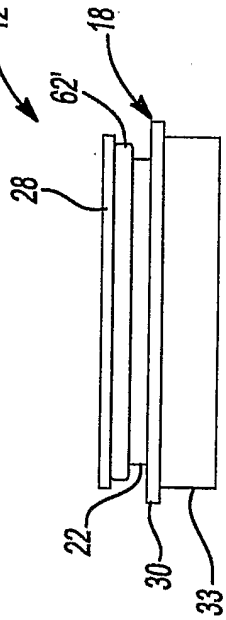


Fig-3



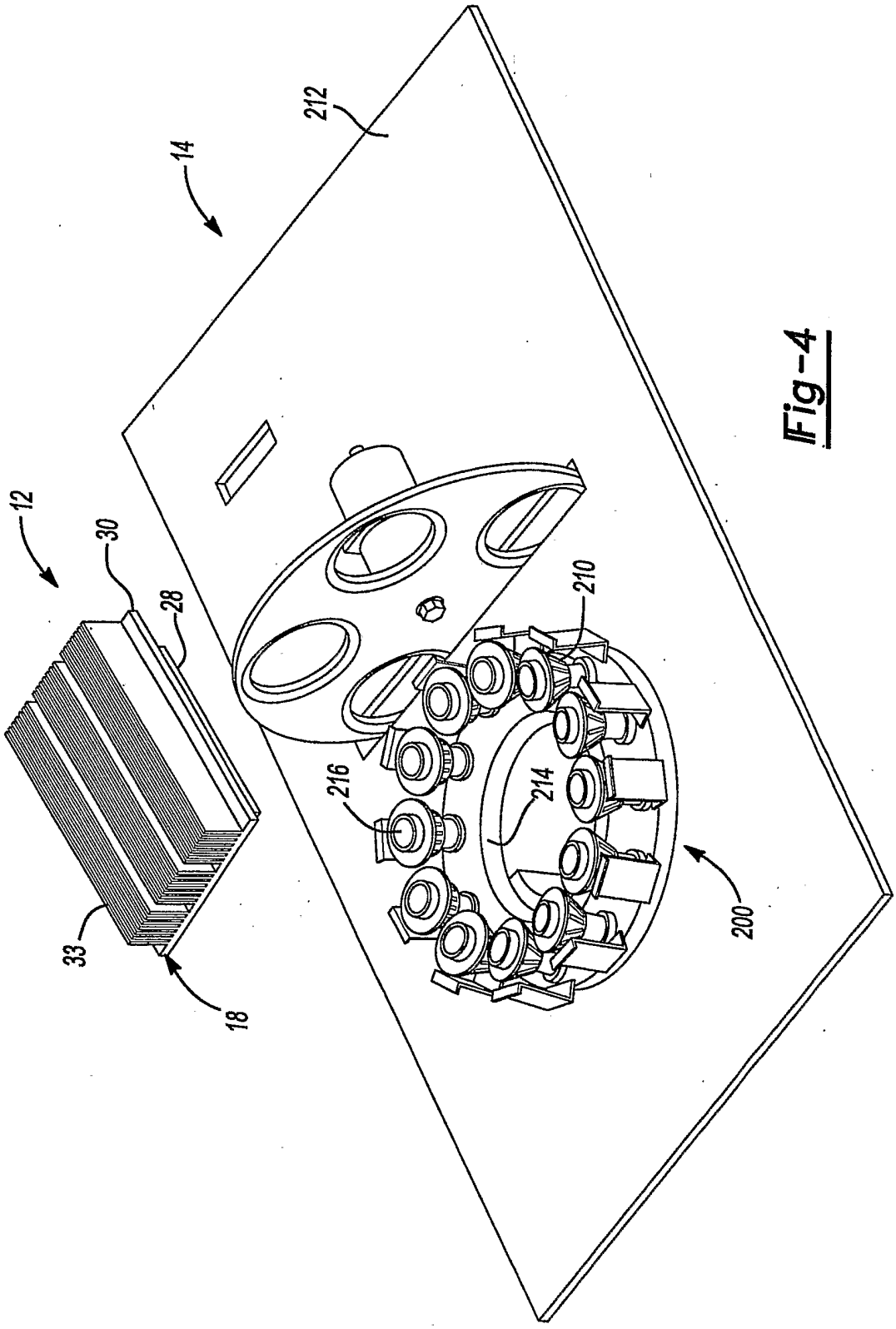


Fig-4

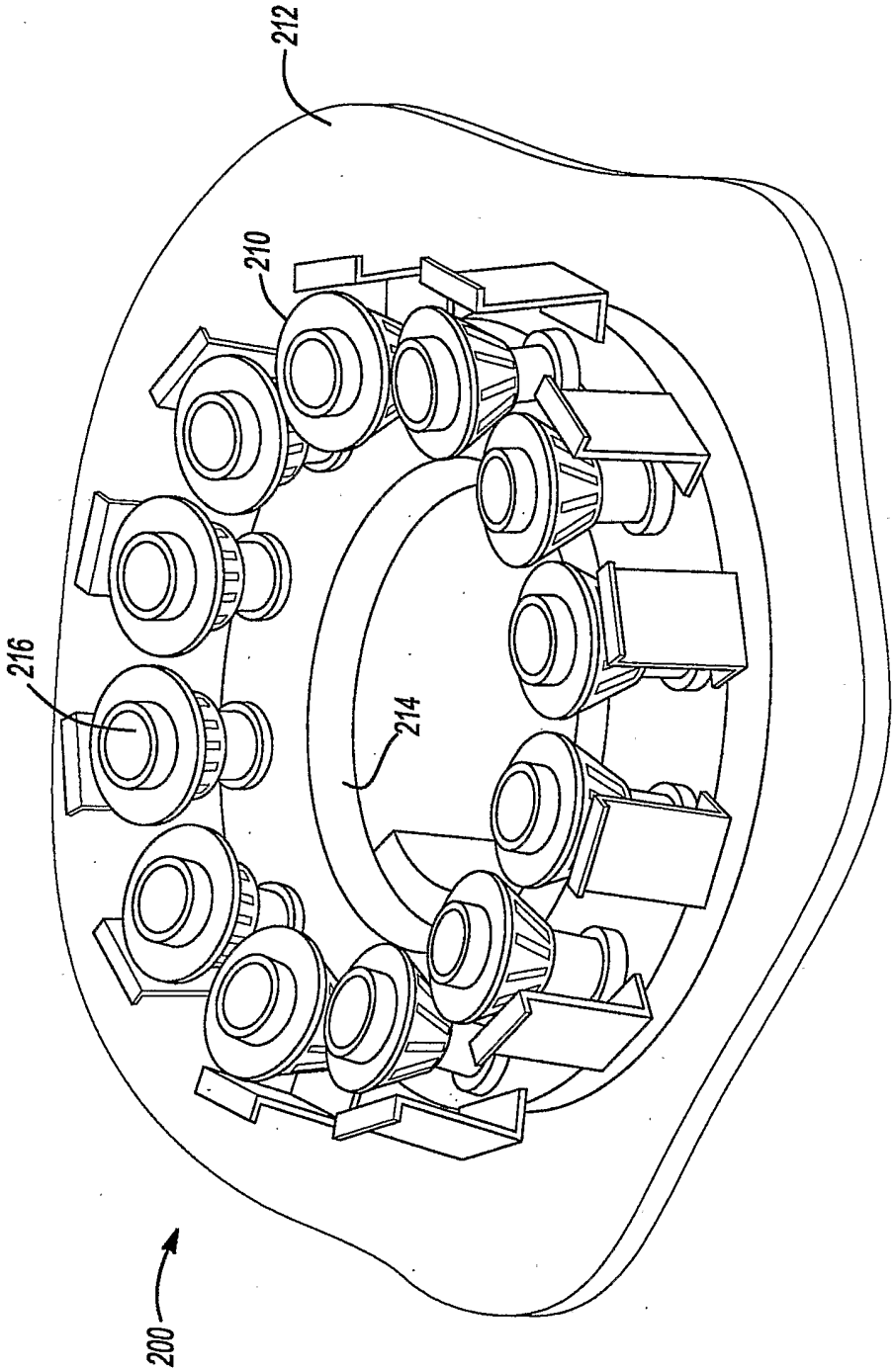


Fig-5

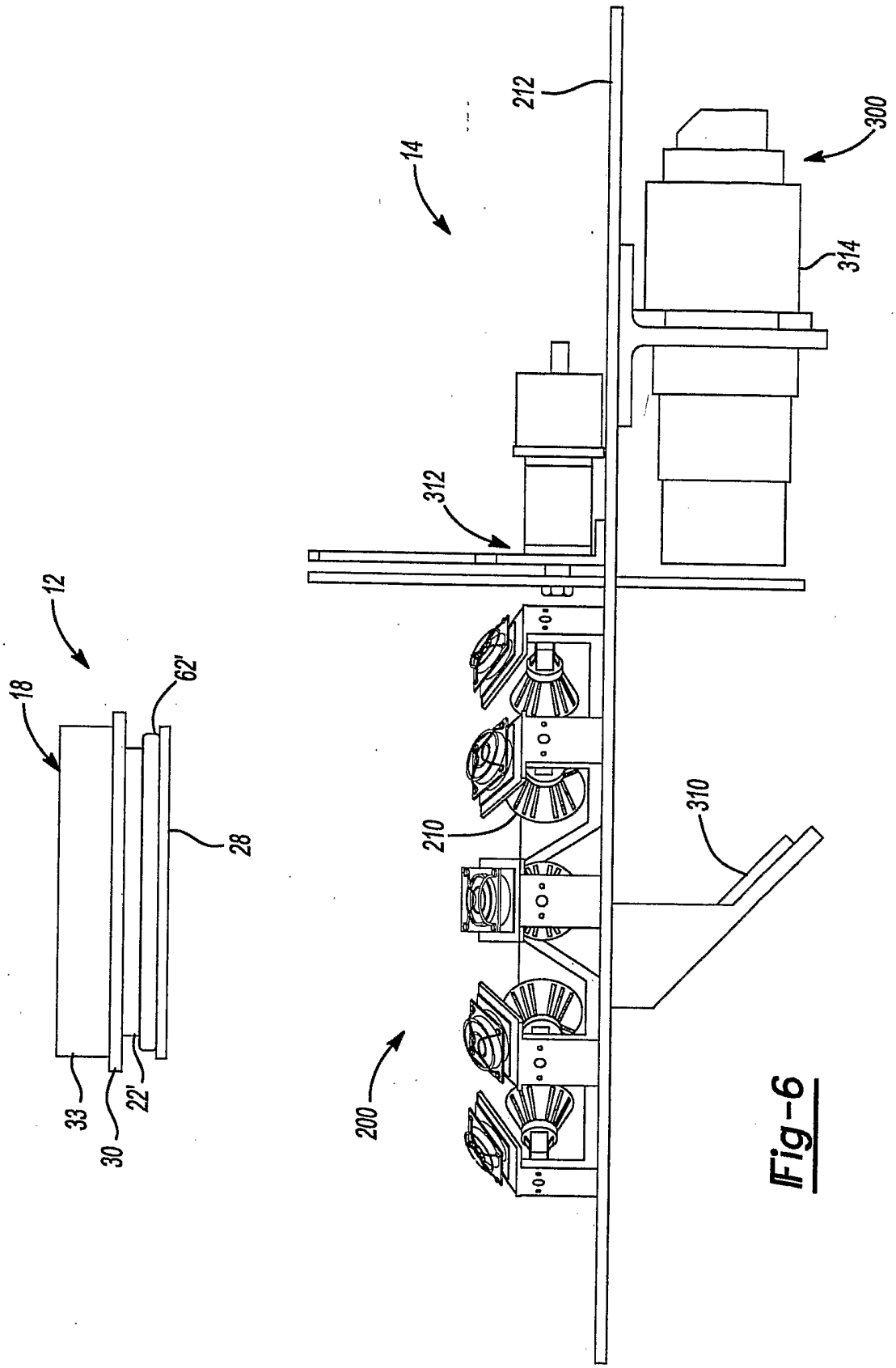


Fig-6

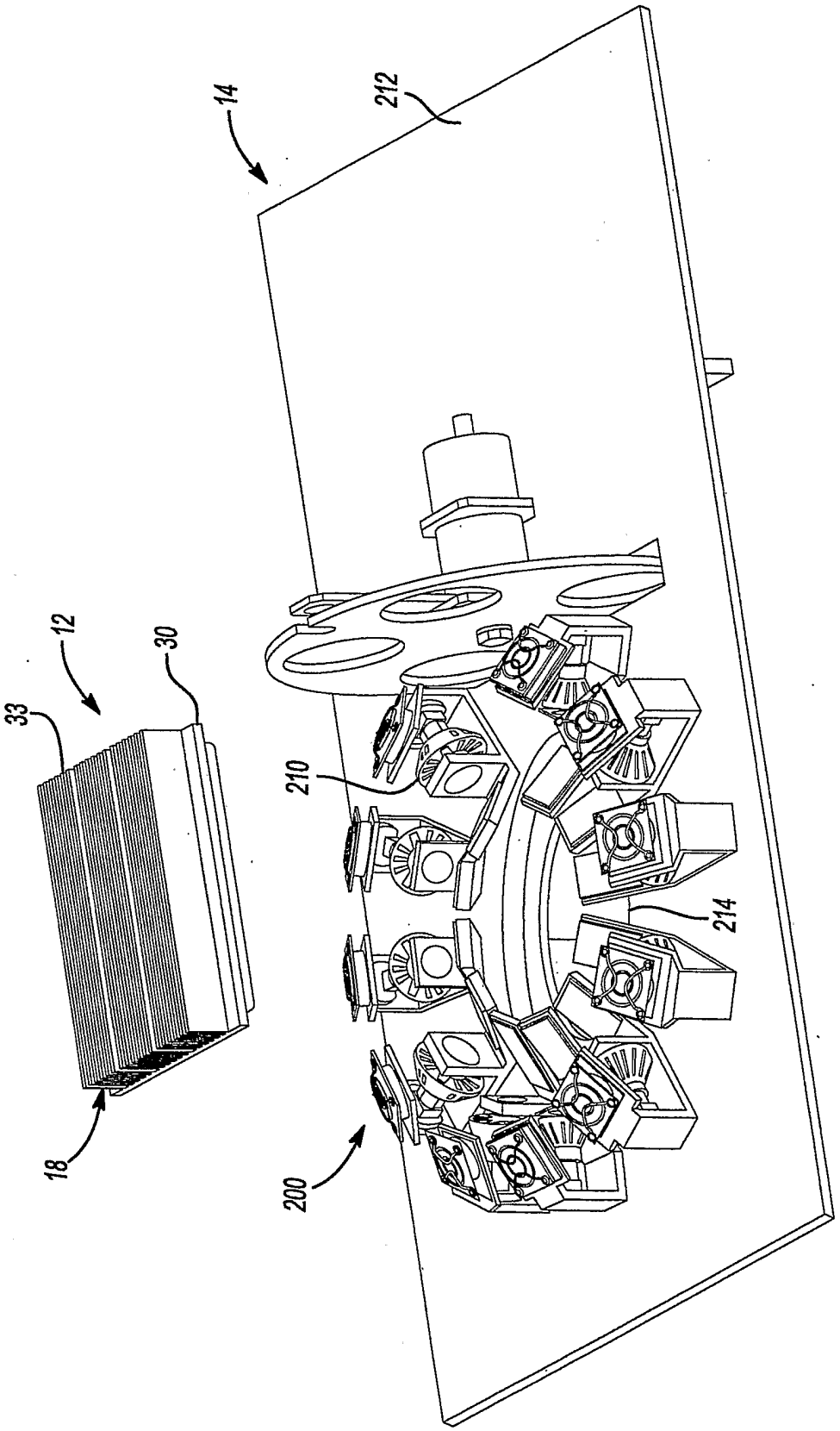


Fig-7

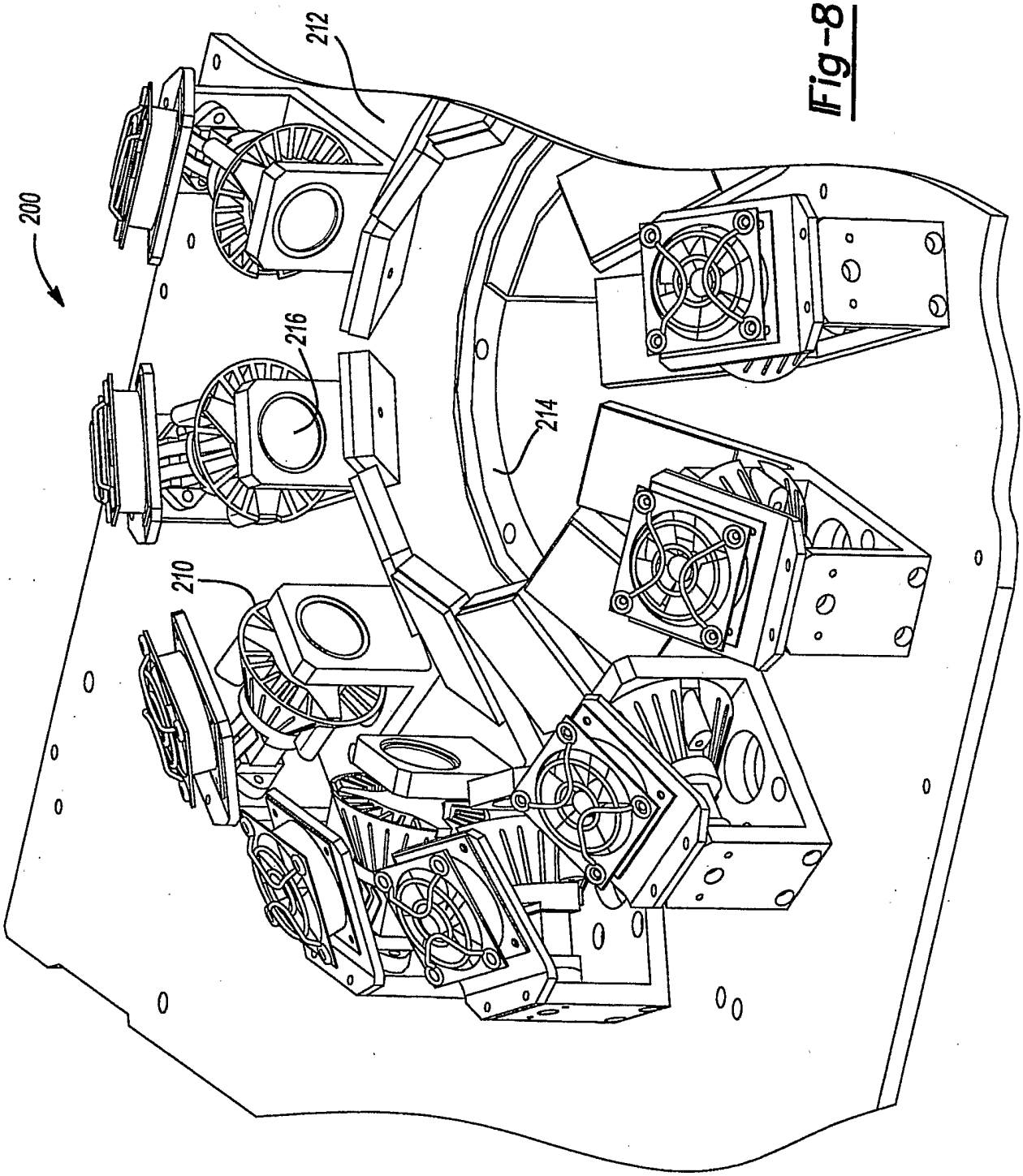


Fig-8

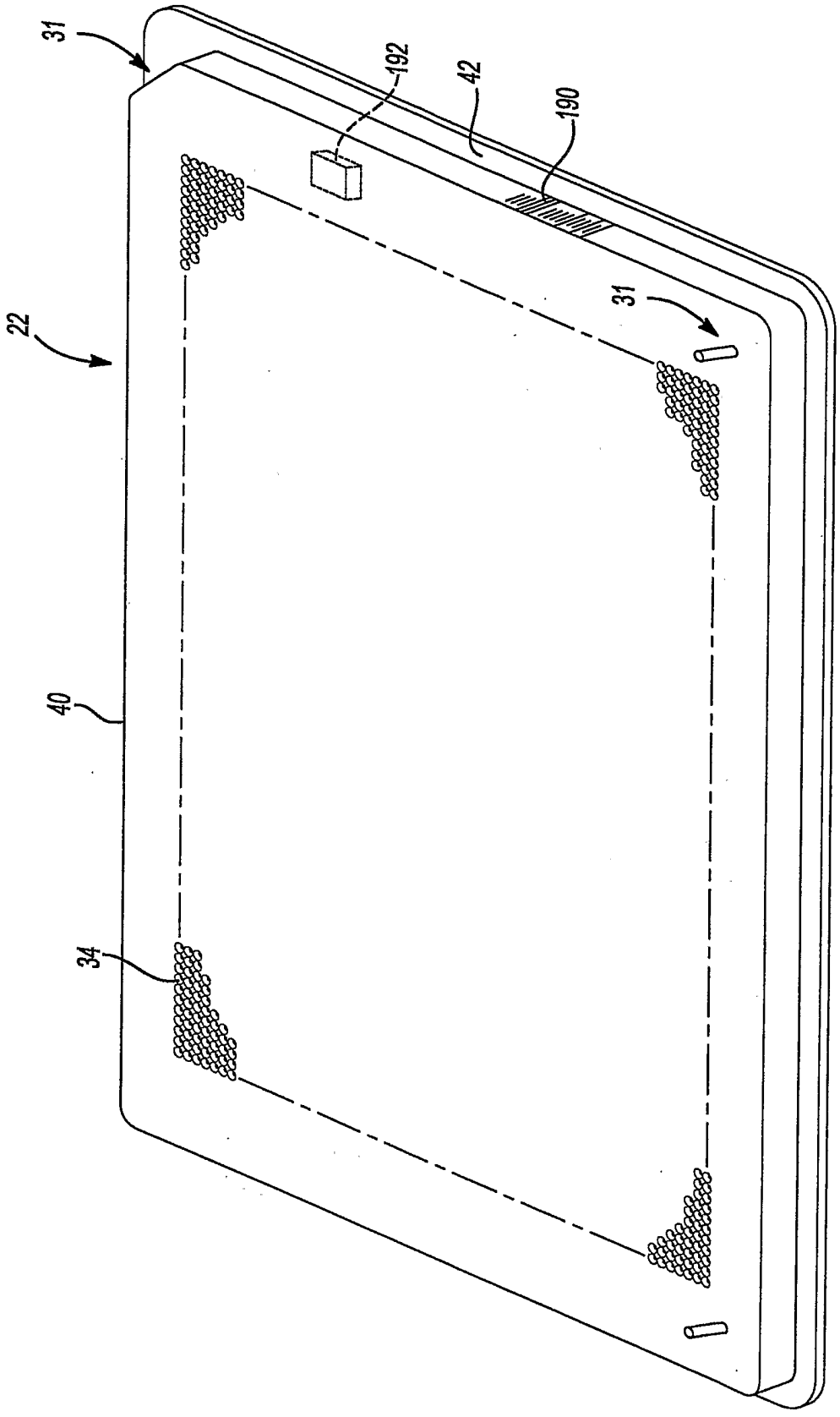


Fig-9

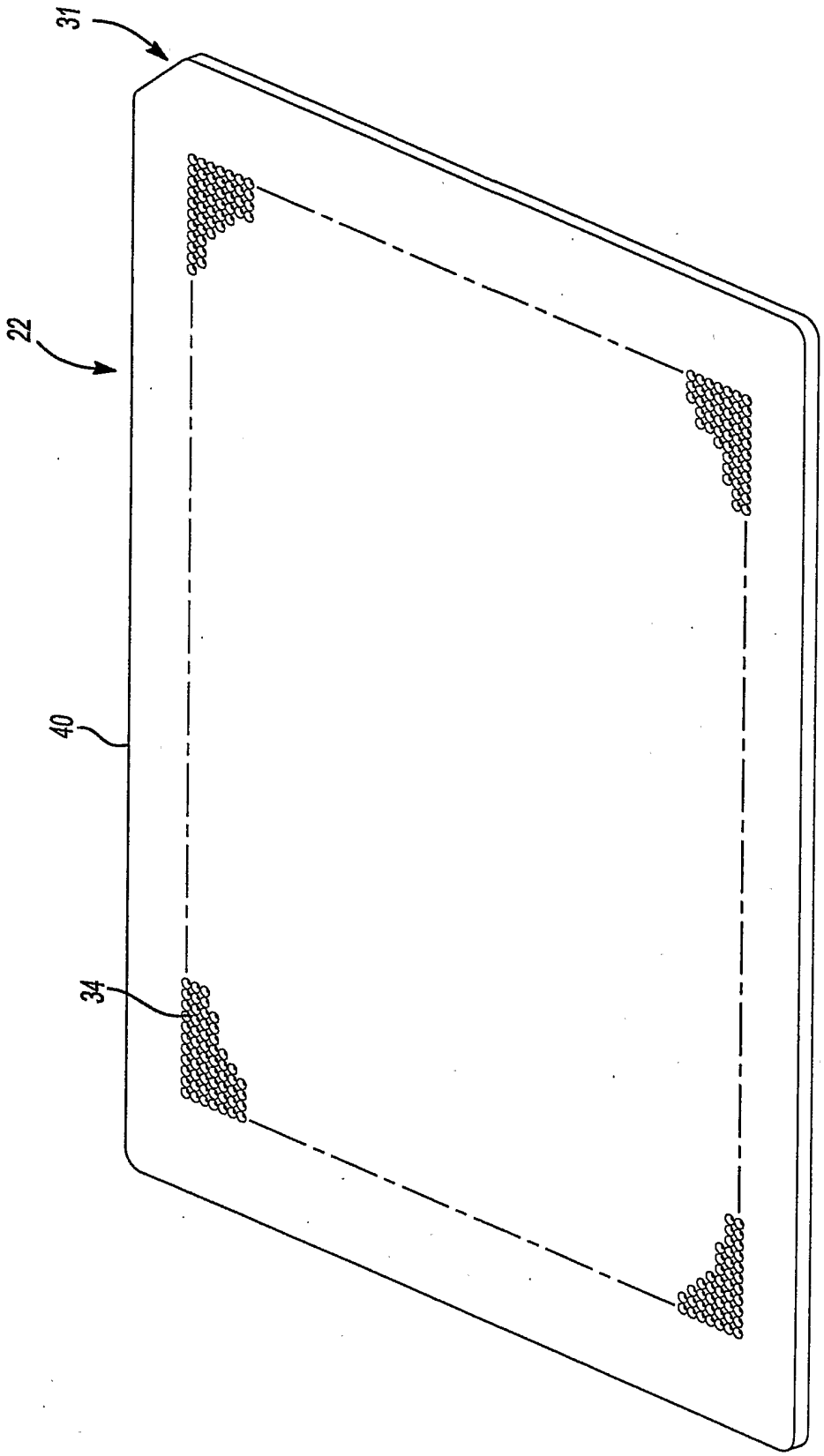


Fig-10

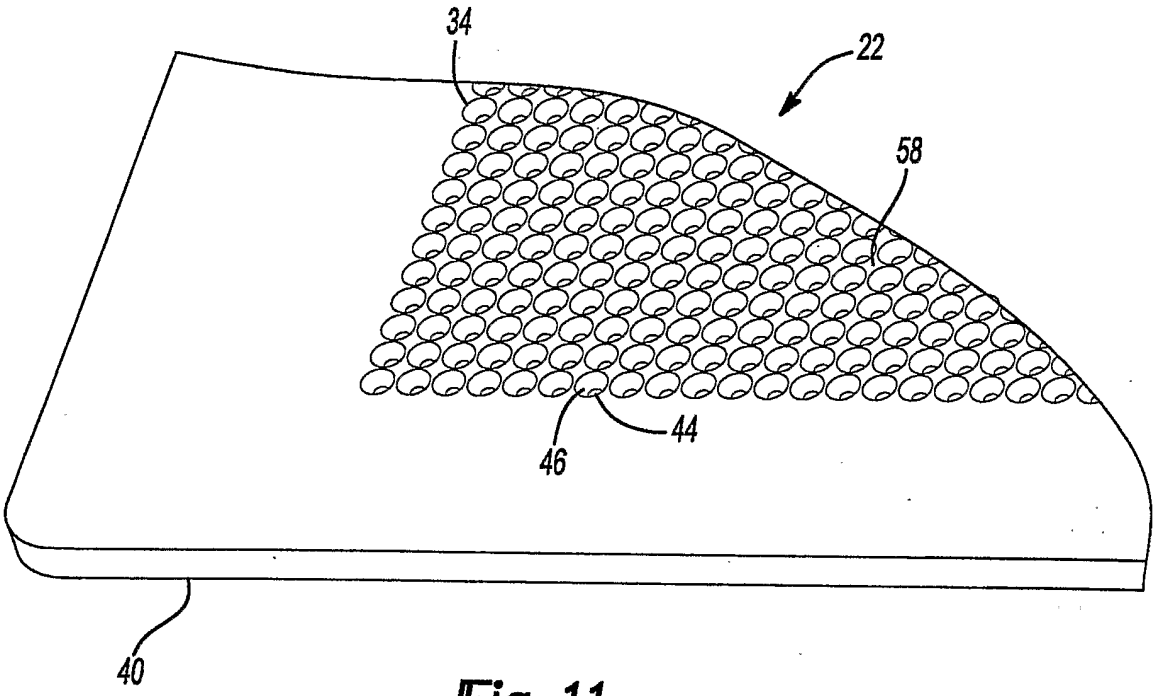


Fig-11

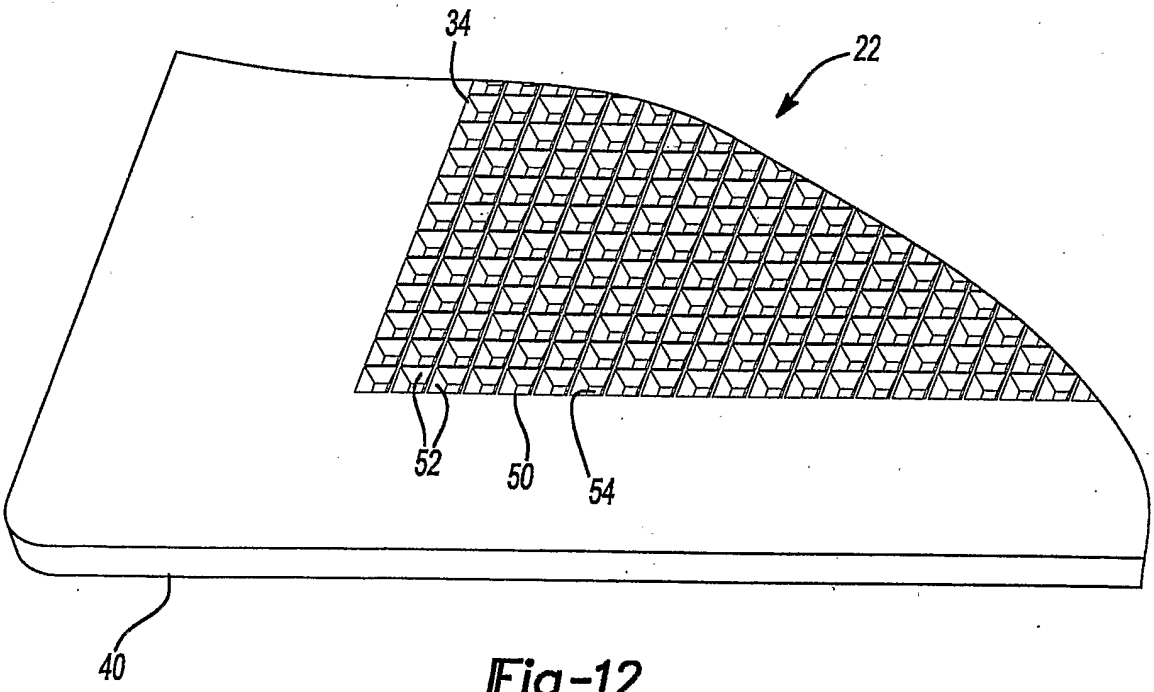


Fig-12

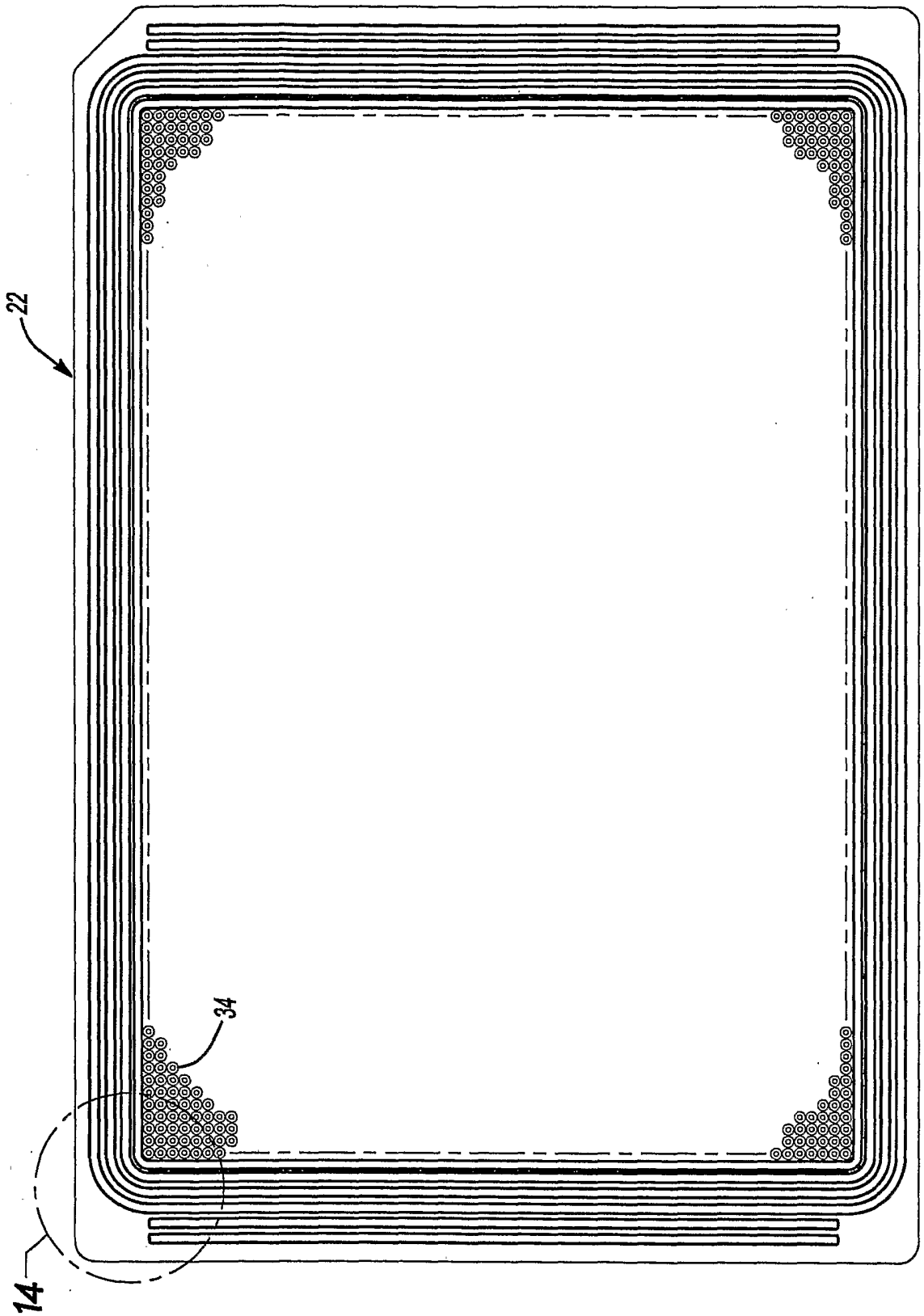


Fig-13

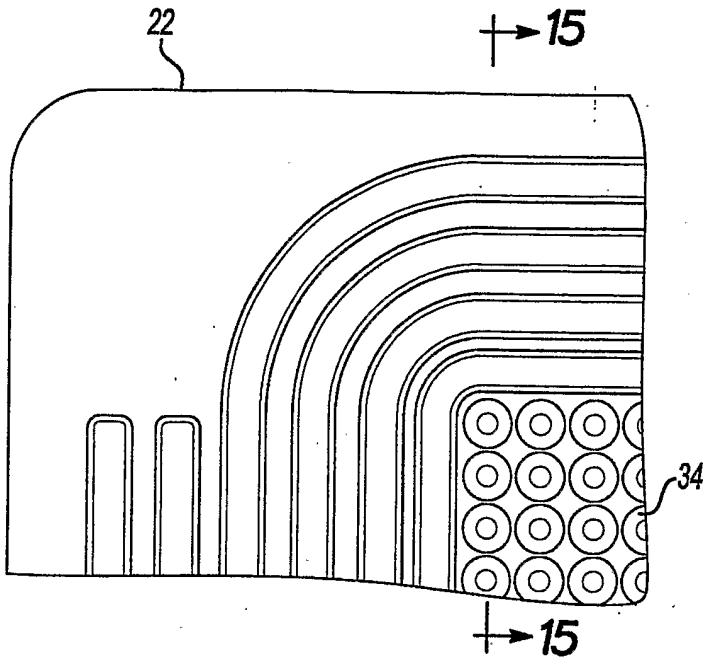


Fig-14

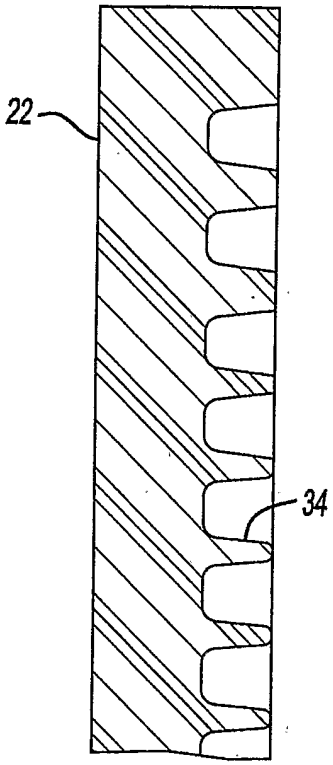


Fig-15

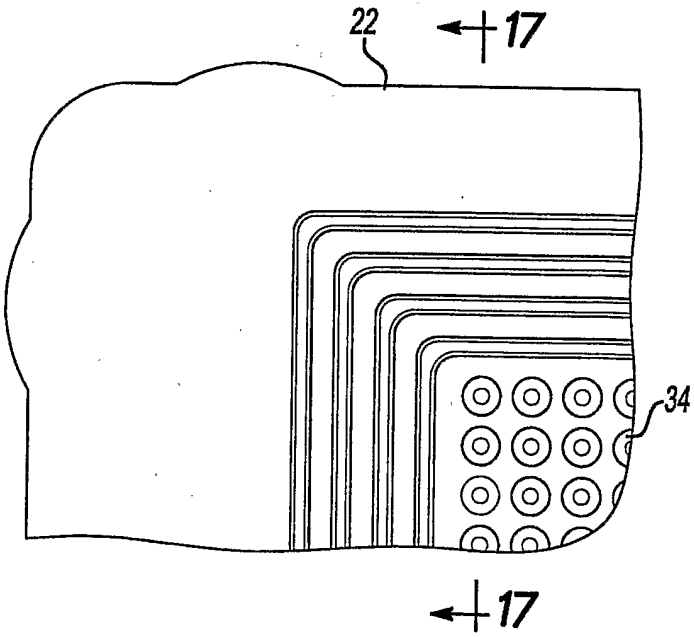


Fig-16

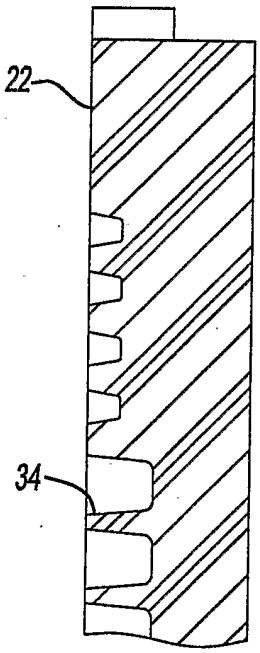


Fig-17

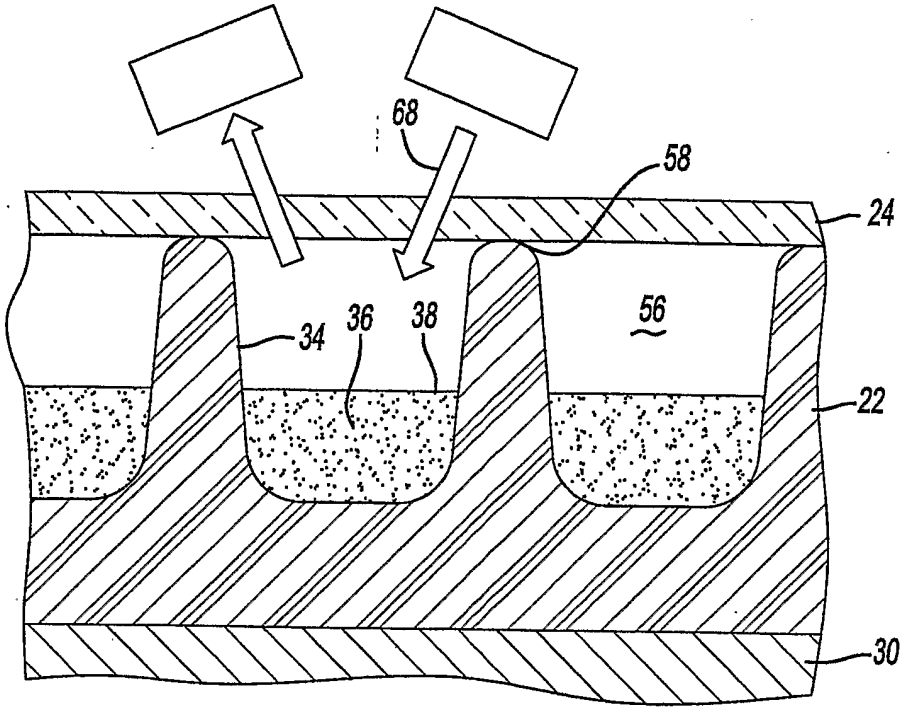


Fig-18

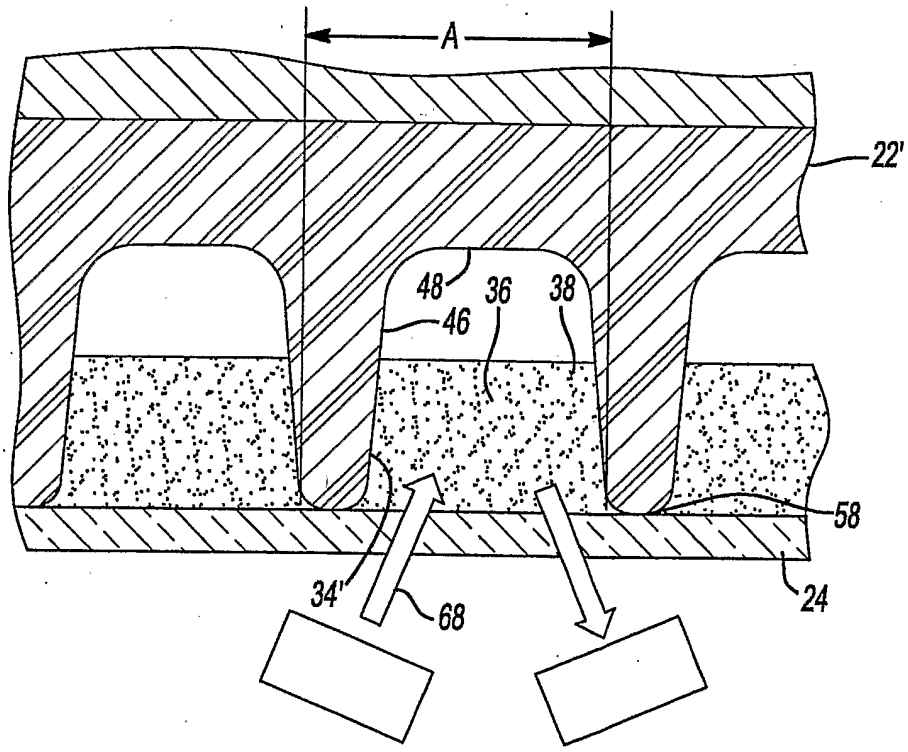


Fig-19

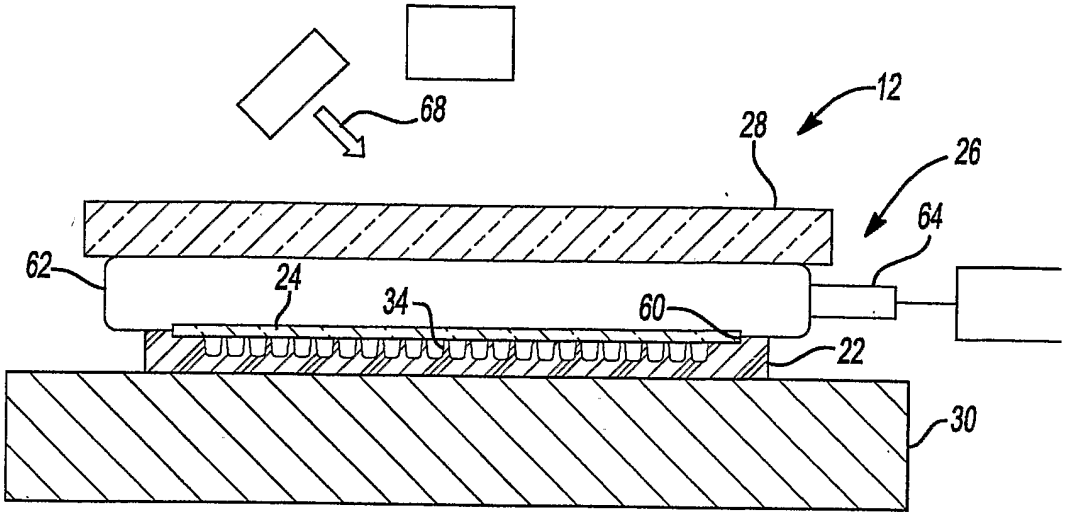


Fig-20

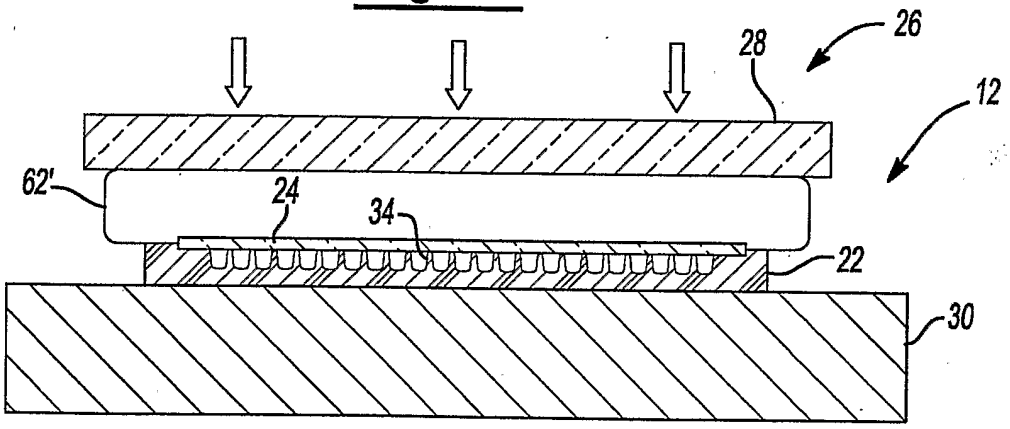


Fig-21

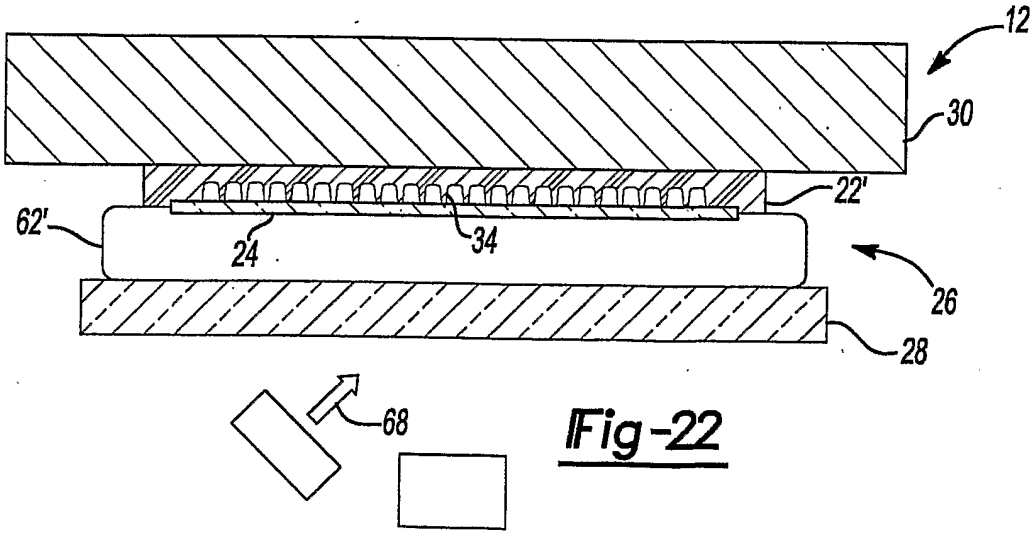


Fig-22

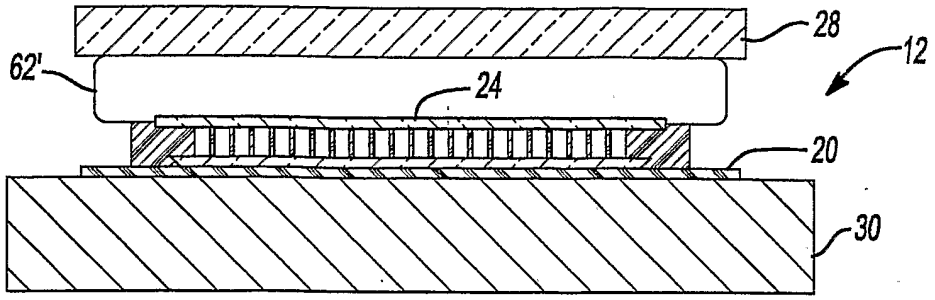


Fig-23

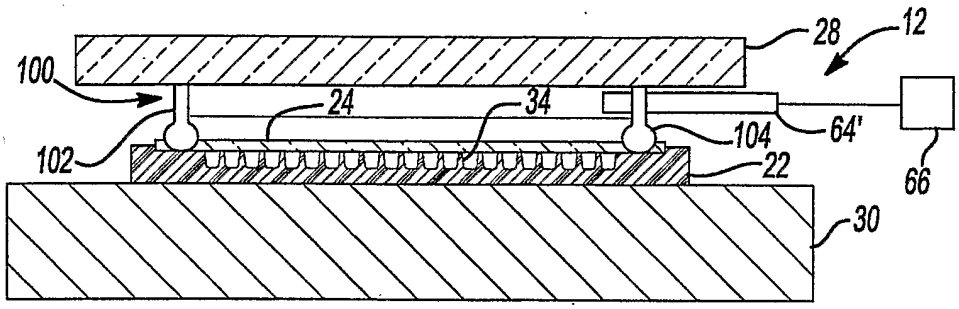


Fig-24

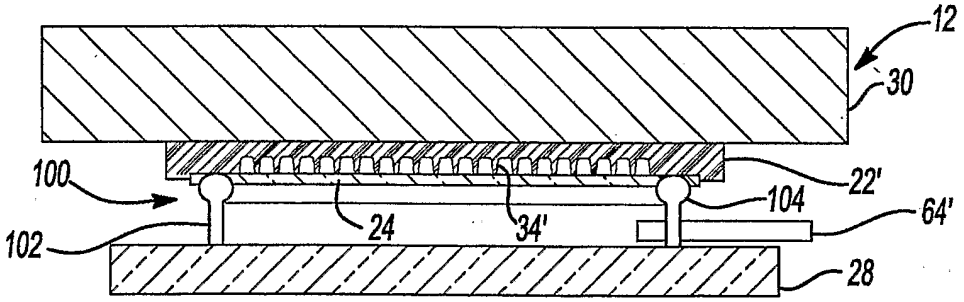


Fig-25

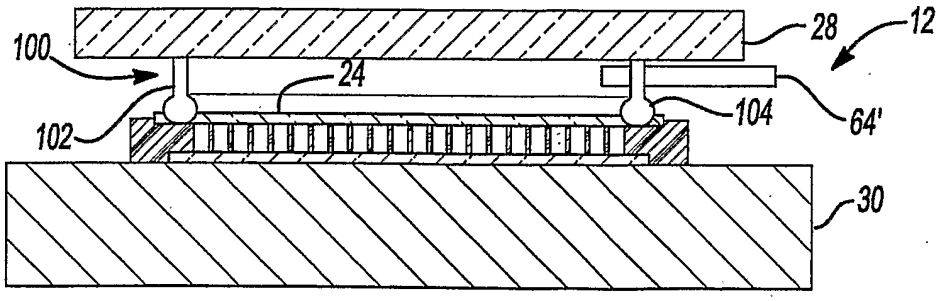


Fig-26

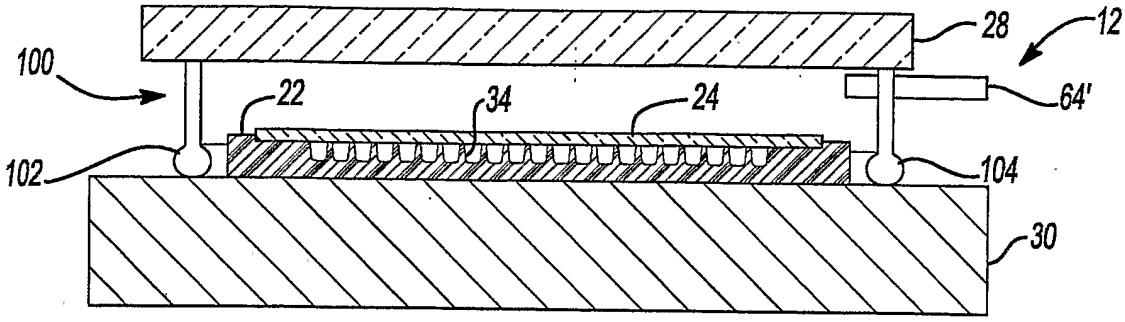


Fig-27

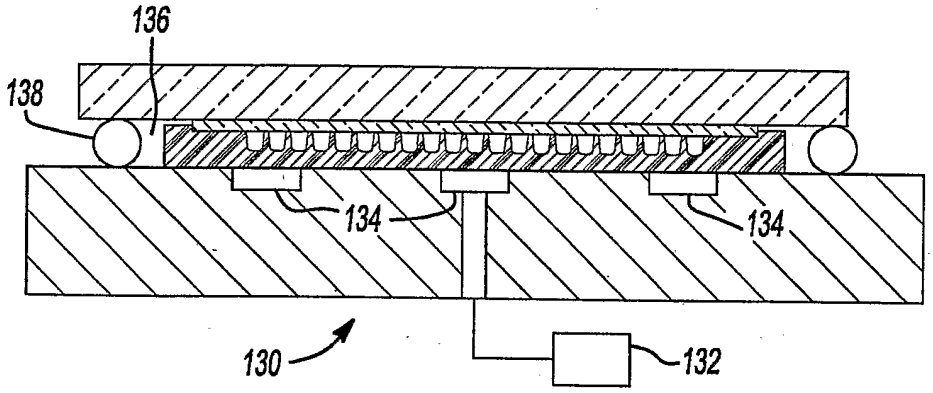


Fig-27A

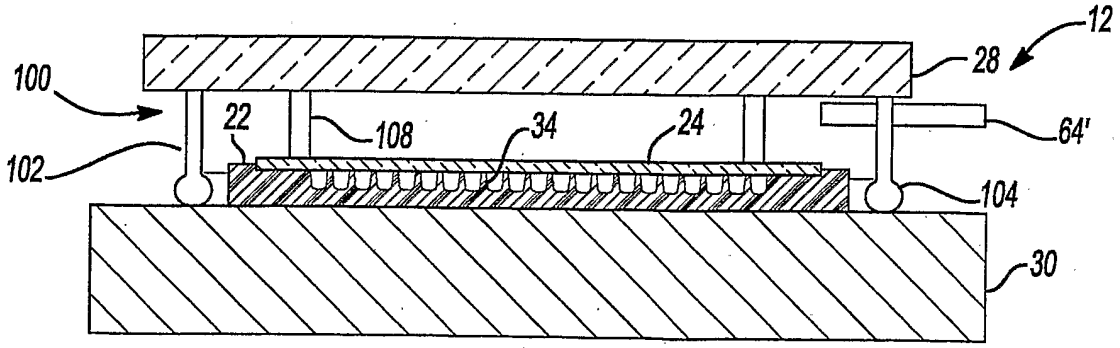


Fig-28

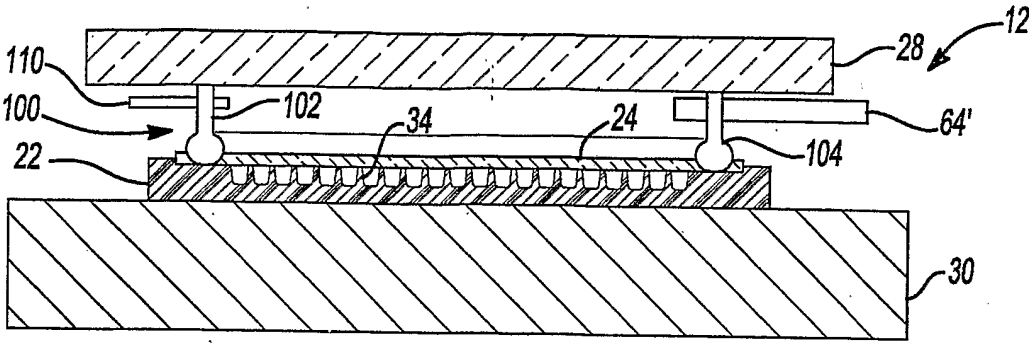

12
28
110
100
102
34
24
64'
22
104
30

Fig-29

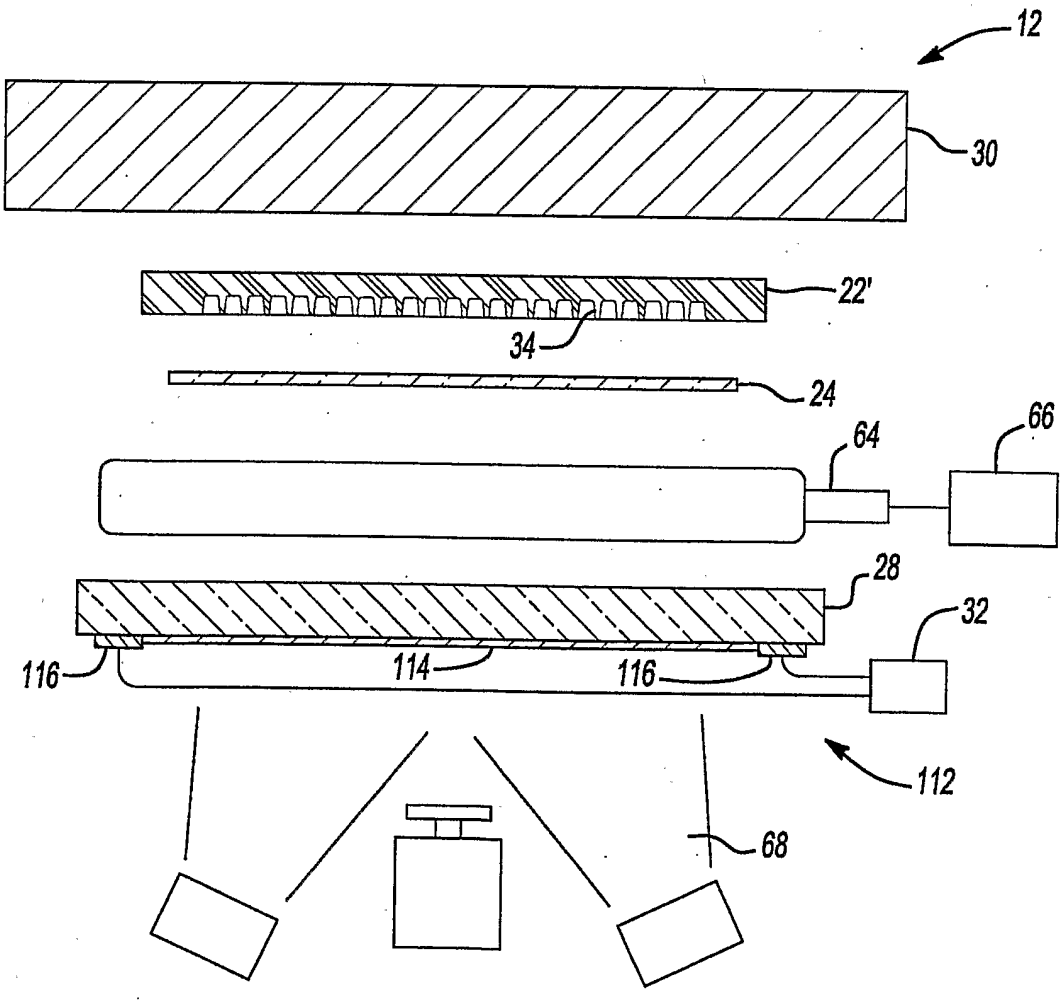

12
30
22'
34
24
64
66
28
32
116
114
116
112
68

Fig-30

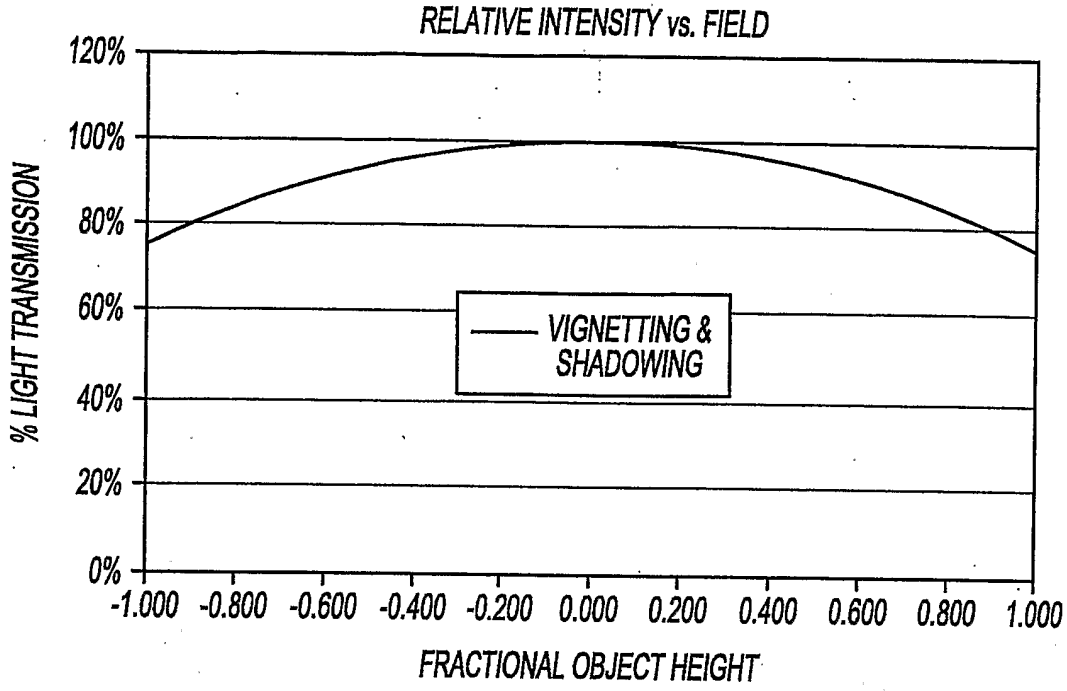

RELATIVE INTENSITY vs. FIELD
% LIGHT TRANSMISSION
120%
100%
80%
60%
40%
20%
0%
VIGNETTING & SHADOWING
-1.000 -0.800 -0.600 -0.400 -0.200 0.000 0.200 0.400 0.600 0.800 1.000
FRACTIONAL OBJECT HEIGHT

Fig-31

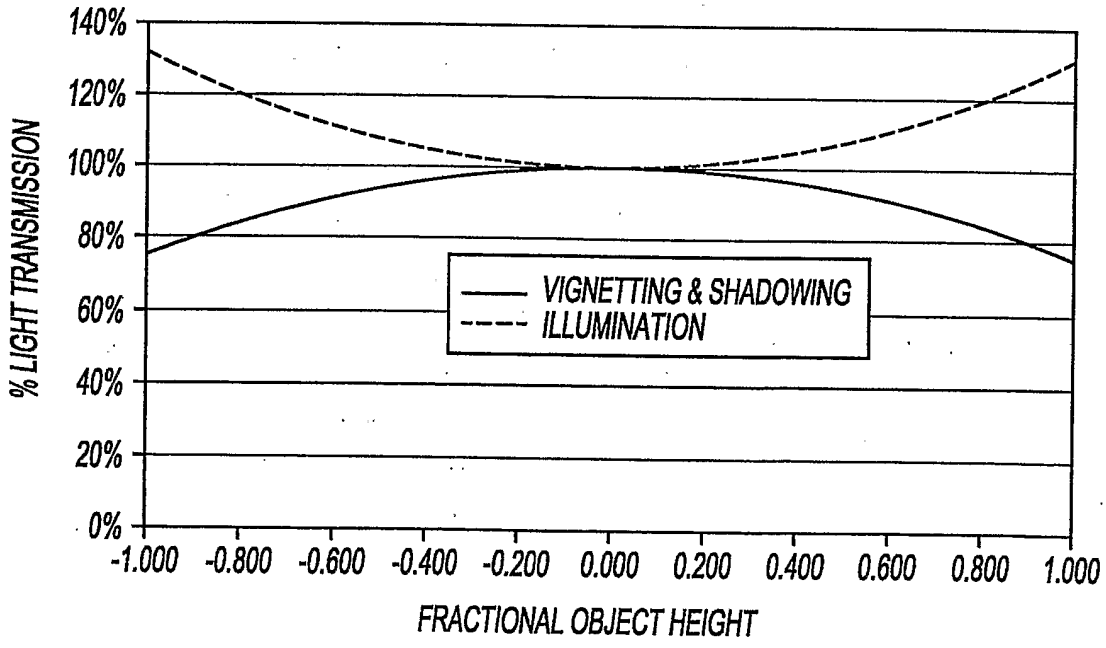

% LIGHT TRANSMISSION
140%
120%
100%
80%
60%
40%
20%
0%
VIGNETTING & SHADOWING
ILLUMINATION
-1.000 -0.800 -0.600 -0.400 -0.200 0.000 0.200 0.400 0.600 0.800 1.000
FRACTIONAL OBJECT HEIGHT

Fig-32

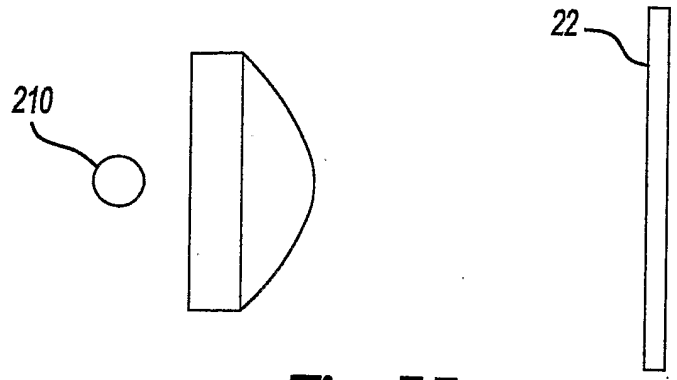


Fig-33

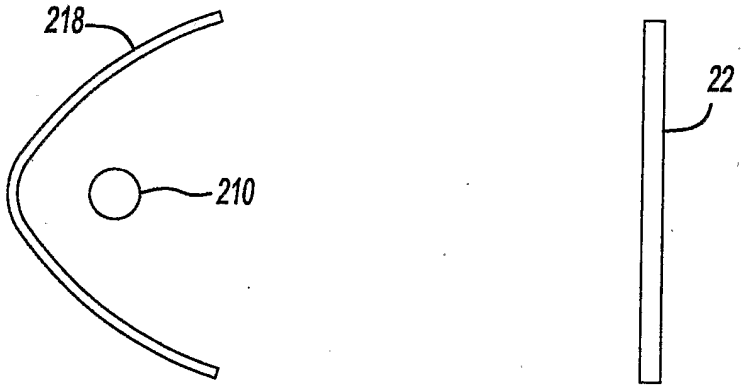


Fig-34

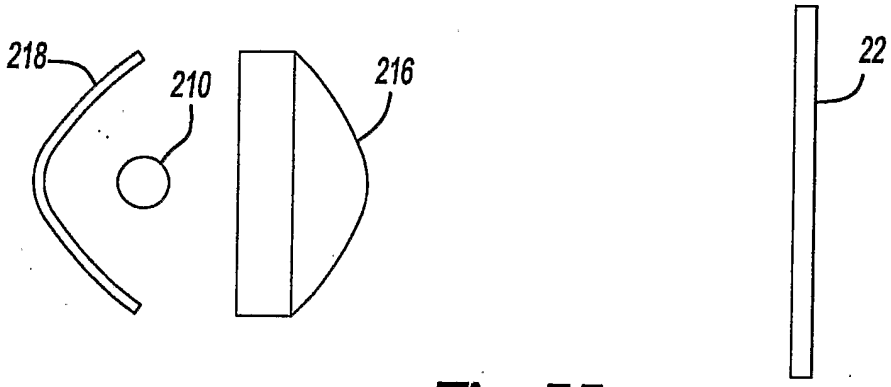


Fig-35

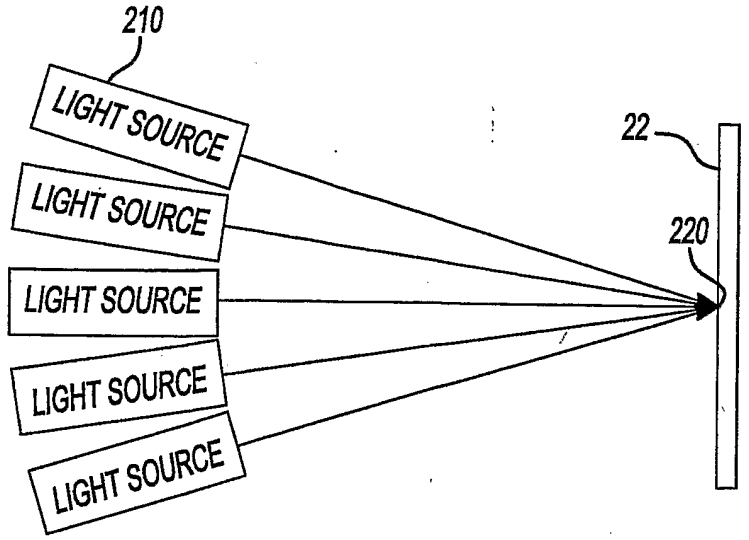

210
LIGHT SOURCE
LIGHT SOURCE
LIGHT SOURCE
LIGHT SOURCE
LIGHT SOURCE
22
220

Fig-36

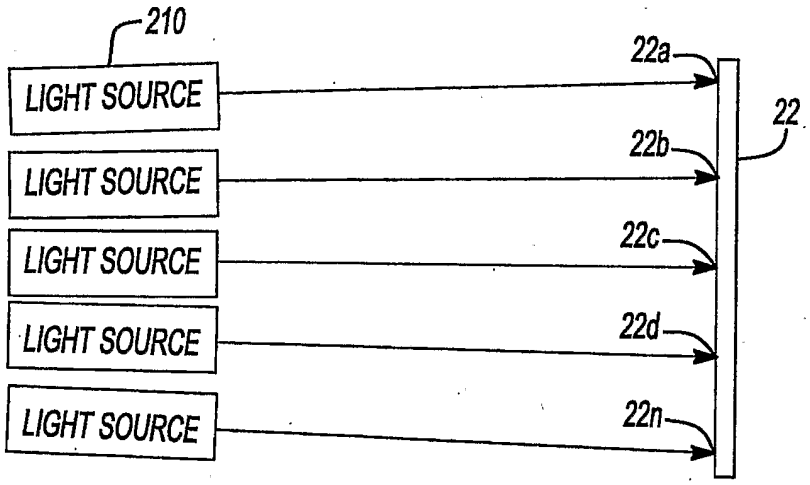

210
LIGHT SOURCE
LIGHT SOURCE
LIGHT SOURCE
LIGHT SOURCE
LIGHT SOURCE
22a
22b
22c
22d
22n
22

Fig-37

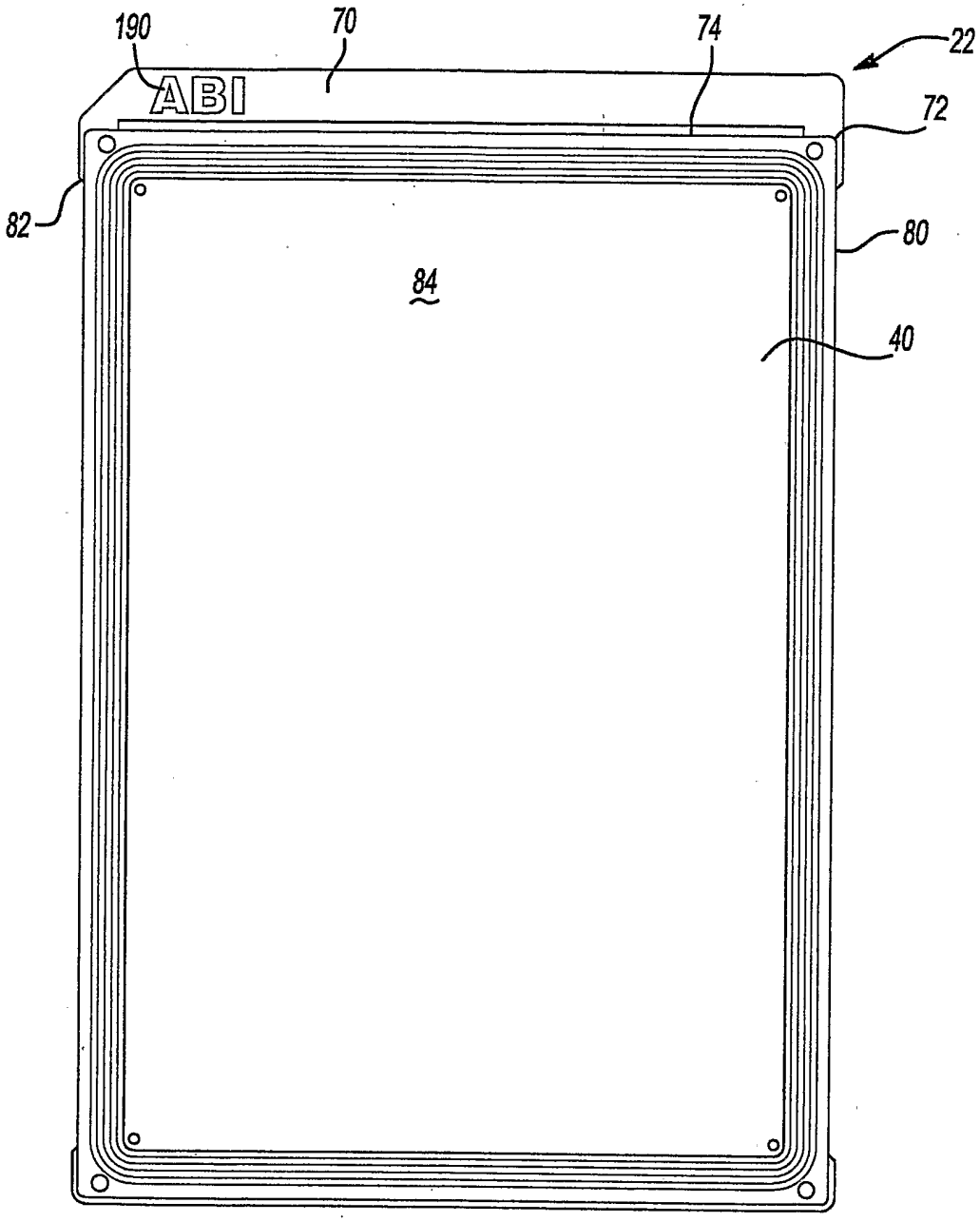


Fig-38

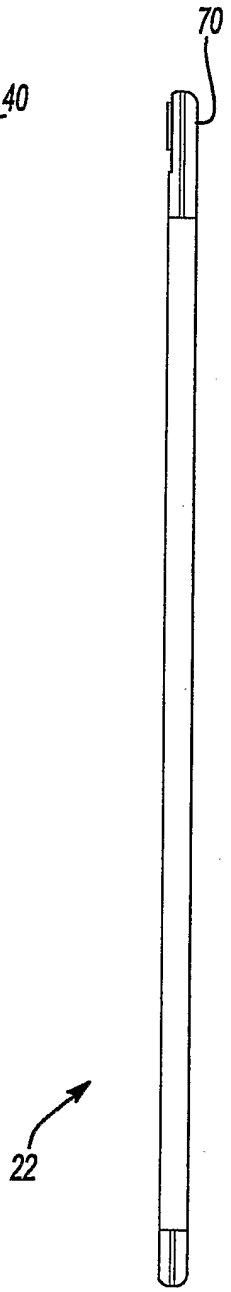


Fig-39

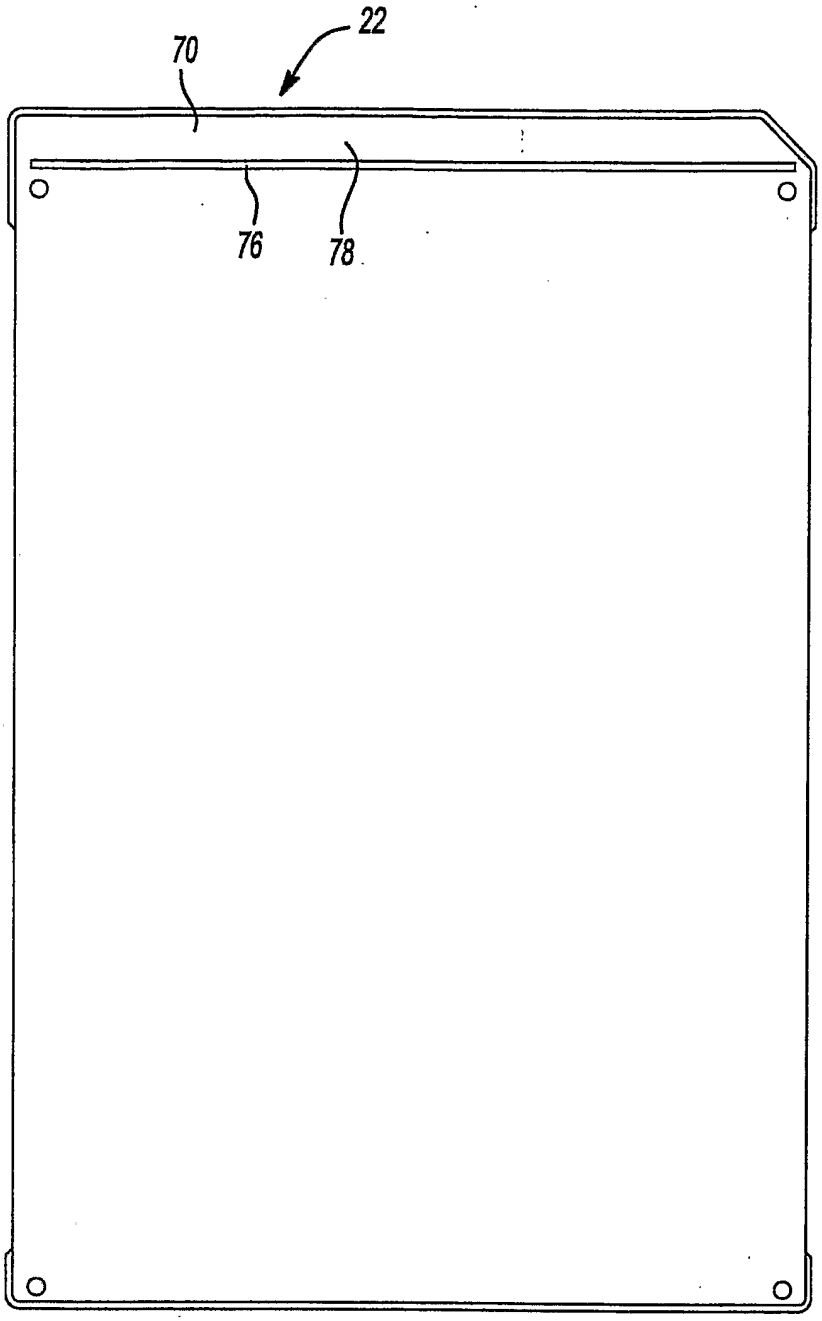


Fig-40

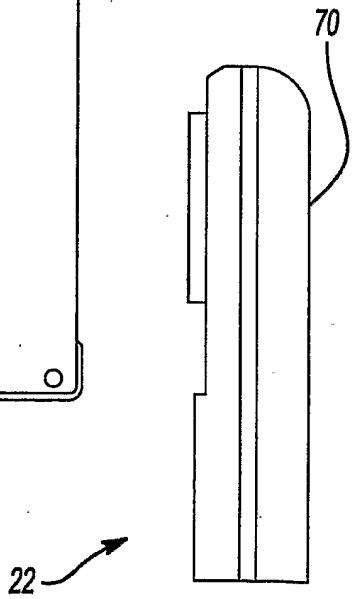


Fig-41

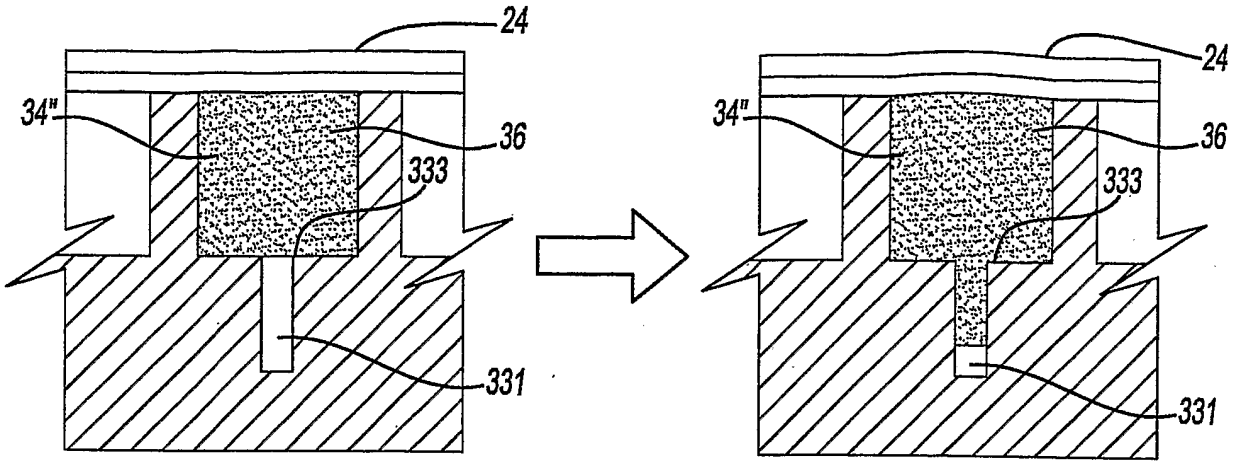


Fig-42

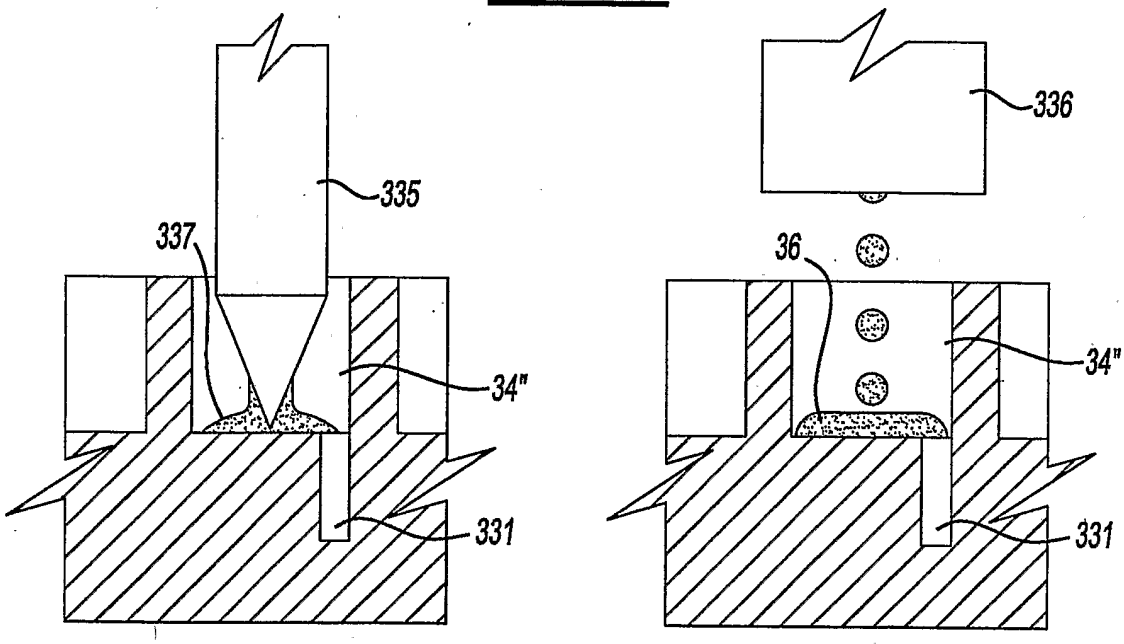


Fig-43

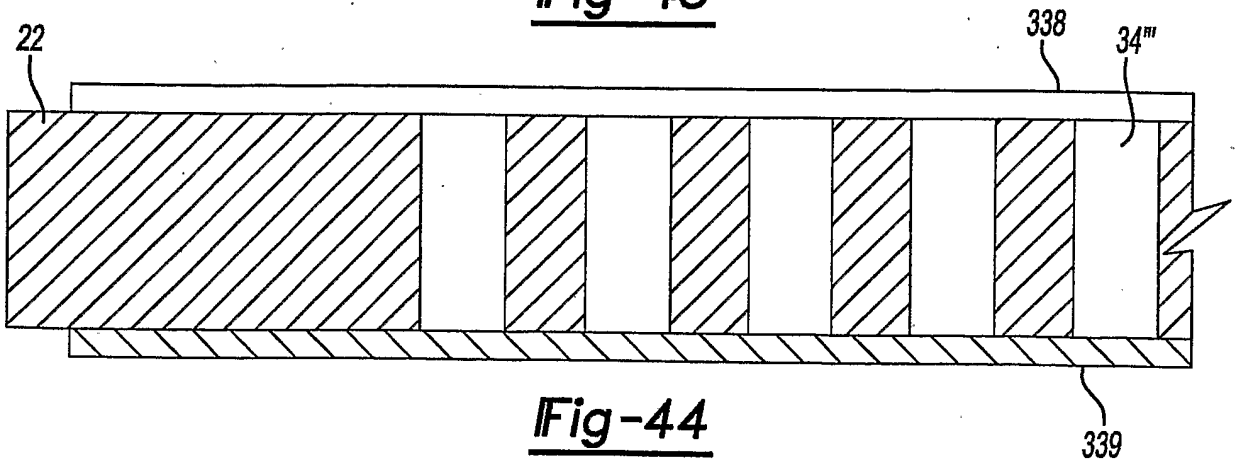


Fig-44

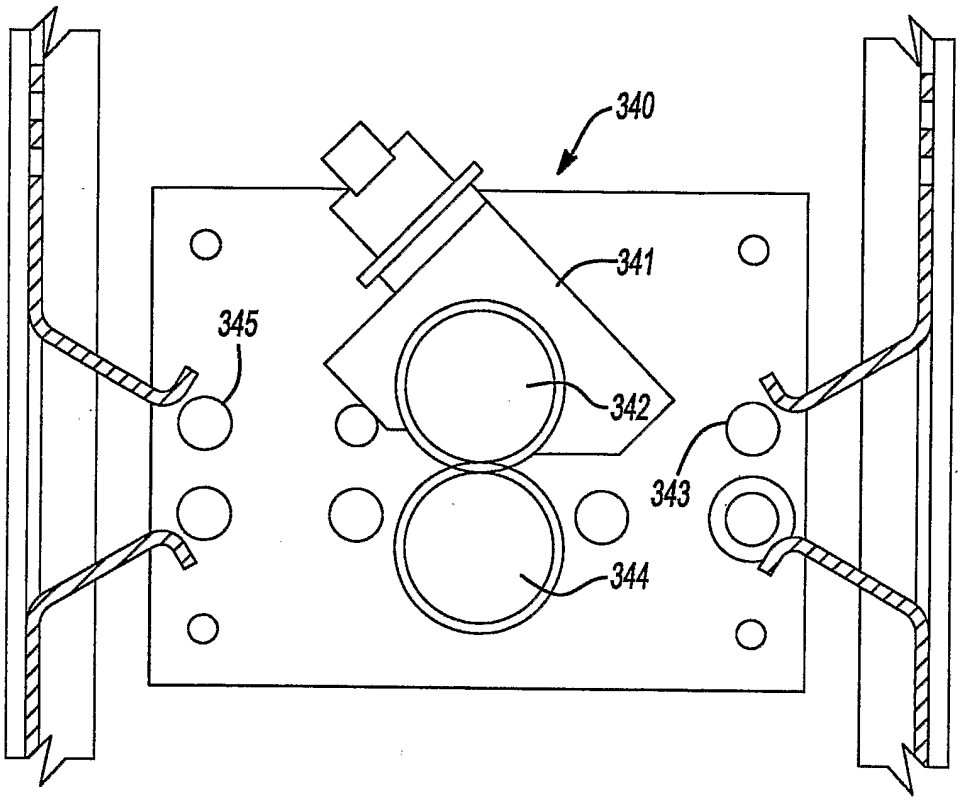


Fig-45

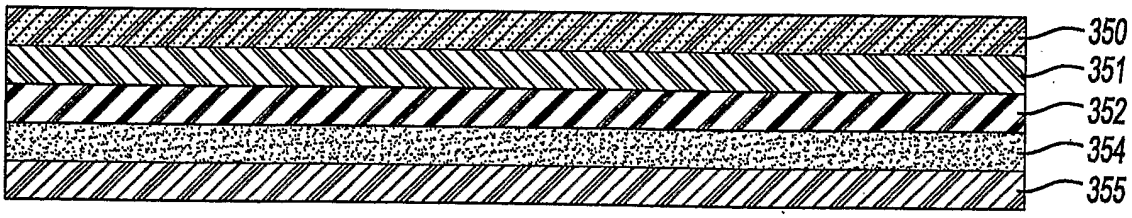


Fig-46

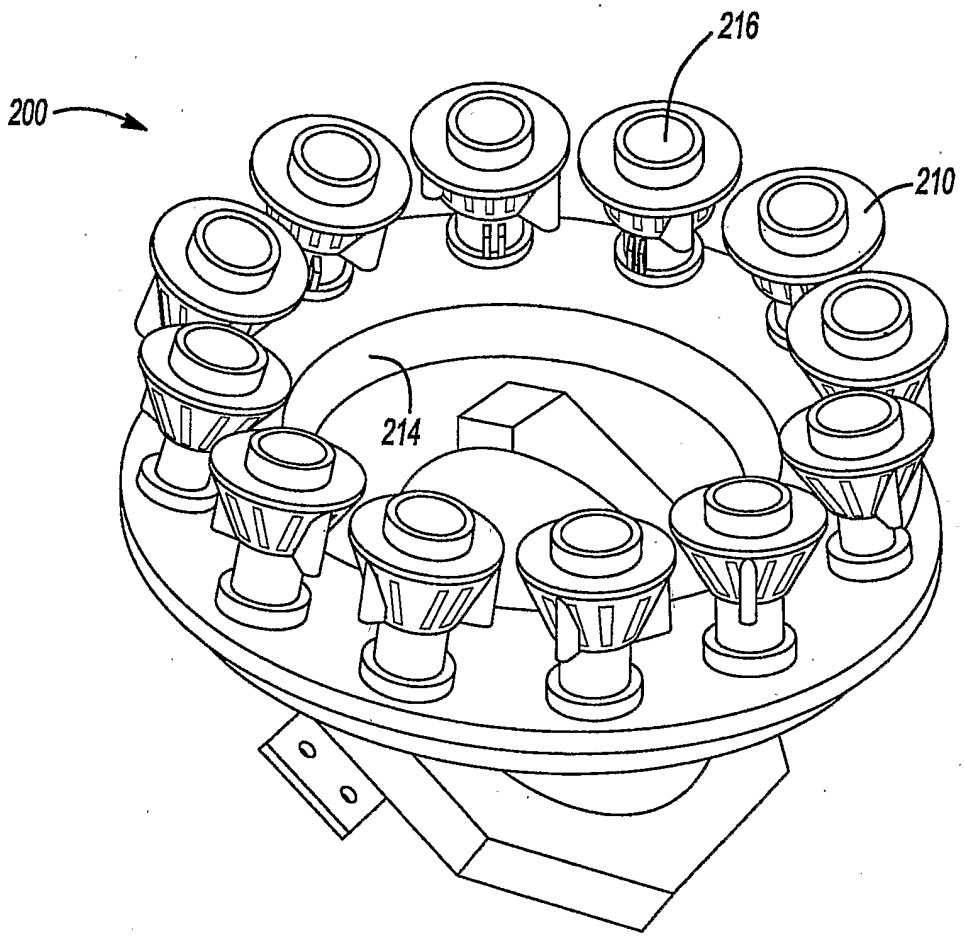


Fig-47

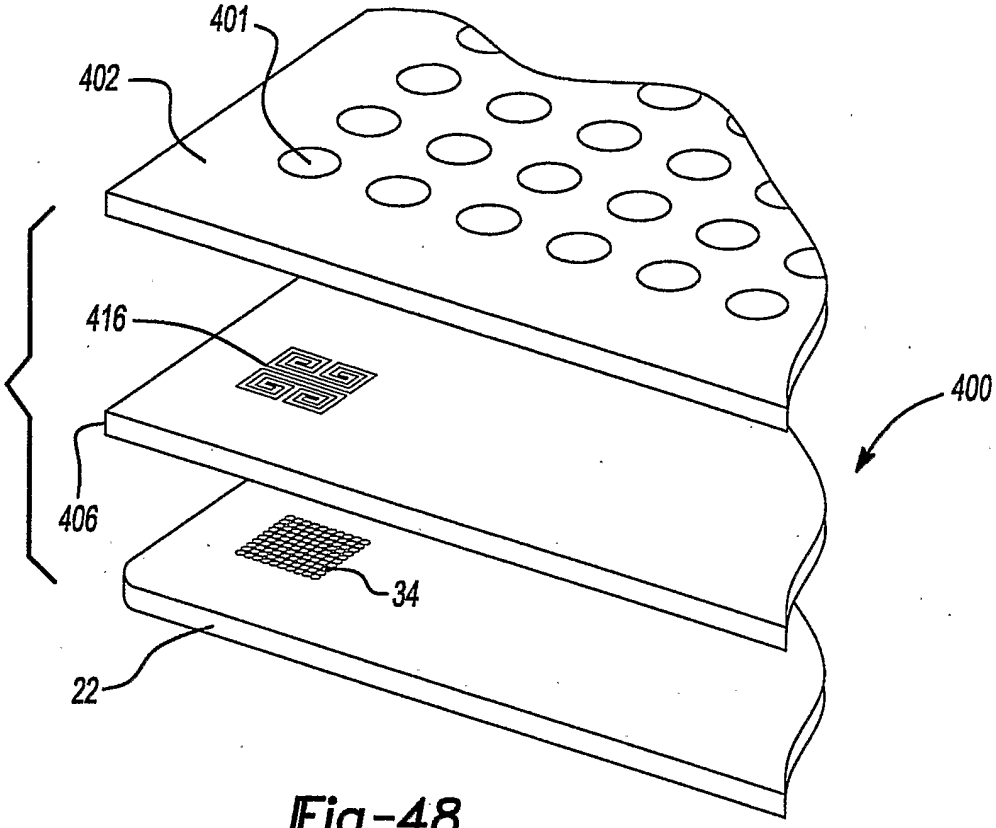


Fig-48

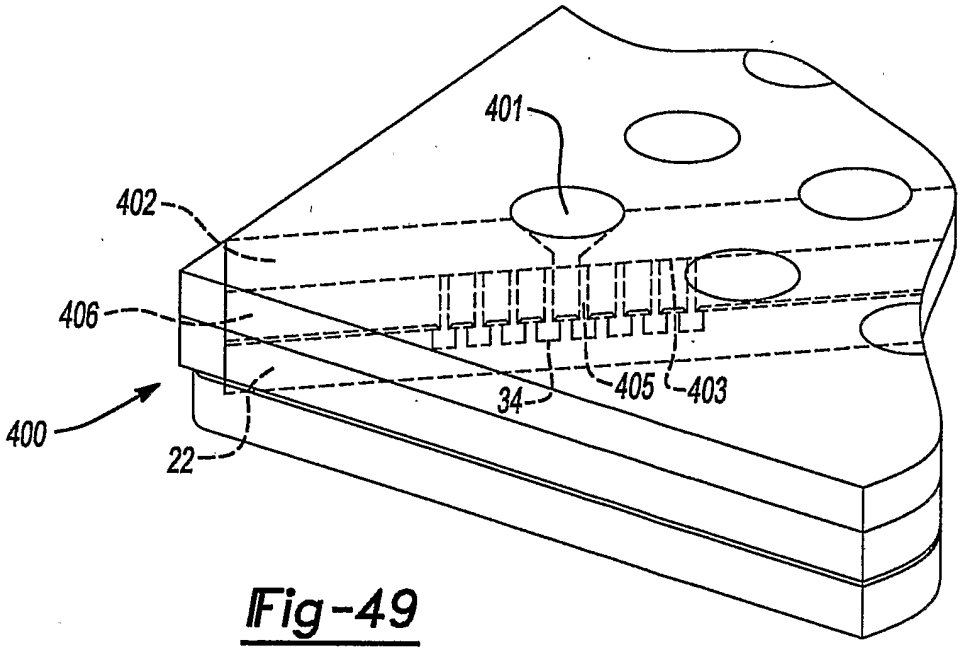


Fig-49

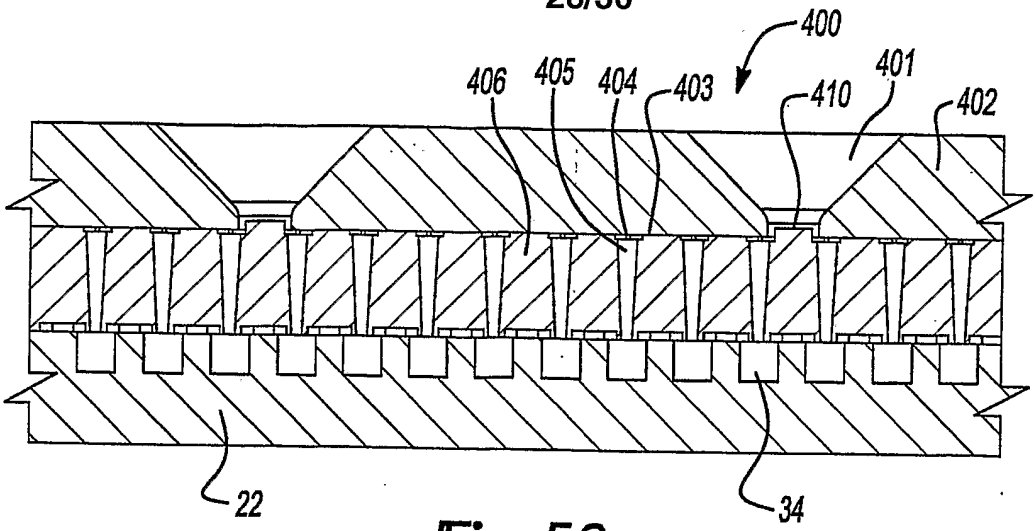


Fig-50

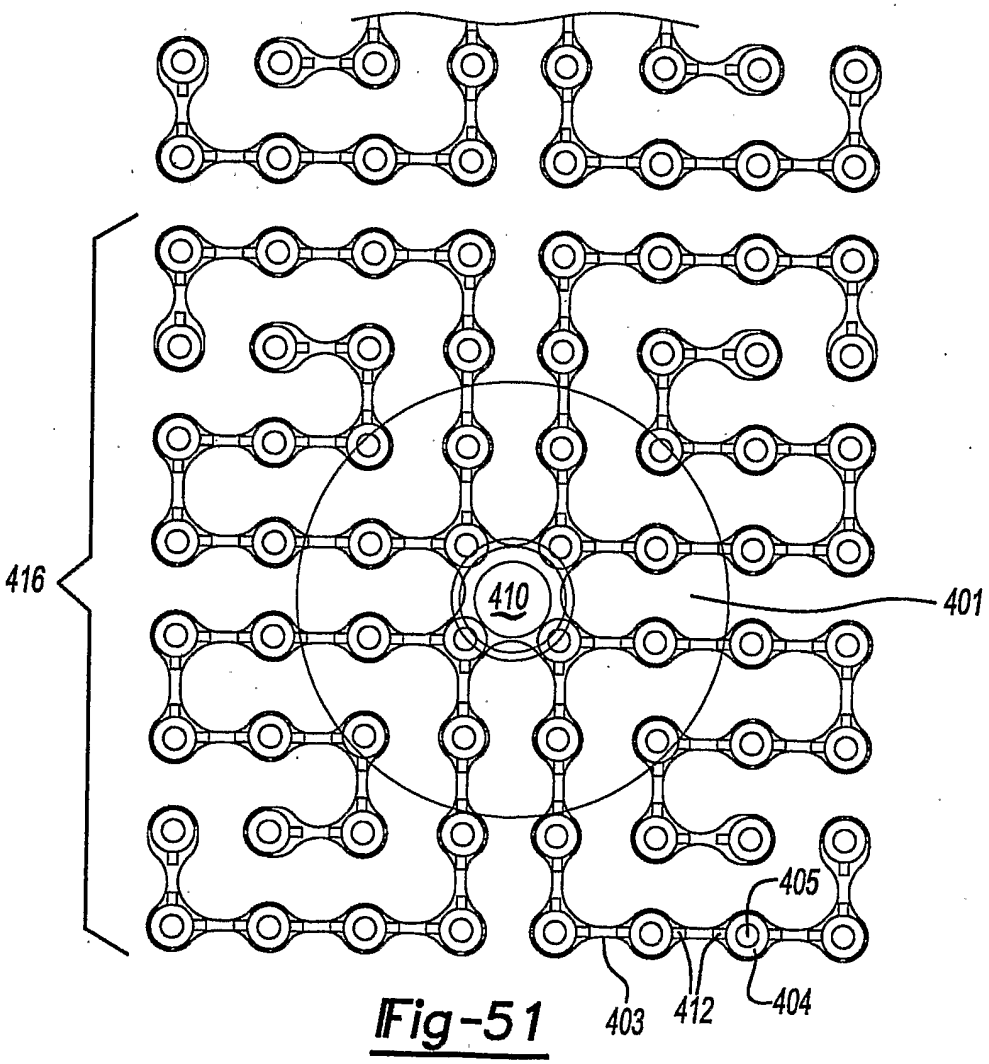


Fig-51

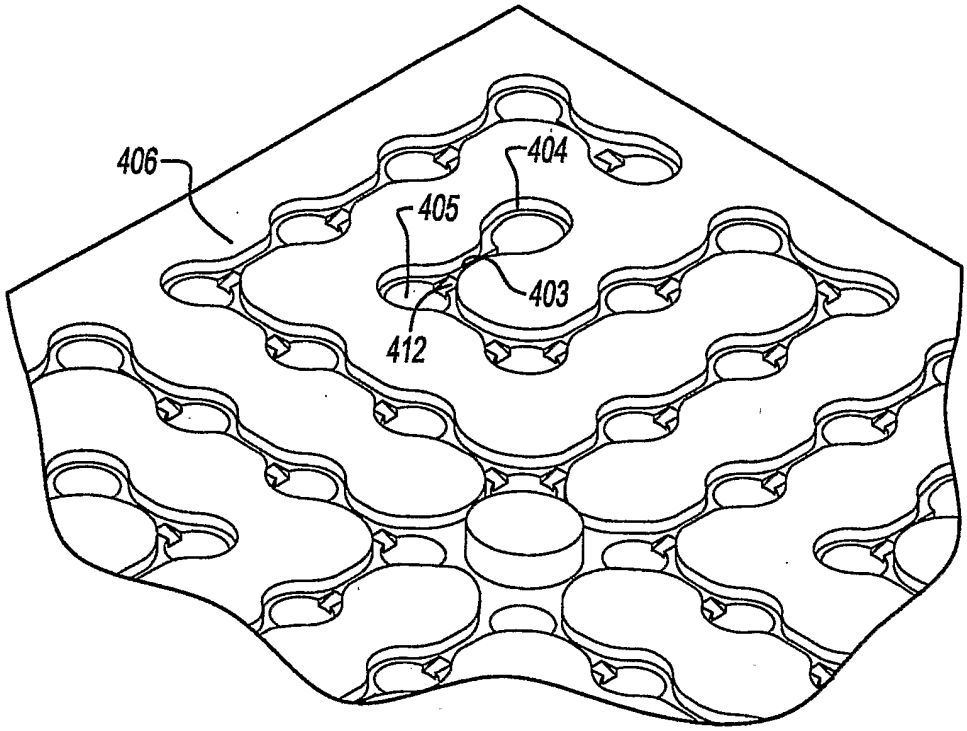


Fig-52

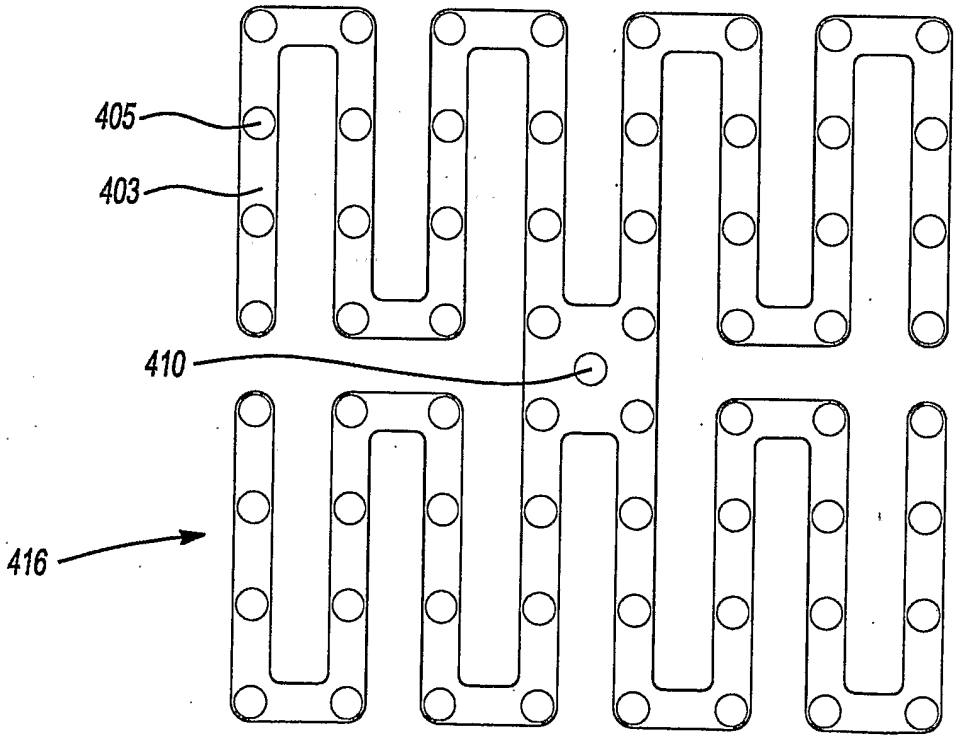


Fig-53

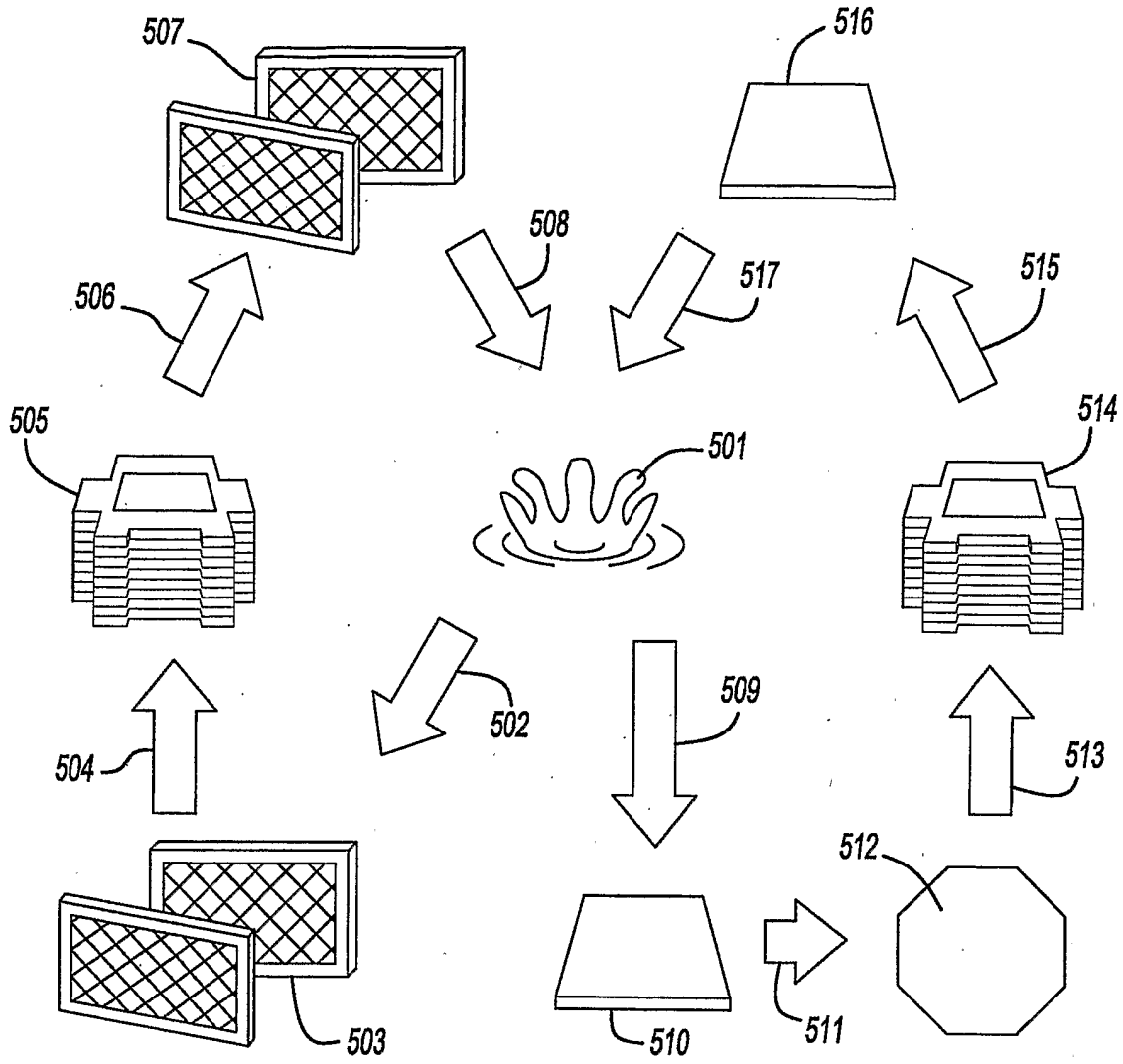

507
516
508
506
517
515
505
501
514
502
509
504
513
512
503
510
511

Fig-54

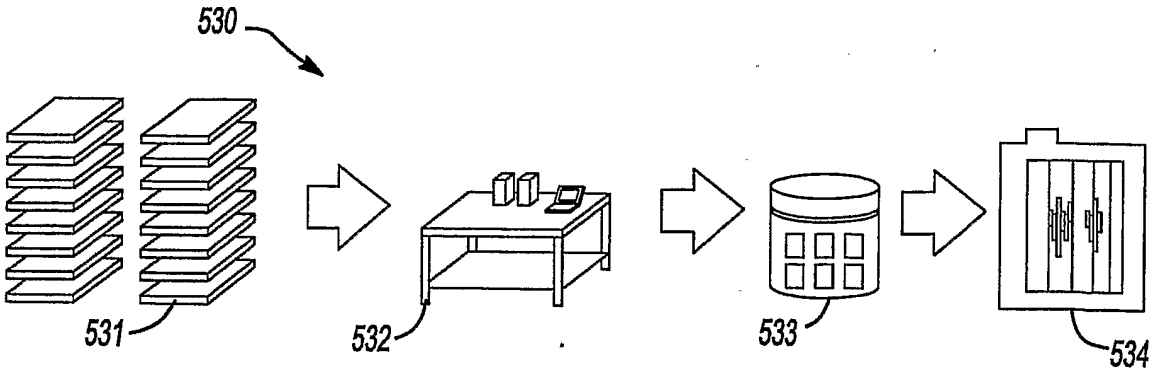

530
531
532
533
534

Fig-55