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(54) Title: SAMPLE-TO-ANSWER MICROFLUIDIC CARTRIDGE

Fig. 1A

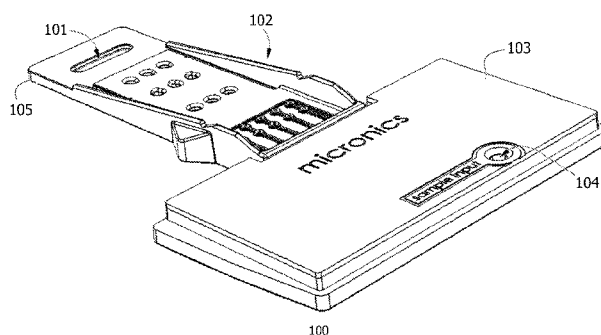
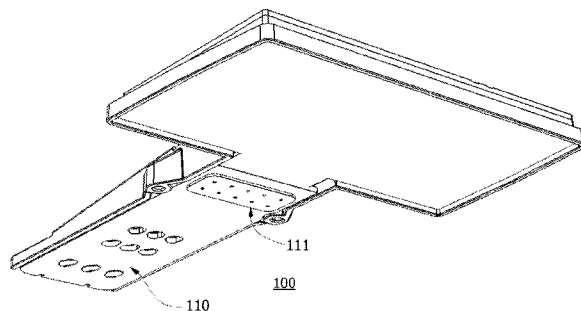


Fig. 1B



(57) Abstract: A microfluidic cartridge and methods for performing a diagnostic, molecular or biochemical assay thereon, where all dried and/or liquid reagents necessary for the assay are contained in the cartridge and the assay requires only the addition of sample. Pneumohydraulic features, chamber and diaphragm technologies are introduced for overcoming the problems of bubble interference and reagent washout during operation of a microfluidic cartridge. The cartridges are inserted into a host instrument for performance of an assay and the cartridge is supplied as a consumable.



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SAMPLE-TO-ANSWER MICROFLUIDIC CARTRIDGE

CROSS-REFERENCE TO RELATED APPLICATION

The present application claims the benefit under 35 USC §119(e) to U.S.
5 Provisional Patent Application No. 61/299,534 filed January 29, 2010, which
application is incorporated herein by reference in its entirety.

BACKGROUND

10 Field

This disclosure is directed to microfluidic devices and methods for
diagnostic, molecular, and biochemical assays and, more particularly, to microfluidic
technologies for dispensing and distributing fluid from on-cartridge reagent reservoirs,
for pumping, heating and mixing, and for rehydrating dried reagents without bubble
15 entrainment and without reagent washout.

Description of Related Art

Microfluidic devices have found increasing use as tools for diagnostic
assays. The devices described by Wilding in US Patent No. 5,304,487 consisted of
20 “mesoscale” channels and chambers formed on reusable silicon substrates which were
infused with fluid reagents from off-cartridge syringe pumps. No consideration was
given to on-cartridge fluid and reagent storage and delivery. However, practical
commercial applications have lead in the direction of “consumable” cartridges—
disposable, single use “sample-to-answer” cartridges that are self-contained for all
25 reagents needed for a particular assay or panel of assays. This is particularly true in the
case of molecular biological assay applications, where contamination associated with
sample carryover or handling absolutely must be avoided.

On board reagents may include both liquid and dry reagent forms. Both
such reagent classes have been subject to certain problems in realization of successful
30 products. Here we address liquid handling issues associated with initial wetout of the
channels and chambers of the cartridge and with rehydration of dried reagents. During
filling and operation of a cartridge containing microfluidic channels and chambers,
particularly those cartridges having a plastic body, liquid wetout is often uneven, such

that air pockets are not infrequently entrained in the fluid column by the advancing meniscus against surfaces and in corners. During pumping and mixing of biological samples, foam and bubbles may form that negatively impact the assay performance of the device. Bubbles may arise due to uneven filling of channels or chambers containing
5 dried reagents. Reagent rehydration, wetout and venting are interlinked with the problem of bubble formation. The problem is exacerbated in more complex fluid networks such as described in US Patent Nos. 6,068,752 to Dubrow and 6,086,740 to Kennedy, for example, and in capillary flow-driven devices such as described by Buechler in US Patent Application No. 2005/0136552 or Wyzgol in US Patent
10 Application No. 2004/024051, which have proved notoriously difficult in plastic body devices.

Bubbles may also arise during heating of a sample liquid due to degassing. It is well known that gas solubility is inversely related to temperature and that solutions which are heated readily become supersaturated. Also a source of
15 bubbles by degassing is cavitation, where a fluid is sheared, such as during mechanical or ultrasonic mixing in microfluidic cavities.

Bubbles interfere with optical interrogation of liquids in microfluidic "cuvettes". The path of light may be altered due to a lensing effect created by the curvature of the gas bubble surface and/or due to the gas bubble refracting the light.
20 Bubbles may also interfere with biochemical reactions by altering solute concentrations at bubble interfaces, by denaturing protein structure, and by impacting bulk heating rate and the homogeneity of temperature in a liquid. For example, in the PCR reaction, in which a thermostable polymerase is used to amplify copies of a target nucleic acid, heating and cooling is uneven in the presence of bubbles in the fluid, reducing the
25 efficiency of the process and limiting sensitivity. The presence of bubbles also reduces the volume of fluid in the reaction chambers, and in assays which rely on detecting analyte in volumes of 10 - 50 uL or less, the presence of a large trapped bubble in a reaction chamber can effectively kill the assay.

In reactions that rely on rate determination, bubbles can drastically
30 interfere with optical determination of slopes and with homogeneous rapid rehydration of dried reagents as is needed to start the reaction with proper availability of substrates. A variety of dried reagents, such as a fluorescent probe, enzyme, buffer or control analyte, may be placed within chambers of a microfluidic device and are needed for

proper conduct of the assay. During wetout, entrapment of one or more bubbles may result in incomplete dissolution and mixing of the dry reagent and the sample, thereby impairing the reaction efficiency and reducing the sensitivity of the test.

Lei, in US Patent No. 6,637,463 proposes varying flow impedance in
5 parallel channels through use of surface tension features and/or cross-sectional area so as to equalize pressure drops, and hence flow, through the multiple flow paths. In one instance, a plurality of exit channels is used to drain fluid from a well so as to avoid formation of recirculating currents or fluid stagnation that would otherwise tend to inefficient washing of fluid and trapping of air bubbles. However, each such feature
10 must be designed by trial and error, and the designs are thus not robust or readily adapted for different assays. Because microscopic variations in dimensions and surface chemistry are difficult to control in microfluidic circuit manufacture, the methods have not been proven a practical solution to the problem of equally dividing flow between parallel subcircuits within a microfluidic card. No description of the use of diaphragms
15 with features for improving wetout was offered.

Ulmanella (US Patent Application No. 2007/0280856) reported efforts to control the meniscus of a fluid filling a microfluidic chamber by physically modifying the bottom surface of the chamber, for example by installing an energy barrier to slow down or stop the leading edge of the meniscus as it crosses the floor of the chamber, or
20 by use of a plurality of grooves or posts on the bottom surface, or by sculpting the depth of the chamber so as to modulate capillary action, or by using a syringe pump, by centrifugation, or by application of a vacuum on the outlet side of the chamber. None of these methods has proved a practical solution to the problem. Capillary action is highly unpredictable and tends to promote formation of air pockets and use of a syringe
25 pump or application of vacuum, as commonly practiced in the prior art, tends to shear the fluid and drive fluid down the path of least resistance, further exacerbating the problem. For example, when two or more microfluidic channels branching from a single inlet are presented to a fluid, such as is useful for splitting a sample or reagent between multiple diagnostic assays pathways in parallel, the fluid may fill the path most
30 readily wetted and leave empty the path having higher fluid resistance. Very tiny differences in resistance between channels lead to preferential wetting of a single channel and no wetting of branching parallel channels, a problem well known to those skilled in the art.

Ulmanella further addresses the effect of dried reagents in wetout of microfluidic chambers and concludes that filling efficiency of chambers containing center-spotted dried reagent was less than 50%, chambers having inlet side spotted reagent were wetted at 65% efficiency, but for chambers having outlet side spotted reagent, the filling efficiency without bubbles increased to 95%. However, positioning of reagent spots with millimeter accuracy during manufacturing is neither a necessary nor a satisfactory means of achieving wetout in the presence of dried reagent spots because it is preferential that the chamber be fully wetted before the reagent is rehydrated so that the concentration of the reagent is not diluted by washout into a downstream channel, as is highly likely if the dry reagent is positioned at the downstream outlet from the chamber!

It is further known that reduction in interfacial and surface tensions in the microfluidic channels or chambers can be achieved, for example, by plasma treatment of the substrate(s) or incorporation of surfactants to decrease hydrophobicity, and by applying a radius to channel intersections. These treatments are also known to improve wettability, but are not effective in eliminating mechanically entrained bubbles and bubbles resulting from thermal degassing, cavitation or stagnation zones. In fact, surfactants can increase the propensity of the gaseous phase to form stable bubbles and foams which can defeat performance of the assay by their persistence. Moreover, the modification of surfaces by processes such as plasma treatment are anticipated to be difficult to control in manufacturing and may be impermanent, degrading progressively during device storage. Therefore it is desirable and is an object of this invention to develop mechanical means and methods for reducing the formation and entrainment of bubbles during initial wetout of assay channels, during rehydration of dry reagents, and for preventing or reducing accumulation and interference of bubbles during operation of the device.

BRIEF SUMMARY

Microfluidic cartridges of the invention, herein termed more generally “devices”, are generally formed of a flexible plastic body which houses fluidic channels and chambers patterned and fluidly intercommunicating according to the needs of a diagnostic or biochemical assay to be performed therein. The assay is conducted by reacting a sample with one or more reagents in one or more steps, typically in one or

more channels or chambers of the device, for times and at temperatures effective in forming a detectable product that indicates the presence or absence of an analyte in the sample. The cartridges are typically consumables; i.e., they are used once and then discarded; and contain all reagents needed for one or more assays.

5 To perform an assay, a device of the invention is inserted into a host instrument which relies on optical detection (or other detection means), such as a spectrophotometer or fluorometer for the detection of a chromogen or fluorophore indicative of the presence, absence, and/or amount of any target analytes of interest. In a preferred embodiment, optical windows in the device are interfaced with detection
10 means in the host instrument. However, the presence of one or more gas bubbles in an optical window may impair the detection of the analyte. Bubbles may also interfere with the reactions required to form a detectable product, such as for example an amplicon or other product of a biochemical or molecular reaction, where a bubble may be responsible for uneven heating of a reaction mixture, inadequate mixing, or
15 incomplete or untimely reconstitution of a dry reagent.

 In use, a sample fluid is introduced into the inventive device, and the fluidly intercommunicating channels and chambers of the device are then wetted with either a biological liquid sample alone, with liquid reagents, or with a mixture of a sample and one or more liquid reagents. The wettable, fluidly intercommunicating
20 aspects of the device are termed the “hydraulic works” of the device and comprise one or more microfluidic subcircuits having channels and chambers. Control of the hydraulics is effected through pneumatically actuated valves, pumps and diaphragms superimposed as a separate, secondary network or manifold of chambers and channels in the device and supplied by external sources of pressurized air and vacuum. This
25 secondary network is termed the “pneumatic works” of the device. Thus the device is composed of a primary “hydraulic network” for conveying a liquid or liquids and a secondary “pneumatic network” for conveying a gas. The pneumatic network provides a) process control and b) positive and negative pressure for driving the liquid or liquids through the hydraulic network, according to valve and pump logic of a host instrument
30 with which the cartridge is interfaced for performing an assay.

 Sample handling and mixing of liquid reagents, including rehydration of any dry reagents disposed within the hydraulic channels and chambers of the device, has been problematic in that bubbles readily become entrained in the fluid during

wetting of the hydraulics. This particularly occurs during initial wetout, where bubbles are engulfed by a meniscus advancing rapidly through the device, and subsequently such as by cavitation or degassing associated with mixing and heating. The present invention addresses this problem through one or more fluid handling mechanisms and methods.

Inventive mechanisms, features and methods include pneumohydraulic diaphragms characterized as:

- 1) an elastic, energy-storing pneumohydraulic diaphragm configured for passively storing a liquid volume under a hydraulic pressure and releasing the liquid volume during wetout of a downstream channel or chamber of the wettable microfluidic subcircuit;
 - 2) a duplexedly layered pneumohydraulic diaphragm having a liquid center for storing and releasing a liquid reagent;
 - 3) a pneumohydraulic diaphragm configured for eliminating headspace from a hydraulic chamber during wetout; or
 - 4) a pair of pneumohydraulic diaphragms comprising a first pneumohydraulic diaphragm interfacing a first hydraulic chamber with valved inlet and a second pneumohydraulic diaphragm interfacing a second hydraulic chamber with valved outlet, and an elevated directly intercommunicating channel between the first and second hydraulic chambers, wherein the pair is configured for reciprocally exchanging fluid through the intercommunicating channel by applying opposing pressure differentials across the first and second pneumohydraulic diaphragms; and
- where the hydraulic chambers and diaphragms are configured for preventing or reducing bubble entrainment or reagent washout during wetout, fill, pumping or rehydration steps of an assay.

In accordance with various exemplary embodiments, one or more liquid reagents are disposed in sealed reservoirs on the device as manufactured. Dry reagents are printed or “spotted” in channels or chambers and are rehydrated at the time of use. The liquid reagents function as buffers, diluents, solvents, eluants, wash reagents, and as rehydrating reagents. In these capacities, the liquids are dispensed as required from their sealed reservoirs into the hydraulics of the device by pneumatic actuation.

In a preferred liquid reagent embodiment, a sealed liquid storage reservoir of the invention is structured as a two-layered diaphragm with a liquid center,

the duplex diaphragm sealedly separating the pneumatics works and the hydraulic works of the device. The duplex diaphragm is composed of two impermeable film layers separated by a liquid center and crimped or fused around the edges and sealed in the device so that the diaphragm separates a hydraulic chamber and a pneumatic chamber. The upper layer, which faces the pneumatics works of the device, is formed of a film having a composition for resisting puncture and the lower layer, which faces the hydraulic works of the device, is composed of a film having a composition that is more susceptible to puncture. Pressurizing the pneumatic side of the diaphragm forces the liquid-filled reservoir against a sharp or "barb" disposed in a fluid receiving basin and punctures the lower layer, but not the upper layer. Following rupture, liquid then flows into the hydraulic chamber and from there into the microfluidic wettable channels of the device. By applying pressure on the pneumatic side of the diaphragm, one or more volumes of reagent can be forced under pressure into the hydraulic works, and by reversing pressure, the fluid can be cause to reflux.

In this aspect, an inventive assay cartridge is characterized as having therein:

a) a duplexedly layered diaphragm sealedly separating a pneumatic chamber of a pneumatic works and a hydraulic chamber of a hydraulic works, the duplexedly layered diaphragm having a first side facing the pneumatic works and a second side facing the hydraulic works, a first layer forming the first side thereof, and a second layer forming the second side thereof, the first and second layers enclosing therebetween a liquid volume as a liquid center;

b) a fluid outlet for receiving and conveying the liquid volume to the downstream microfluidic subcircuit; and

c) a sharp or "barb" disposed in the hydraulic chamber, the sharp for selectively rupturing the second layer and for releasing the liquid volume into the hydraulic works when the duplexedly layered diaphragm is piercingly urged into contact with sharp by application of a pressure differential across the diaphragm.

Surprisingly, the liquid may be released from the on-board reagent reservoir in a series of smaller liquid volumes by the action of serial pulses of pneumatic pressure applied to the first layer of the diaphragm, which remains intact.

Optionally the first layer of the duplexedly layered diaphragm is a rupture-resistant layer and the second layer is a rupture-sensitive layer. The liquid center may contain a liquid reactant, a buffer, a rehydrating fluid, a solvent, or a diluent. On-board storage of liquid is useful for, for example, rehydrating a dry reagent disposed
5 in a downstream chamber or channel, for rinsing a solid phase, for eluting a target analyte or analytes from a solid phase substrate, for making a dilution, for making a chromatographic separation, for actuating or stopping a reaction, or for detecting the target analyte or analytes, and minimizes the possibility of carry-over contamination. Optionally the liquid volume is degassed and the duplexedly layered diaphragm is gas
10 impervious. Advantageously, any entrained bubbles are likely to be resorbed in degassed liquids, and degassed liquids are not susceptible to degassing on heating, such as is useful for thermocycling in PCR.

While the devices are generally planar, they may be mounted in the host instrument in a canted position (i.e. angularly with respect to a ground plane), typically
15 at about 15 degrees from flat and are vented at a downstream aspect of each microfluidic subcircuit. As a liquid sample or reagent is introduced upstream into the hydraulic subcircuitry, air is displaced downstream and is vented. The liquid sample and reagents progressively fill and move through the device. By canting the card at an angle of 10 to 35 degrees, air in the device during priming (termed here "wetout") is
20 found to be more readily displaced from the hydraulic works. By careful management of the advancing meniscus during initial fill of the canted card, the problem of bubble entrainment, particularly during fill, is substantially reduced or prevented.

Thus optionally, the hydraulic works may be configured for operation when mounted at an angle of 10 – 35 degrees relative to the ground plane on a tilted
25 stage of a host instrument and at least one hydraulic chamber is configured with an outlet and intercommunicating channel positioned superiorly relative to that chamber for venting a gas or discharging a bubble from the chamber.

In another aspect of the invention, entrainment of bubbles during wetout is limited by a filling mechanism that involves passive relaxation of an elastically
30 stretched or distended pneumohydraulic diaphragm. This passive mechanism was found to be superior to fill by capillarity and to fill by positive displacement pump action or vacuum. A liquid is first forced under pressure into a specially designed manifold having a "pneumatic chamber" stacked on top of a "hydraulic chamber",

where the two chambers are separated by an elastic diaphragm stretched over the roof of the hydraulic chamber. Optionally, liquid may instead be aspirated into the lower chamber, but advantageously, the upper pneumatic chamber is vented and open to atmospheric pressure. The position of the two chambers, while termed “upper” and “lower” or “top” and “bottom” chambers for purposes of explanation, is relative, and is not limiting on the operation of the device. As a liquid volume enters the liquid-receiving chamber, the diaphragm is stretched to hold the volume and resiliently stores the energy of deformation, a form of potential energy having a returning force and a spring constant. Diaphragm material and deformation conditions are chosen so that the “elastic limit” of the material is not exceeded. Then by opening a valve to a downstream channel or channels, the distendedly stretched diaphragm returns to its relaxed state and fluid gently fills the downstream fluid structures without entrainment of bubbles in the advancing meniscus.

This mechanism and method has proved startlingly advantageous where flow is split into multiple channels. By providing an upstream staging manifold with multiple liquid-receiving chambers having elastic diaphragms, each with separately valved outlets that are opened in synchrony, the hydraulic pressure for initiating and sustaining liquid flow into multiple downstream fluidic subcircuits in parallel is segregated or “quantized” so that the flow into all channels is essentially equal and sufficient. Total pressure and volume per downstream channel can be precisely calibrated by selection of the spring constant and the deformation of the elastic diaphragm member so that the restoring flow of liquid into the downstream channel is the volume required to fill the downstream channel to a desired mark; the displaced volume delivered by each diaphragm of the staging manifold is neither insufficient nor in excess for the fluidic operation of splitting flow equally among multiple parallel channels or subcircuits, a necessary fluidic operation in devices intended for multiple assays in parallel. This is a technological advance in the art. Any air downstream is readily displaced by the advancing meniscus and is conveyed to a downstream vent by this means.

In this aspect, an inventive assay cartridge includes:

- a) a staging manifold having a plurality of chambers, wherein each chamber of the plurality of chambers is separated into a hydraulic chamber and a pneumatic chamber by an elastic, energy-storing pneumohydraulic diaphragm sealedly

mounted therebetween, such that a liquid volume admitted through an inlet into each hydraulic chamber in series or in parallel distends each energy-storing pneumohydraulic diaphragm according to an isobaric pressure proportionate throughout said staging manifold to the displacement volume thereof;

5 b) the inlet is valvedly closeable for equilibrating the hydraulic pressure throughout the staging manifold after filling is complete; and,

 c) a plurality of vented downstream channels in parallel, wherein one the channel of the plurality of channels is in fluidic communication with each hydraulic chamber of the staging manifold, each vented downstream channel having a
10 valve for closing during filling and pressurization and for opening during draining and depressurization, whereby the hydraulic pressure of the elastic, pneumohydraulic diaphragm in a distended state is passively converted to the work of advancing a meniscus during initial wetout of the plurality of vented downstream channels equally in parallel.

15 More generally, wetout or 'priming' is improved by harnessing the mechanical properties an elastic, pneumohydraulic diaphragm in a fluidly distended state to do the work of advancing a meniscus through a wettable downstream microfluidic circuit fluidly connected thereto and thereby displacing any gas therein to a downstream vent without bubble entrainment. This principle is particularly
20 advantageous in equally splitting a fluid into a plurality of downstream microfluidic subcircuits in parallel. In this way, multiple assays may be conducted in parallel and a single sample may be split equally for parallel assays having separate downstream detection means. Surprisingly, the mechanical properties of the elastic diaphragm can be calibrated to fill one or more downstream microfluidic subcircuits to a mark, as is
25 useful in reconstituting a defined mass of a dried reagent in a defined volume, for example.

 Microfluidic devices may typically also include at least one dried reagent disposed within the downstream hydraulic network. These reagents are typically spotted or printed during manufacture. During an assay, the dried reagents are
30 rehydrated by sample or by contact with a liquid reagent dispensed as described above. Serendipitously, we have found that the passive liquid wetting mechanism and method described here is advantageously suited to the rehydration of dry reagents without entrainment of bubbles, another technological advance in the art.

In a related embodiment, we have found that by providing pneumatically actuated diaphragms in downstream chambers where dried reagents are spotted, the diaphragms overlying those reagent spots can be pressurized so as to a) temporarily seal the reagent zone (typically central to and on the floor of the chamber) from contact with bulk fluid during the chamber wetting process and b) remove or expel essentially all of the headspace above the dried reagent. When deformed so as to fill the hydraulic chamber, the diaphragm is not fully sealed around the periphery of the chamber. Liquid entering the chamber around the diaphragm is shunted around the lower edges of the chamber and readily displaces any residual air, which is vented from the hydraulics during filling. By relaxing or by reversing the pressure differential across the diaphragm, additional fluid is readily aspirated into the chamber without the formation or entrapment of gas bubbles. Reagents are rehydrated only after the downstream outlet of the chamber is valvedly closed, thereby reducing reagent losses to washout. The reduced dead volume of the dry reagent chambers is thus turned to advantage. Happily, in this way, dry reagent spots can be precisely reconstituted with a desired volume of rehydrating reagent or sample, ensuring that the biological activity of the reagent is quantitatively correct for the assay conditions, a useful refinement in art.

Thus the invention also may feature at least one microfluidic subcircuit having a downstream reaction chamber with upstream inlet and downstream vent, the downstream reaction chamber containing a dried reagent spot or spots, further characterized in that the pneumohydraulic diaphragm is configured to operate with a first position wherein the diaphragm is distended against the floor of the chamber so as to displace headspace air and form a protective temporary tent around and over the reagent spot or spots during wetout, and a second position wherein the diaphragm is relaxedly positioned or aspirated against the roof of the chamber so as to fill the chamber with the liquid volume and uncover and dissolve the reagent spot at full strength without bubble entrainment or reagent washout. The dried reagent spot may be a buffer, an enzyme, a co-enzyme, a co-factor, a polymerase, a primer, a molecular beacon, a probe, a fluorophore, a dehydrogenase, an oxidase, a reactant, a chromogen, a substrate, an antibody, an antigen, or a control.

Also claimed is a method for wetting a microfluidic cartridge while limiting bubble entrainment therein, which comprises:

- a) pumping a liquid volume through an inlet and into a plurality of hydraulic chambers forming a staging manifold of a microfluidic card so that an elastic pneumohydraulic diaphragm overlying the liquid volume in each said hydraulic chamber is stretchedly distended, thereby isobarically pressurizing the liquid volume in the plurality of hydraulic chambers;
- b) valvedly opening an outlet from each of the hydraulic chambers of the staging manifold, each outlet with fluidic connection to a vented downstream microfluidic subcircuit; and
- c) splitting the liquid volume substantially in equal measure into each said wettable downstream microfluidic subcircuit by passive relaxing the distended elastic diaphragm—without bubble entrainment.

Wetting a microfluidic device by passive relaxation of an elastic diaphragm is readily distinguished from wetting by capillary action or by active pumping, and has proven surprisingly advantageous in overcoming difficulties with bubble entrainment as are known in the art.

Also claimed is a method for wetting a microfluidic cartridge which contains dried reagent spots, while limiting bubble entrainment therein, which comprises:

- a) pumping a liquid volume through an inlet and into a plurality of hydraulic chambers of a microfluidic card so that an elastic pneumohydraulic diaphragm overlying the liquid volume in each the hydraulic chamber is distended, thereby isobarically pressurizing the liquid volume;
- b) pressurizing a second diaphragm in a plurality of downstream reaction chambers, each downstream reaction chamber containing a dried reagent spot, the second diaphragm forming a protective temporary tent for sealing around and over the reagent spot and for displacing headspace air from the downstream reaction chamber;
- c) valvedly opening an outlet from each the hydraulic chamber, each the outlet with fluidic connection to one of the plurality of downstream reaction chambers;
- d) wetting the downstream reaction chamber around the temporary tent and displacing any residual air from the reaction chamber by allowing the

distended elastic pneumohydraulic diaphragm to relax, the liquid volume forming an advancing meniscus;

e) optionally closing a valve downstream from the downstream reaction chamber;

- 5 f) lifting the temporary tent and conveying a remaining part of the liquid volume into each reaction chamber, thereby dissolving the reagent spot at full strength without bubble entrainment or reagent washout. The temporary tent is lifted by relaxing or by reversing the pressure differential across the second diaphragm member.

In another method, pairs of chambers with pneumohydraulic diaphragms
10 may be used to aid wetout and reagent dissolution for PCR, and for reciprocally pumping fluid when interconnected in series by a channel. By application of alternating positive and negative pneumatic pulses to a first diaphragm in a first chamber, a second diaphragm in a second chamber is driven in synchrony. The second diaphragm may be an elastic diaphragm that functions in accommodating and elastically storing the pulsed
15 energy of the first diaphragm. Mixing is readily achieved by conveying a liquid volume back and forth between the two chambers. By providing each hydraulic chamber with a thin heat exchange film and suitable contact heating elements, "two-zone" PCR is readily achieved. In an improved device, the intercommunicating channel between the chambers is contoured and elevatedly positioned so that bubbles are gravitationally
20 urged to clear the chambers during initial wetout and pumping, and will trap any additional bubbles that form during heating. The intercommunicating channel is preferably configured and contoured to be operated at a tilt of 10 – 35 degrees and is positioned on the high side of the paired chambers so as to reduce interference from bubbles. Fluid is cycled between a first chamber at a denaturing temperature of a target
25 nucleic acid and a second at an annealing temperature. The plastic body of the device limits parasitic heat capacitance of the device during PCR. Nucleic acid amplification at rates of 8 seconds or less per thermal cycle is readily achieved.

For PCR, amplification reagents are provided with the device. Typically the first chamber contains a first reagent or reagents and the second chamber contains a
30 second reagent or reagents. Typically the reagents are spotted in a centric or pericentric zone in each chamber. During initial wetout, the diaphragms in the chambers are inflatedly distended to press down on and cover the reagents so as to limit rehydration and any washout that would otherwise occur as the meniscus of the rehydrating fluid or

sample dissolves the spotted reagents and carries them downstream with the solvent front. After initial wetout, a suction pressure may be applied to the diaphragm so as to aspirate a fluid into the chamber and dissolve the reagents therein. Alternatively, an upstream chamber may be pressurized so as to hydraulically inflate the downstream chamber and dissolve the reagents. Fluid direction of flow may be reversed one or more times so as to improve mixing and rehydration.

Thus the invention may also include a cartridge for use with a host instrument having thermal interfaces for "two-zone thermocycling" and a pneumatic interface with pneumatic means for driving and controlling a PCR amplification. The device works by reciprocating pneumohydraulic action of paired diaphragms in two interconnected hydraulic chambers so as to cyclically denature and anneal a target nucleic acid, the cartridge advantageously having one or more wettability features of the invention for improving wetout of the chambers with liquid without entrainment of bubbles. The device is also advantageously for dissolving reagents in a fixed volume without washout losses during wetout, ensuring that primers, buffers and other reagents are at a fixed strength when reconstituted.

Thus the various aspects of the invention offer novel utility in operation of microfluidic cartridges for diagnostic and biochemical assays and are found to be advantageous as mechanisms and methods for limitation of the bubble interferences that have been a longstanding source of problems with these devices.

In the following description, certain aspects and embodiments of the invention will become evident. It should be understood that these aspects and embodiments are merely exemplary and explanatory and are not restrictive of the invention. Other features and advantages will become apparent from the detailed description which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A and 1B show two perspective views of a disposable, single-use, sample-to-answer microfluidic cartridge of the invention, the cartridge containing all reagents for an assay and requiring only introduction of a biological sample.

FIG. 2 demonstrates insertion of the assay cartridge in a host instrument for performance of an assay thereon.

FIG. 3A is a detailed view of the cartridge as inserted in a mechanism of a host instrument. The mechanism includes a heating manifold shown in FIG. 3B and a pneumatic control interface.

FIG. 4 is an exploded view of disposable microfluidic cartridge with liquid center foil diaphragm packs carrying liquid reagents.

FIG. 5A is a perspective view of a microfluidic circuit for extraction of a nucleic acid target from a biosample; FIG. 5B is a schematic of the extraction process.

FIGS. 6A-G provide views of a reagent reservoir formed of a bilayered duplex diaphragm with liquid reagent center and a sharp or “barb” for puncturing and releasing the liquid into the hydraulic works of the microfluidic device.

FIGS. 7A and 7B show a microfluidic cartridge canted with a tilt as mounted in a host instrument.

FIGS. 8A and 8B show a worms-eye view of a network of channels and chambers for performing PCR on a microfluidic cartridge; the positions of dry reagents are also marked.

FIGS. 8C and 8D illustrate an alternative cartridge configuration in worm’s-eye view.

FIGS. 9A-9L schematically depict a passive initial wetout mechanism with staging manifold.

FIGS. 10A-10C depict the operation of a staging manifold whereby reagents are rehydrated in preparation for PCR. The operational sequence is continued in FIGS. 10D-G.

FIGS. 10D-10G illustrate a PCR amplification using dual chambers with reciprocating diaphragm action.

FIG. 11 describes the steps of a method for extracting nucleic acids from a sample, where a bilayered duplex diaphragm with liquid center is used to dispense the reagents.

FIG. 12 describes the steps of a method for priming the microfluidic channels of a hydraulic works with liquids dispensed from a bilayered duplex diaphragm with liquid center.

FIG. 13 describes the steps of a method for rehydrating dry reagents without bubble entrainment.

FIGS. 14A and 14C are worm's eye views of a network of microfluidic channels and chambers for reverse-transcriptase-mediate PCR. FIG. 14B is a detail view of an in-line chamber for production of cDNA.

FIGS. 14D and 14E illustrate an alternative configuration of a cartridge
5 with modified features.

FIG. 15 illustrates use of optical windows of a detection chamber of a device of the invention for monitoring a fluorescent endpoint.

FIG. 16 depicts more detail of a two piece microfluidic card assembly for performing PCR and a pneumatic interface with gasket for interfacing the cards with
10 a compatible host instrument.

FIG. 17 is a plot showing a positive and negative fluorescence assay in the detection chambers of the cartridge, including multiple scans of the sample while increasing the temperature of the reaction mix.

FIGS. 18A and 18B analyze the pooled data of FIG. 17. Scans of a
15 molecular beacon-amplicon duplex demonstrate a FRET melting curve capability of the cartridge when interfaced with a compatible host instrument.

FIG. 19 depicts FRET data for amplicons obtained with a device of the invention when used in a host instrument compatible therewith.

20 DETAILED DESCRIPTION

Although the following detailed description contains specific details for the purposes of illustration, one of skill in the art will appreciate that many variations and alterations to the following details are within the scope of the claimed invention. The following definitions are set forth as an aid in explaining the invention as claimed.

25

Definitions

A "cartridge" is an analytical device designed for operation by insertion into a host instrument. The host instrument supplies the pneumatic pressure, pulses, and detection means for performance of the assay. The cartridge contains hydraulic
30 works and pneumatic works, and may include embedded microfluidic "cards" with embedded microfluidic channels and chambers. Sample and reagent liquids are conveyed in a hydraulic network of the cartridge or card; fluid flow is controlled and driven by a pneumatic network that interfaces with the hydraulics at selected junctions,

channels and chambers. Typically, the body of the cartridge or card is made of a flexible plastic and may be formed by lamination, molding or a combination thereof. Plastics may include, but are not limited to, polycarbonate, polyethylene terephthalate, cyclic polyolefins, acrylates, methacrylates, polystyrene, graft and block copolymers, and composites thereof. A preferred cartridge is made from rollstock and includes dry reagents printed thereon.

“Hydraulic works” of a device: includes the network or networks of intercommunicating channels and chambers that are intended to be wetted by sample or liquid reagents in the course of an assay. The hydraulic networks are configured with microfluidic subcircuits for performing the steps of an assay.

“Pneumatic works” of a device: includes the network or networks of pneumatically actuated valves, pumps and diaphragms and interconnecting circuitry and manifolds that are useful for powering and controlling the hydraulics of the device. The pneumatic works of the cartridge device interface with positive and negative pressure sources on the host instrument and with valves, diaphragms, pumps and other pneumatically actuated elements that control and drive liquids in the hydraulic network.

“Microfluidic works” of a device: include the hydraulic works formed of a network or networks of internal channels and chambers wetted in the course of the assay and the pneumatic works formed of valve control and pump driving circuits powered by positive and negative pressure sources on the host instrument.

The microfluidic works may be divided into microfluidic subcircuits, where each subcircuit comprises channels and chambers for performing a particular function on a liquid sample or reagent. The microfluidic subcircuits may be organized into serial subcircuits (such as for extraction, amplification and detection of a nucleic acid target or targets) and parallel subcircuits and networks such as for simultaneous assay for multiple targets on a single sample by splitting the sample.

“Top”, “bottom”, “up”, “down”, “above”, “below”, “upward”, “downward”, “superior to”, “floor”, “roof”, and so forth are indications of relative position and not absolute position, unless reference is made to a specific frame of reference, such as the “ground plane”, which is taken as orthogonal to an intersecting plumb line.

“Wetout” (“wet out”) refers to the initial hydration of a plastic surface interior to the hydraulic works of a cartridge. Because of interfacial tension effects,

initial wetout can involve overcoming a substantial energy barrier and is a major factor in resistance to capillary flow in these devices.

“Target analyte”: or “analyte of interest”, or “target molecule”, may include a nucleic acid, a protein, an antigen, an antibody, a carbohydrate, a cell component, a lipid, a receptor ligand, a small molecule such as a drug, and so forth. Target nucleic acids include genes, portions of genes, regulatory sequences of genes, mRNAs, rRNAs, tRNAs, siRNAs, cDNA and may be single stranded, double stranded or triple stranded. Some nucleic acid targets have polymorphisms, single nucleotide polymorphisms, deletions and alternate splice sequences, such as allelic variants. Multiple target domains may exist in a single molecule, for example an immunogen may include multiple antigenic determinants. An antibody includes variable regions, constant regions, and the Fc region, which is of value in immobilizing antibodies. Target analytes are not generally provided with the cartridge as manufactured, but are contained in the liquid sample to be assayed; in contrast, “control analytes” are typically provided with the cartridge or are routinely present in a sample of a particular type and are assayed in order to ensure proper performance of the assay. Spiked samples may be used in certain quality control testing and for calibration, as is well known in the art.

“Means for Amplifying:” of which the grandfather technique is the polymerase chain reaction (referred to as PCR) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, Ausubel et al. (Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. 1989), and in Innis et al., (“PCR Protocols”, Academic Press, Inc., San Diego Calif., 1990). Polymerase chain reaction methodologies require thermocycling and are well known in the art. Briefly, in PCR, two primer sequences are prepared that are complementary to regions on opposite complementary strands of a target sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, e.g., Taq polymerase. If the target sequence is present in a sample, the primers will bind to the target and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the template to form reaction products, excess primers will bind to the template and to the reaction products and the process is repeated. By adding fluorescent intercalating agents, PCR products can be detected in real time.

Other amplification protocols include LAMP (loop-mediated isothermal amplification of DNA) reverse transcription polymerase chain reaction (RT-PCR), ligase chain reaction ("LCR"), transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA), "Rolling Circle",
5 "RACE" and "one-sided PCR", also termed "asymmetrical PCR" may also be used, having the advantage that the strand complementary to a detectable probe is synthesized in excess.

These various non-PCR amplification protocols have various advantages in diagnostic assays, but PCR remains the workhorse in the molecular biology
10 laboratory and in clinical diagnostics. Embodiments disclosed here for microfluidic PCR should be considered representative and exemplary of a general class of microfluidic devices capable of executing one or various amplification protocols.

Typically, nucleic acid amplification or extension involves mixing one or more target nucleic acids which can have different sequences with a "master mix"
15 containing the reaction components for performing the amplification reaction and subjecting this reaction mixture to temperature conditions that allow for the amplification of the target nucleic acid. The reaction components in the master mix can include a buffer which regulates the pH of the reaction mixture, one or more of the natural nucleotides (corresponding to A, C, G, and T or U—often present in equal
20 concentrations), that provide the energy and nucleosides necessary for the synthesis of nucleic acids, primers or primer pairs that bind to the template in order to facilitate the initiation of nucleic acid synthesis and a polymerase that adds the nucleotides to the complementary nucleic acid strand being synthesized. However, means for amplification also include the use of modified or "non-standard" or "non-natural" bases such as
25 described in US Patent No. 7,514,212 to Prudent and US Patent Nos. 7,517,651 and 7,541,147 to Marshall as an aid to detecting a nucleic acid target.

"Means for detection": as used herein, refers to an apparatus for displaying an endpoint, ie. the result of an assay, which may be qualitative or quantitative, and may include a machine equipped with a spectrophotometer,
30 fluorometer, luminometer, photomultiplier tube, photodiode, nephelometer, photon counter, voltmeter, ammeter, pH meter, capacitive sensor, radio-frequency transmitter, magnetoresistometer, or Hall-effect device. Magnifying lenses in the cover plate, optical filters, colored fluids and labelled probes may be used to improve

detection and interpretation of assay results. "Labels" or "tags" include, but not limited to, dyes such as chromophores and fluorophores; and chemoluminescence as is known in the prior art. QDots, such as CdSe coated with ZnS, decorated on magnetic beads, or amalgamations of QDots and paramagnetic Fe₃O₄ microparticles, are a convenient method of improving the sensitivity of an assay of the present invention. Fluorescence quenching detection endpoints are also anticipated. A variety of substrate and product chromophores associated with enzyme-linked immunoassays are also well known in the art and provide a means for amplifying a detection signal so as to improve the sensitivity of the assay, for example "up-converting" fluorophores.

"Molecular beacon": is a single stranded hairpin-shaped oligonucleotide probe designed to report the presence of specific nucleic acids in a solution. A molecular beacon consists of four components; a stem, hairpin loop, end labelled fluorophore and opposite end-labelled quencher. When the hairpin-like beacon is not bound to a target, the fluorophore and quencher lie close together and fluorescence is suppressed. In the presence of a complementary target nucleotide sequence, the stem of the beacon opens to hybridize to the target. This separates the fluorophore and quencher, allowing the fluorophore to fluoresce. Alternatively, molecular beacons also include fluorophores that emit in the proximity of an end-labelled donor. 'Wavelength-shifting Molecular Beacons' incorporate an additional harvester fluorophore enabling the fluorophore to emit more strongly. Current reviews of molecular beacons include Wang K et al, 2009, Molecular engineering of DNA:molecular beacons. *Angew Chem Int Ed Engl*, 48(5):856-870; Cissell KA et al, 2009, Resonance energy transfer methods of RNA detection, *Anal Bioanal Chem* 393(1):125-35 and Li Y, et al, 2008, Molecular Beacons: an optimal multifunctional biological probe, *Biochem Biophys Res Comm* 373(4):457-61. Recent advances include Cady NC, 2009, Quantum dot molecular beacons for DNA detection. *Methods Mol Biol* 554:367-79.

Fluorescence nucleic acid assays include amplification with tagged primers and probe-based detection chemistries. Fluorescent products can be assayed at the end of the assay, or by measuring the amount of amplified product in real time. While not limiting, TaqMan Probe (Applied Biosystems) which relies on displacement and polymerase-mediated hydrolysis of a 5' reporter dye with 3' quencher construct, FRET hybridization probes, dual oligo FRET-based probes (Roche), minor groove binder-conjugated hybridization probes (MGB probes, Applied Biosystems), Eclipse

probes, Locked NA Probes (Exiqon/Roche), Amplifluor primer chemistries, Scorpions primer chemistries, LUX primers, Qzyme primers, RT-PCR, among others, are all suitable in the present invention. Fluorescent probes include intercalating probes, such as Syber Green® (Molecular Probes), ethidium bromide, or thiazole orange, FRET probes, TaqMan® probes (Roche Molecular Systems), molecular beacon probes, Black Hole Quencher™ (Biosearch Technologies), MGB-Eclipse® probes (Nanogen), Scorpions™ (DxS Ltd) probes, LUX™ primer-probes (Invitrogen), Sunrise™ probes (Oncor), MGB-Pleiades (Nanogen), and so forth. Recent advances in probe technologies are reviewed by Lukhtanov EA et al, 2007, Novel DNA probes with low background and high hybridization-triggered fluorescence, Nucl Acids Res 35:e30, for example. Reverse transcriptase is used to analyze RNA targets and requires a separate step to form cDNA. Recent advances include Krasnoperov LN et al. [2010. Luminescent probes for ultrasensitive detection of nucleic acids. Bioconjug Chem 2010 Jan 19 epub].

In addition to chemical dyes, probes include green fluorescent proteins, quantum dots, and nanodots, all of which are fluorescent. Molecules such as nucleic acids and antibodies, and other molecules having affinity for an assay target, may be tagged with a fluorophore to form a probe useful in fluorescent assays of the invention.

“FRET” (Fluorescence Resonance Energy Transfer) – is a fluorescence technique that enables investigation of molecular interactions. It depends on the transfer of energy from one fluorophore to another fluorophore (ie. a donor and a quencher) when the two molecules are in close proximity such as when hybridized. Recent advances include Carmona AK et al, 2009, The use of fluorescence resonance energy transfer (FRET) peptides for measurement of clinically important proteolytic enzymes, Ann Acad Bras Cienc 81(3):381-92.

Unless the context requires otherwise, throughout the specification and claims which follow, the word “comprise” and variations thereof, such as, “comprises” and “comprising” are to be construed in an open, inclusive sense, that is as “including, but not limited to”. Reference throughout this specification to “one embodiment”, “an embodiment”, “one aspect”, or “an aspect” means that a particular feature, structure or characteristic described in connection with the embodiment or aspect may be included one embodiment but not necessarily all embodiments of the invention. Furthermore, the features, structures, or characteristics of the invention disclosed here may be

combined in any suitable manner in one or more embodiments. “Conventional” is a term designating that which is known in the prior art to which this invention relates. “About” and “generally” are broadening expressions of inexactitude, describing a condition of being “more or less”, “approximately”, or “almost” in the sense of “just about”, where variation would be insignificant, obvious, or of equivalent utility or function, and further indicating the existence of obvious minor exceptions to a norm, rule or limit.

Description of the Drawings

Turning to the figures, FIGS. 1A and 1B show two perspective views of a disposable, single-use, sample-to-answer microfluidic cartridge of the invention, the cartridge containing all reagents for an assay and requiring only introduction of a biological sample. In this representative embodiment, the cartridge **100** includes a protective chassis or body **102** with coverplate **103** for convenience in handling. The coverplate includes and contains an inlet port **104** for addition of sample. The projecting nose **105** of the cartridge is inserted into a docking bay of a host instrument (FIG. 2). The projecting nose of the cartridge body includes optical window cutout **101** that aligns with a backside mirror of the docking bay for reflective transillumination and fluorescence detection, while not limited thereto, of a target analyte when inserted into the host instrument. Also on the underside of the cartridge is a thermal interface **110** for heating zones of the microfluidic cartridge and a disposable gasket **111** for sealedly seating the cartridge to a pneumatic control interface of the host instrument in the docking bay. The cartridge body may include microfluidic cards as shown in FIG. 16; however microfluidic works may optionally be integral to the cartridge body.

FIG. 2 demonstrates reversible insertion (double arrow) of the assay cartridge **100** in a docking bay **201** of a host instrument **200**. Performance of an assay is controlled with an operator interface generally as shown. Optical window **101** aligns with a detection apparatus inside the chassis **202** of the host instrument.

FIG. 3A is a detailed view of the cartridge as inserted into a mechanism of a host instrument. An inclined mounting plate **300** is used to angle the mechanism (and the cartridge) at a fixed angle theta (cf. FIG. 7B), which aids in venting air and entrainment of bubbles during initial wetout. The host instrument includes an optics assembly with track-mounted scanning detector head **303** and motorized clamping

mechanism **304** for interfacing with optical window **101** of the cartridge. The optics assembly and docking bay are mounted as part of a floating stage that is bolted to the inclined mounting plate but is suspension-mounted so that the cartridge may be clamped against the thermal control module **310** and pneumatics interface ports **330** shown in FIG. 3B. Further description of a host instrument, docking bay, and optics package is provided in copending World Patent Appl. Publ. No. WO 2010/088514, titled "PORTABLE HIGH GAIN FLUORESCENCE DETECTION SYSTEM," which is incorporated herein in full by reference.

A thermal control module **310** and pneumatic control interface **330** with ten pneumatic ports are shown in more detail in FIG. 3B, which includes a partial view of inclined mounting plate **300**. The underside of a cartridge (which is sealed with a thin layer of a heat-conductive polymer as a thermal interface) contacts the upper surfaces of first, second, third and fourth "zone" heating elements (**311**, **312**, **313**, **314**). A fan **315** is provided for cooling. The top face of the first heating element is provided with a mirror face **320** and operates in conjunction with the optics of host instrument for transillumination and capturing reflected light and/or fluorescent emissions through the optical window **101** of the cartridge when aligned in the docking bay.

FIG. 4 is an exploded view of a disposable microfluidic cartridge **100** with on-board liquid reagents in frangible liquid reservoirs. Each reagent reservoir is a bilayered duplex diaphragm pack carrying a liquid reagent. The cartridge chassis supports reagent reservoirs (**421**, **422**, **423**, **424**) in separate wells **426** within the housing. The cartridge as illustrated here is a cartridge designed for PCR and includes four liquid reagents. Optical window cutout **101** on the anterior nose **105** of the cartridge chassis **102** is again shown. Also inside the chassis under the coverplate **103** is an adsorbent pad **430** for sequestering liquid wastes generated in the assay. The cartridge **100** is disposable and is sealed to prevent loss of biohazardous waste. The sample inlet **104** on the coverlid **103** of the device is the sole externally accessible fluid port in the device. All reagents (including any dry reagents and any liquids reagents or rehydrating fluids) are provided within the structure of the device.

On the underside of the cartridge chassis, two "cards" containing microfluidic works are provided, an "outboard card" **410** and an "inboard card" **400**. These cards are built up of laminated and/or molded layers and contain hydraulic and pneumatic networks designed for a PCR assay. They are generally flexible and made of

plastics such as polyethylene terephthalate and polycarbonate, although not limited thereto. Disk **409** is a glass solid phase adsorbent used in the extraction of nucleic acids from the sample. A seal patch **425** is needed to seal the hydraulic works of the outboard card after installation of the solid phase disk **409**.

5 The outboard card **410** contains a fluidic circuit that works in conjunction with liquid reagent reservoirs **421**, **422**, **423**, **424** and solid phase adsorbent disk **409** to extract nucleic acids by the protocol outlined in FIG. 5B. The inboard card **400** receives purified nucleic acids via the fluidic interface (overlapping tongues **411a/411b** for forming a card junction) between the two cards **400** and **410** and
10 conducts amplification and detection within the hydraulic network of microfluidic channels embedded in the card. The inboard card includes thin surface films that form a detection window **101a** sealing the top and bottom of detection chambers enclosed within the card body. These chambers contain less than 50 uL of fluid and are heated by contact with the heating blocks of FIG. 3B. Gasket **111** is provided for sealing the
15 pneumatic control interface to the undersurface of the inboard card at card tongue **411b**, which connects to and extends the pneumatic distribution manifold of the host instrument within the microfluidic device.

FIG. 5A is a perspective view of the outboard card **411**, which interfaces with the cartridge chassis and liquid reagent reservoirs for extraction of a nucleic acid
20 target from a biosample; FIG. 5B is a schematic of the extraction process. In the extraction process, which is based on the Boom method (US Patent No. 5,234,809), the sample is first mixed with a lysis buffer, consisting of a mixture of a chaotropic agent and a detergent, and contacted with solid phase adsorbent **409**. Following washing with multiple aliquots of wash buffer, which are conveyed to waste, the adsorbed nucleic
25 acids **501** are eluted with a dilute buffer solution and transferred (open arrow, to FIG. 7A) through a fluidically communicating port system under tongue **411a** to a staging manifold on the inboard card **400**. The liquid contents of this staging manifold are used for nucleic acid amplification as described below. In each step of the extraction, a liquid reagent is required. Each liquid reagent is stored in a bilayer foil diaphragm with
30 a liquid center and the liquid is released under control of a pneumatic actuator that impels the two-layer diaphragm against a sharp, which ruptures (only) the lower layer of the diaphragm and forces the liquid into the hydraulic works of the cards. This process is illustrated in FIGS. 6A-6G.

FIGS. 6A-6G provide various views of a reagent reservoir pouch **600** formed of a bilayered (i.e. two-layered) diaphragm (layers **602**, **603**) with liquid reagent center **601** and a “sharp” **610** or “barb” disposed below the reservoir, the sharp tip pointing upwards against the lower of the two diaphragm layers **603a/603b**, in a sealed internal chamber **615** formed with well **426**. The sharp member **610** is shaped for puncturing and releasing the liquid contents into the hydraulic works of the microfluidic cartridge or card.

FIG. 6A illustrates a fluid-filled pouch or reservoir consisting of two diaphragm layers surrounding a liquid center. The two layers are illustrated in a cross-section through the pouch in FIGS. 6B and 6C. Layers **602** and **603** enclose liquid center **601**. The two layers are sealed at the edges **604**. Foil coated layers of polyester and other plastics were used in forming the diaphragm layers **602**, **603**. Top layer **602** is generally tough, flexible and resists puncture. Contrastingly, bottom layer **603** is designed to be punctured by sharp **610** and to release its contents into the microfluidic works of the cartridge via reagent outlet channel **611** (FIG. 6D), shown here not to scale. FIG. 6B describes a biconvex reservoir with diaphragm layers **602a** and **603a** surrounding liquid center **601a** with sealed edge **604a**, FIG. 6C describes a planoconvex reservoir with diaphragm layers **602b** and **603b** surrounding liquid center **601b** with sealed edge **604b**, each having particular advantages in assembly and use.

In FIG. 6D the reagent reservoir is shown mounted as a duplex diaphragm enclosing a liquid center **601** in a reagent chamber **615** of the cartridge housing. Lip seals **605** isolate the pneumatic works **606** from the hydraulic works **612**. While not limited thereto, lip seals **605** may be formed by gluing with a UV-actuated adhesive or other sealing method known in the art. When pressurized by air through pneumatic control port **607**, the lower surface of the duplex diaphragm assembly (**600**) is pressed against sharp **610** so that the bottom film layer **603** is ruptured, but not top film layer **602** (FIGS. 6B-6C). In this way, the mechanism becomes a micro-dimensioned pneumatic diaphragm-actuated liquid dispenser. Surprisingly, once the liquid center is pierced, serial pneumatic pulses may be used to force successive microliter volumes of liquid through outlet channel **611** and into the hydraulic works. The reagent outlet channel **611** is in fluidic communication with channels and chambers of the hydraulic network involved in assay reactions dependent on wetting, mixing, eluting and so forth. Plastic cover layers **616** and **617** seal the chamber **615**.

FIGS. 6E – 6G provide detailed views of the sharp member **610**, which is designed so that puncture of lower film layer **602** is not self-sealing around the contour of the sharp. FIG. 6E is a face elevation view; FIG. 6F is a side elevation view, and FIG. 6G is a CAD-generated isometric view. While not limited to the precise form and detailed dimension shown, the sharp is formed as a bisected cone **620** or frustrum of a cone with a barb tip **621**, a planar first face **622** that is modified by the molded addition of a protruding convex 2nd facet **623** and a recessed concave 3d facet **624**, which forms the mouth of outlet channel **611**. The delicately molded concavity (concave 3d facet **624**) in the projecting tip of the sharp, particularly in combination with the male convexity of the 2nd facet **623**, confounds the tendency of the film layer to close the rupture in diaphragm **603**, thus ensuring operation as what is essentially a pneumatically actuated “spigot” formed for piercing and draining the liquid centered diaphragm. The spigot remains open and fluid flows freely in response to controlled pneumatic pressure applied via port **607**. Pan **625** aids in draining the fluid of the reservoir into outlet channel **611**.

After extensive experimentation, the piercing action of the sharp was found to be most advantageously effective when the barb tip **621** of the frustrated cone was brought to a radius of from 0.004 to 0.0045 inches, and a preferred radius for this feature as determined to be 0.004 inches (four thousandths of an inch). Sharps outside the range were not found to be as effective by comparison. A microfluidic cartridge of the invention optionally may be characterized as having a sharp for piercing a reagent reservoir where the sharp is a frustrum section of a cone, the cone formed with a tip for selectively piercing a puncture sensitive layer of a duplex diaphragm, the tip having a cutting point with radius of 0.0040 to 0.0045 inches.

The frustrum section of the cone is provided with a planar first facet, a convex second facet formed on the planar first facet, and a concave third facet formed on the concave second facet, the concave third facet forming a mouth of a fluid outlet descending therefrom for draining the released liquid into the hydraulic works.

In a preferred embodiment of the reagent reservoir with liquid center, the first layer of the duplexed layered diaphragm is rupture resistant and the second layer, proximate to the sharp, is rupture sensitive. The first layer may be a laminated polymer with outer nylon film configured to be puncture resistant and the second layer may be a laminated polymer with outer polyethylene terephthalate film configured to be puncture

susceptible. Suitable polymer layers may also contain a sandwiched metallized layer, and are available for example from Technipaq Inc (Crystal Lake, IL), with a laminated polyethylene/metal/polymer backing sandwich trilayer structure. An opposable polyethylene film between the two diaphragm members of the fluid pouch is useful to permit heat sealing. UV-activated glues may be used to form a seal or gasket for assembling the diaphragm in a cartridge housing.

FIGS. 7A and 7B show the inboard microfluidic card **400** canted with a tilt as mounted in a host instrument. The card is inclined at about 15 degrees (θ) on its side as detailed in FIG. 7B, which is a sectional view through three detection chambers enclosed in the card. The tilt of the card is configured so air in the card is buoyantly directed to one or more venting ports during wetout and fill, and any bubbles that do arise are trapped in upstream channels and chambers of the card and are limited in entry into the heated zones and detection chambers of the card. Fluid **501** from the nucleic acid elution operation of FIG. 5B enters the inboard card as shown and is routed into a network of microfluidic channels and chambers described in the following figure. A tilt of 10 to 35 degrees has been found to be useful in reducing interference by bubble entrainment and may be implemented for automated assay systems by configuring the host instrument to accommodate a canted stage whereupon a microfluidic card or cartridge is supported during the assay. A vibration assist may also be provided to further isolate bubbles from critical paths. These features also aid in removing air during initial wetout, thus reducing the overall air available for bubble formation.

FIGS. 8A and 8B show a “worms-eye” view of a network **800** of channels and chambers for performing PCR as within a microfluidic card **400**. The illustration depicts the appearance of the internal wettable surfaces forming a microfluidic subcircuit, but depth of the channels and chambers is exaggerated for clarity. As shown in FIG. 8A, where three channels **a**, **b** and **c** are depicted, eluate **501** (containing any nucleic acids of a sample) is ported into the card through via **801** and enters a three-chambered staging manifold **802'**, the purpose of which is to split the fluid into three downstream channels equally and to gently and evenly urge the fluid into downstream chambers **804** and **805** while avoiding entrainment of bubbles during initial wetout of internal plastic surfaces. Valves **811** are initially closed. The mechanism of FIG. 8B depicts a single channel.

The splitting of a liquid volume **501** between multiple channels initially was found to be problematic because of uneven wetting, but is desirable so that multiple amplifications or assays can be performed in parallel. As reduced to practice, during the first stage of the filling process, liquid **501** enters three chambered manifold **802'**
5 under pressure. Each of chambers **802a**, **802b**, **802c** is bisected horizontally by an elastic diaphragm (see FIGS. 91-9L, **900**) that segregates the fluid contents from an interfacing pneumatic chamber (i.e., the vented upper cavity in a stack of two cavities separated by a diaphragm) and passively stretches during fill. During this step, pressure is equalized between the multiple channels. During the fill, air beneath the diaphragms
10 exits through vent **803**, which contains as a sanitary feature a gas permeable, liquid impermeable filter membrane that seals when wetted. Continued pressurization inflates the diaphragms in chambers **802**, so that when released by opening valves **811** (and all downstream valves thereto), the pressurized liquid flows evenly into the three (or more) parallel channels as urged by a restorative spring force or pressure exerted by the elastic
15 diaphragm **900**, which is distended during filling of chambers **802**. Because the restorative pressure can be precisely controlled and limited, and is a function of the spring constant of the diaphragm, and because the displacement volume of the elastic diaphragms can be precisely controlled, the extent of wetout or "priming" of the downstream channels can be precisely calibrated in the manner of volumetrically filling
20 a pipet, a clear advance in the art. Elastomeric diaphragms were achieved with polyurethane, polyvinylidene chloride, and/or polyester as diaphragm material. One such material is Saranex™ (Dow Chemical), which is a polyvinylidene chloride extruded sheet sandwiched between polyolefin layers as a composite thin film. Other materials may be used.

25 Advantageously, the passively stretching diaphragms **900** (FIGS. 9A-9L) of each chamber **802** thus become an energy storing device for distributing fluid into one or more parallel downstream channels without entrainment of bubbles. By knowing the downstream volume, the energy in the stretched diaphragms may be adjusted so that each parallel channel is filled to a mark, as in a volumetric pipet, the fill
30 volume generally falling short of the detection chambers **806** and final valve structure **812** in each branch, but fully wetting chambers **804** and **805**. During wetout, all downstream structures are cleared of air ahead of a steadily advancing meniscus via terminal vent **807**, which may be operated under sanitary conditions by capping with a

hydrophobic liquid impermeable gas-permeable membrane in the manner illustrated for vent **803**, if desired. Because the flow of liquid during relaxation of the diaphragms is not forced by pneumatic overpressure, does not depend on capillary flow, and is finite, the advance of the meniscus is progressive and orderly, limiting entrainment of air pockets in its wake. This is a technological advance in the art, permitting precise filling of parallel downstream networks without entrainment of bubbles. The method is facilitated by the tilt of the card and by removing corner radii (as are sometimes associated with localized increases in surface tension that may impede wetting) from junctions of channels and chambers.

10 In a further refinement of this method, chambers **804** and **805** are also fitted with internal diaphragms. Unlike the passively flexing diaphragm of chamber **802**, the pneumatic face of the diaphragms of chambers **804** and **805** are not vented to atmosphere and can be driven by positive pneumatic pressure or negative pneumatic pressure supplied from a pressure manifold, thus serving as pumps. During the fill cycle, the diaphragms are “tented” or “inflated” downward to occupy volume of the lower hydraulic chamber so as to reduce or eliminate any dead volume of the chambers. Liquid seeping past these diaphragms on the outside bottom edges of the chambers fully wets the chambers and displaces any residual air. Then upon releasing the diaphragms after closing valve **812**, liquid is aspirated from upstream to fill and make up the volume of the chamber.

In a further refinement of this method, dry reagents are placed in chambers **804** and **805**, the nature of the dry reagents relating to the nature of the assay to be performed. The reagents are generally spotted near the center of the chamber. During initial wetout, the diaphragm is fully tented downward to occupy the volume of the lower hydraulic chamber so as to reduce the dead volume therein and protectively covers and protects the dry reagent spots from dissolution and washout as the chamber residual dead volume is wetted. After valve **812** is closed and the chamber is flooded with liquid by reversing pressure differential across the diaphragm, the reagent dissolves rapidly and at full strength.

30 The positions of dry reagents are marked in FIG. 8B. As can be seen, dry reagents having specific functions are placed in designated chambers. Dry reagent spot **821** contains for example master mix and primers that are advantageously mixed with and denatured in the presence of target template. This chamber **804** is

preferentially heated at a temperature sufficient for the denaturing of template nucleic acid. Chamber **805** contains for example dried polymerase **822** and is at a temperature suitable for annealing of primers and target and for initiation of polymerization. In the detection chamber **806**, dry reagent spot **820** contains probes such as, for example,
5 “molecular beacons” or intercalation dyes which are used to detect amplicon produced in the amplification. Detection chamber **806** is bounded at a “top” and a “bottom” by thin film optical windows and is reflectively transilluminated for fluorometric detection of amplified target. Synergically, the bottom thin film layer is also effective in heat transfer from the mirror faced heating element shown in FIG. 3B, with which the card
10 interfaces during the assay, and can thus be termed a “thermo-optical window”, such as is useful in assaying by thermal melting curve as will be described below.

FIGS. 8C and 8D describe an alternative cartridge **830** for PCR. In this cartridge, sample **501** entering the cartridge under pressure at sample inlet **831** is split at trifurcation **833** and fills each of three chambers **832a**, **832b**, **832c**, which are
15 independently vented at hydrophobic vents **834**. Each chamber **832** contains an elastic pneumohydraulic diaphragm, which when stretched during fill exerts a pressure on the liquid volume contained in the chamber. The chambers may be filled by injecting a series of pressurized volumes from an upstream pump. Fluid flow into the three branches of the distribution manifold is not necessarily split equally, but volume and
20 pressure in each chamber (**832a**, **832b**, **832c**) become isobaric and equalized as the staging manifold equilibrates. During the fill process, downstream valves **835** are closed.

After pressurization of the staging manifold **836** is completed and equilibrated, valves **835** are opened so that the elastic diaphragm of chambers **832** can
25 relax while passively urging the liquid contents into amplification chambers **837** and **838**. During this process, the diaphragm elements of chambers **837** and **838** are inflated to occupy the lower hydraulic chamber so that headspace is removed and any dried reagents in the chambers are protected from being washed away by the advancing meniscus. PCR amplification is performed as described for FIGS. 8A and 8B.
30 Downstream valve **839** is opened to convey any amplification products through an antechamber **840** to a detection chamber **846** by pressurizing diaphragms in both chambers **837** and **838** while valve **835** is closed. Any air is flushed out of the system through terminal vent **847**. Advantageously, dried probe **841** printed or spotted in the

antechamber is dissolved and mixed with amplicon prior to injection into the detection chamber, which improves the transparency of the thermo-optical window bounding the detection chamber and reduces or prevents autofluorescence of certain dyes useful as molecular beacons or FRET probes. By operating the card at a tilt angle θ as described in FIG. 7, air is advantageously purged to a vented port superiorly disposed on the detection chamber.

As can be seen in FIG. 8D, the inlet, outlet, and venting ports of detection chamber **846** and the amplification chambers **837** and **838** are asymmetrically placed. When the cartridge or card is canted on a tilted stage of the host instrument (referencing FIGS. 3A and 7B), communicating ports (**842**, **843**) between the amplification chambers and at the terminal venting port (**848**) associated with the detection chamber are elevated relative to the chambers themselves and are contoured to overcome any surface tension effects of the geometry. Air in the system is thus preferentially flushed from the system by the advancing liquid during wetout, which fills the lower aspects of the chambers first, and any bubbles generated by heating-associated degassing of the liquid during PCR are preferentially trapped between the amplification chambers so as to not interfere with heat transfer, and do not enter the detection chamber.

In more methodological detail, FIGS. 9A-9L present a simplified chronology and schematic of the steps or stages of passive initial wetout with staging manifold. Cross-sectional and plan views are shown so that the progress of the advancing meniscus may be seen. In the first view, FIG. 9A, the diaphragm **900** in the staging manifold chamber **802** is shown to be upwardly distended, turgid with a liquid reagent entering from the left through open valve **910**, and the pneumatic face of the diaphragm is vented at **905** to atmosphere. Downstream chamber **903** is dry, valve **911** is closed. The initial dry state of reagent spot **905** is monitored in plan view in FIG. 9B on the right.

In FIG. 9C, both valves **910** and valve **911** are closed and valve **912** is open for venting. Diaphragm **900** is pressurized and is tented down over reagent spot **905**; the footprint of the diaphragm in contact with the base of chamber **903** is illustrated by a dotted line **902a** in FIG. 9D.

In FIG. 9E, valve **910** remains closed and valves **911** and **912** are open. As shown in plan view in FIG. 9F, an advancing meniscus begins to enter chamber **903**.

In FIG. 9G, liquid continues to wet chamber **903** and fill any dead volume on the periphery of the chamber around the collapsed roof formed by the diaphragm. This process continues as shown by snapshot in FIG. 9I. The progressive deflation of the passively stretched diaphragm **900** in chamber **802** is shown on the left
5 in timelapse snapshots in FIGS. 9G, and 9I. It can be seen in FIG. 9K that pressure applied across diaphragm **902** can be reversed when valves **910** and **912** are closed so that liquid is aspirated from the staging manifold chamber **901** and fills chamber **903**, dissolving dried reagent **905**. Complete dissolution is shown figuratively in FIG. 9L. During this process, air has been effectively displaced from the wetted areas, first by
10 elimination of deadspace in chamber **903**, then by the progressive elimination of residual air by the relaxation of diaphragm **900**, and finally by sealing the purged system and aspirating the contents of chamber **802** into chamber **903** to solubilize and to be mixed quantitatively as a reagent solution. The volume of liquid filling downstream chambers can be precisely controlled by configuring a displacement
15 volume of elastic diaphragm **900** and chamber **802**; the rate of passive downstream fluid wetout is controlled by selecting a spring constant for the elastic diaphragm **900**.

FIGS. 10A – 10C demonstrate a further advantageous use of the above inventive mechanism for priming a PCR reaction, where a system having two zones for thermal cycling of the nucleic acid substrate and polymerase is demonstrated. All
20 fluidic systems are contained in a card or cartridge body **1000**. Staging manifold chamber **1001** is vented to atmosphere and contains a passive elastic diaphragm **1002** capable of storing a pressurized liquid, which enters from the left through valve **1003**. As shown in FIG. 10B, this diaphragm **1002** becomes distended during fluid entry and valve **1003** is then closed. Diaphragms **1011** and **1021** in chambers **1010** and **1020**,
25 respectively, are pressurized to form a protective tent over dried reagent spots **1012** and **1022**, and to displace deadspace air from the chambers as shown in FIG. 10B. Downstream valves **1023** and **1033** are open at this stage so that displaced air is vented from the system via terminal vent **1034**. In FIG. 10C, valve **1004** is opened and liquid enters the two chambers where PCR will occur, first in amounts sufficient for priming
30 the chambers. This sequence is continued in FIGS. 10D through G. Fluid is introduced in an amount sufficient to fill the denaturation hot chamber **1010** but no more; applying a vacuum to diaphragm **1011** aids this process. FIG. 10D shows that the “hot” or “denaturing” chamber **1010** is under vacuum and liquid has been aspirated to fill the

chamber. After any dry reagent **1012** is quantitatively dissolved and nucleic acid denaturation is sufficient, the liquid contents of chamber **1010** are transferred to the second chamber **1020** at a temperature suitable for annealing of primers so that polymerase-mediated extension may begin upon dissolution of reagent spot **1022**. FIG. 10E shows liquid pumped from the hot chamber to the “annealing” chamber by reversing the pressure differential across the two diaphragms. This process is again reversed in FIG. 10F, demonstrating the reciprocating pumping action of the two diaphragms in forcing the liquid back and forth between the hot zone at **1010** (which is contacted with heating block **313**, FIG 3B) and the annealing zone at **1020** (which is contacted with heating block **312**). This reciprocating pneumohydraulic action is the basis of nucleic acid amplification by thermocycling in the apparatus. Finally, the fluid with any amplicons is ejected into the detection chamber as shown in FIG. 10G. The detection chamber as shown here is fitted with a pair of optical windows **1035**.

In a more complex configuration, an additional temperature station and associated thermal interface with thermal block **314** (FIG. 3B) is used, for example, for reverse transcriptase mediated synthesis of cDNA prior to a PCR-type amplification process. Thus additional chambers may be useful and the geometry and configuration may be varied, *mutatis mutandi*, by logical extensions of the teachings of the invention. During wetout, diaphragms in each chamber are used to reduce initial deadspace volume. Subsequently, application of pressure differentials across the diaphragms are used to harness serial diaphragm assemblies and valve elements as pumps and mixing elements for hydraulic movement of fluid volumes through the hydraulic works of the devices. A first embodiment of a reverse-transcriptase device is shown in FIGS. 14A-14C, and will be described in more detail below.

FIGS. 11 – 13 summarize the steps of the methods described above. FIG. 11 describes the steps of a method for extracting nucleic acids from a sample, where a bilayered duplex diaphragm with liquid center is used to dispense the reagents. After starting the host instrument, a liquid sample is placed in a microfluidic cartridge and the cartridge is inserted in the docking bay of the instrument. The host instrument reads a bar code on the microfluidic cartridge indicating the type of assay to be run. The liquid sample is aspirated into a mixing chamber and cell lysis buffer is dispensed and mixed with the liquid sample. To dispense the lysis buffer a “liquid-centered diaphragm” is urged by pneumatic actuation against a sharp, rupturing the lower layer

of the diaphragm and pumping the liquid into the card. The sample lysate is then contacted with a solid phase nucleic acid adsorbent positioned in a chamber in the card and the depleted sample lysate is directed to on-board waste. Ethanolic solution is dispensed from a second liquid-centered diaphragm member and used to wash
5 contaminants from the solid phase adsorbent. The wash step may be repeated. The washes are sequestered to on-board waste. The solid phase matrix is briefly dried under a stream of air to remove residual solvent. Elution buffer is then dispensed from a final liquid-centered diaphragm reservoir and contacted with the solid phase matrix. The eluate with eluted nucleic acids **501** is then transferred to a staging manifold for entry
10 into a detection subcircuit. In the example provided here, a nucleic acid assay with PCR amplification is conducted on the eluate. Other nucleic acid amplification methods are known in the art and, as would be understood from the teachings and drawings herein, may be practiced by reconfiguration of the various components of the devices of the invention

15 FIG. 12 describes steps of a method for “priming” (i.e., wettingly loading) channels and chambers of a hydraulic works with liquids dispensed from a bilayered duplex diaphragm with liquid center **601** as pictured in FIG. 6D. After eluting a nucleic acid extract **501** with an elution buffer released by rupturing a reagent reservoir containing the buffer, the fluid can be oscillated when contacting solid phase
20 absorbent **409** (FIG. 4) so as to efficiently take up adsorbed nucleic acids. The eluate is then pumped under pressure into a staging chamber of a microfluidic card so that an elastic diaphragm which covers the chamber becomes distended and stores the potential energy. The staging chamber inlet is sealed and pressure throughout the staging manifold equalizes rapidly. A downstream valve to each channel is then opened. All
25 downstream fluid channels and chambers are vented during this operation, which is useful to wet out or prime the downstream wettable surfaces. Advantageously, as the elastic diaphragm relaxes, releasing its stored energy, the elasticity of the diaphragm gently but firmly forces a liquid volume into the downstream channels and chambers equally in parallel, the advancing meniscus displacing any residual air without bubble
30 entrainment, an advance in the art. The liquid volume is split into branching parallel fluid pathways in this way.

FIG. 13 describes steps of a method for rehydrating dry reagents without bubble entrainment or reagent washout. During manufacture of a cartridge of the

invention, a dried reagent spot is printed in the center of a reagent chamber. The reagent chamber is configured with an overlying pressurizable diaphragm. A liquid sample is added and the cartridge is inserted into the docking bay of a host instrument, which supplies pressure and valve commands for operation of the cartridge. The sample is first pumped under pressure into an unvented staging chamber, which distends an elastic energy-storing diaphragm covering the liquid in the staging chamber. The aforementioned downstream reagent chamber is vented and the pressurizable diaphragm therein is pressurized so as to form a protective temporary seal around and over the dried reagent spot. The downstream valve of the staging chamber is then opened; the elastic diaphragm relaxes and elastic energy of the diaphragm's recovery gently forces sample fluid into the downstream reagent chamber, displacing any residual air around the protective temporary seal. Finally, the pressure differential across the pressurizable diaphragm is reversed or relaxed, uncovering the reagent spot and aspirating a full volume of liquid into the reagent chamber so that the reagent spot advantageously dissolves at full strength in the sample fluid without bubble entrainment, an advance in the art.

FIGS. 14A and 14C are worm's eye views of a network of microfluidic channels and chambers for reverse-transcriptase-mediated PCR. A device having three parallel channels **a**, **b**, and **c** is shown. Sample **501** is split between the channels so that three (or more) separate multiplex assays may be performed in parallel, for example. Unlike previously depicted embodiments, here a reagent **1220** is printed in a channel **1205** rather than in a diaphragm-actuated chamber. The passive wetting principle articulated in FIG. 9, however, is retained: liquid is expelled into the channel by the passive relaxation of an energy storing diaphragm that had been primed by an upstream pump. This principle is effective in limiting entrained air and in balancing fluid flow in parallel channels branching from a common staging manifold, where each channel provided with a discrete passive diaphragm. Devices utilizing this passively driven wetting principle, as realized herein, are an advance in the art, overcoming deficiencies associated with both capillary-wetted and actively-wetted devices of the prior art.

In one embodiment, FIGS. 14A and 14C show a network **1200** of channels and chambers for performing rtPCR within another microfluidic card of the invention. Depths of the channels and chambers in this "worms-eye" view are exaggerated for clarity. Eluate **501** (containing any nucleic acids of a sample) is ported

into the card through via **1201** and enters a fluidly interconnected three-chambered staging manifold **1202**, the purpose of which is to split the fluid into three downstream fluid pathways equally and to gently and evenly urge the fluid through downstream valves **1204**, reagent channel **1205**, valve **1206** and into chamber **1207** while avoiding
5 entrainment of bubbles during initial wetout of internal plastic surfaces. Valves **1204** are initially closed.

During the first stage of the filling process, liquid **501** enters the poly-chambered manifold **1202** under pressure. Each chamber **1202** is bisected horizontally by an elastic diaphragm (see FIG. 9, **900**) that segregates the fluid contents from a
10 vented upper pneumatic cavity in the chamber and passively stretches during fill. During the fill, air beneath the diaphragms exits through vent **1203**, which contains as a sanitary feature a gas permeable, liquid impermeable filter membrane that seals when wetted and allows an increase in pressure, distending the diaphragms. Continued pressurization inflates the diaphragms in chambers **1202** with liquid, so that when
15 released by opening valves **1204** (and all downstream valves thereto), the pressurized liquid flows evenly into the three (or more) parallel downstream channels as urged by a restorative force exerted by the elastic diaphragms. The restorative pressure can be controlled and limited, and is a function of the spring constant of the diaphragm. The volumetric displacement of the elastic diaphragms can be controlled, so that the extent
20 of wetout (or "priming") of the downstream channels is calibrated in the manner of volumetrically filling a pipet q.s. to a mark. The capacity to equally split a sample is advantageous in performing assays in parallel in a microfluidic device and has been problematic when attempted by capillary flow and by suction or positive displacement methods (such as a syringe pump) because there is no assurance that flow in each of the
25 channels will progress at an equal rate. Surprisingly, using the principle of wetout driven by passive relaxation of mated diaphragms in a staging manifold, this problem is advantageously solved for multiple parallel channels.

Chambers **1207** and **1208** are fitted with internal diaphragms that interface between a hydraulic chamber and a pneumatic chamber. However, unlike the
30 passively flexing diaphragm of chamber **1202**, the pneumatic faces of the diaphragms of chambers **1207** and **1208** are not vented to atmosphere and can be actively driven by positive pneumatic pressure or negative pneumatic pressure supplied from an external source, thus serving as pumps. During the fill cycle, the diaphragms are fully distended

down into the hydraulic cavity to as to reduce or eliminate any dead volume of the chambers. Liquid seeping past these diaphragms on the outside bottom edges of the chambers fully wets the chambers and displaces any residual air. Then upon releasing the diaphragms after closing valves **1209**, liquid is aspirated under suction pressure
5 from upstream and fills the entire volume of hydraulic chamber **1207**, air having been entirely flushed from the system.

In a variant, one of the pneumatic chambers is vented to atmosphere, and is slaved to the action of the unvented diaphragm. The two chambers are isolated from the remaining circuit elements by valves. When the active diaphragm is pulsed with
10 positive pressure, liquid is forced to the adjoining chamber; when the active diaphragm is pulsed with negative pressure, liquid is aspirated from the adjoining chamber. Optionally, the passive diaphragm may be an elastic diaphragm.

In a further refinement of this method, dry reagents are placed in chambers **1207** and **1208** and in channel **1205**. The reagents are generally spotted on
15 the floor of a hydraulic chamber or channel where the breadth of the passageway permits access by a printing head. The reagent **1220** spotted in channel **1205** comprises a reverse transcriptase and nucleotide substrates in a suitable buffer. Typically a PCR master mix and suitable primers are provided as reagent spot **1221** in chamber **1207**. Spot **1222** is a dehydrated *Taq* reagent spot. Spot **1223** includes optional detection
20 reagents, such as a fluorescent probe. Multiple separate spots may be printed using a roll-type or sheet-type process in each chamber or channel.

RNA target in the eluate **501** is converted to cDNA by the action of reverse transcriptase, generally at a temperature of 20 to 45 °C. This action is effected within channel **1205** in the elution buffer, and is depicted in more detail in FIG. 14B,
25 where valves **1204** and **1206** are separated by a modified channel segment **1205** containing a dried reagent spot **1220**. The reagent, for example a reverse transcriptase, is dissolved in sample transiting the specially modified channel segment. Substrates and any cofactors for full enzyme activity are also provided.

During initial wetout, diaphragms in chamber **1207** and **1208** are fully
30 distended down into the hydraulic chamber so as to reduce the dead volume therein and the covering provided by the diaphragm protectively seals the underlying dry reagent spot or spots from premature dissolution and washout during wetting. Vent **1211** is open to exhaust air that is displaced by entry of the fluid, generally as a smoothly

advancing meniscus. After valve **1209** is closed and the chamber **1207** is filled with liquid by reversing pressure across the diaphragm (i.e. aspirating the liquid into the chamber), valve **1206** is also closed. Any spotted reagent dissolves rapidly and at full strength, without dilution, essentially as described with respect to FIG. 8 for a direct
5 PCR process, where there was no need to form a cDNA from an RNA target. Reconfiguration of the device is thus flexible and may be adapted to a variety of molecular assay processes.

Chamber **1207** is preferentially heated at a temperature sufficient for the denaturing of template nucleic acid. Chamber **1208** contains for example dried
10 polymerase **1222** and is at a temperature suitable for annealing of primers and target and for initiation of polymerization. Hot start of PCR is initiated for example by dissolution of a *Taq* polymerase reagent spot **1222** in chamber **1208**. Then, by alternating pressure applied to the diaphragms of the two chambers **1207** and **1208**, fluid may be moved back and forth from denaturing to annealing conditions by a
15 reciprocating pneumohydraulic action of the diaphragms, and chain elongation and amplification has been found to be successful in generating amplicons during this process. In the detection chamber **1210**, dry reagent spot **1223** contains probes such as, for example, “molecular beacons” which are used to detect any amplicon produced in the amplification. As before, detection chamber **1210** is bounded on top and bottom by
20 thin film optical windows and is reflectively transilluminated for fluorometric detection of amplified target. Synergically, the bottom thin film layer is also effective in heat transfer from the mirror faced heating element shown in FIG. 3B, with which the card interfaces during the assay, and can thus be termed a “thermo-optical window”, such as is useful in assaying or confirming amplicon identification by thermal melting curves as
25 will be described below (FIGS. 17, 18A and 18B).

FIGS. 14D and 14E describe an alternative cartridge **1230** for PCR. In this cartridge, eluate **501** entering the cartridge under pressure at sample inlet **1231** is split at trifurcation **1233** and fills each of three chambers **1232a**, **1232b**, and **1232c**, which are independently vented at hydrophobic vents **1234**. Each chamber **1232**
30 contains an elastic pneumohydraulic diaphragm, which when stretched or distended exerts a pressure on the liquid volume contained in the chamber. If needed, the chambers may be filled by injecting a series of pressurized volumes from an upstream pump; fluid flow into the three branches of the distribution manifold is not necessarily

split equally, but volume and pressure in each chamber (**1232a**, **1232b**, **1232c**) become equalized as the staging manifold equilibrates. During the fill process, downstream valves **1235** are closed.

After pressurization of the staging manifold **1236** is completed, valves **1235** and **1237** are opened so that the elastic diaphragm of chambers **1232** can relax while passively urging the liquid contents into reverse transcription channel **1238**. Reverse transcription is conducted under buffer, substrate and temperature conditions adapted for reverse transcriptase; buffer and any enhancers are generally supplied as a dried reagent spot **1257** in chambers **1238**. The sample is then urged into amplification chambers **1247** and **1248**. Each amplification chamber is fitted with a pneumohydraulic diaphragm. During this process, the diaphragm elements of chambers **1247** and **1248** are inflated under pneumatic pressure so that headspace is removed and any dried reagents in the chambers are protected from being washed away by the advancing meniscus by the inflated diaphragms, which are tented into the hydraulic chambers to cover the reagent spots. PCR amplification is performed on cDNAs made by reverse transcription as described for FIGS. 14A and 14C. Downstream valve **1249** is opened to convey any amplification products through an antechamber **1250** to a detection chamber **1251** by pressurizing diaphragms in both chambers **1247** and **1248** while valve **1237** is closed. At each stage, any air in the system is flushed out through terminal vent **1252**. Advantageously, dried probe **1253** printed or spotted in the antechamber **1250** is dissolved and mixed with amplicon prior to injection into the detection chamber, which improves the transparency of the thermo-optical window bounding the detection chamber and reduces or prevents autofluorescence of certain dyes useful as molecular beacons or FRET probes.

As can be seen in FIG. 14E, the inlet, outlet, and venting ports of detection chamber **1251** and the amplification chambers **1247** and **1248** are asymmetrically placed. When the device is canted on a tilted stage of the host instrument (referencing FIGS. 3A and 7B), communicating ports (**1254**, **1255**) between the amplification chambers and at the terminal venting port (**1256**) associated with the detection chamber are elevated relative to the chambers themselves and are contoured to overcome any surface tension effects of the geometry. Air in the system is thus preferentially flushed from the system by the advancing liquid during wetout, which fills the lower aspects of the chambers first, and any bubbles generated by heating-

associated degassing of the liquid during reciprocal pumping for PCR are preferentially trapped between the amplification chambers so as to not interfere with heat transfer, and do not enter the detection chamber.

Alternatively, reverse transcriptase cDNA and amplification may be performed in one of the cartridges of FIG. 8. This is achieved by spotting reverse transcriptase (MMLV-RT, AMV-RT) and substrates in first amplification chamber 804 and by first incubating at 40 to 50 °C. Nucleic acids are extracted in the presence of an RNAase inhibitor. Suitable buffers for one-pot rtPCR are described in the literature and result in what is essentially a pre-amplification of RNA targets, thus improving sensitivity and the range of detectable molecular targets. Buffer systems for one-pot rtPCR are described for example by Young [Young et al. 1993. Detection of Hepatitis C virus RNA by a combined reverse transcription-polymerase chain reaction assay. J Clin Microbiol 31:882-86] and by others. Generally an RNAase inhibitor is added.

FIG. 15 illustrates use of optical windows of a detection chamber of a device of the invention for monitoring a fluorescent endpoint. An objective lens 1520 is used to transilluminate a detection chamber 1500 holding a liquid sample 1501. The detection chamber is bounded by an upper optical window 1502 and a lower optical window 1503. The chamber rests on a mirror face 320 of a heating block 311, the heating block thus fulfilling dual functions of reflecting back the optical path for reflected light rays absorbed or emitted by chromogens or fluorophores in the chamber and for modulating the temperature of detection chemistry in the fluid. This configuration has value for example in FRET detection and for confirmation of detection of nucleic acid targets.

Photons emitted by a target molecule 1510 may be emitted in a cone that is capture by the objective lens or may be reflected from mirror face 320, thus forming a virtual image 1511 of the target molecule, and again are captured by the objective lens, increasing sensitivity. The detection chamber is thus mirrored by a “virtual detection chamber” (dotted lines) in the body of the heating block 311. Advantageously, bubbles 1505 forming in the detection chamber are gravitationally urged away from the center of the chamber by the inclination angle theta at which the device is disposed in the docking bay within the host instrument (see FIG. 7B). Synergically, the mirror-smooth surface 320 also improves heat transfer, and lower optical window 1503 also serves as a heat transfer film. The heat transfer film is

advantageously very thin and is forced into thermal contact with heating block **311**. A preferred heat transfer film is described in US Patent Nos. 7,544,506 and 7,648,835, which are coassigned, but may also include cyclic polyolefin films of similar dimensions, for example. The assembly thus functions as a thermo-optical window, achieving improved heating and optical interrogation of any amplicons or other detectable species present.

FIG. 16 depicts a representative level of complexity of the microfluidic works for performing PCR and a pneumatic interface with gasket for interfacing the microfluidic works with a host instrument. Shown for purposes of illustration are fluidic channels and chambers comprising a hydraulic works with microfluidic subcircuits and a pneumatic works for operating a molecular detection assay, exemplary details of which have been described here. An outboard card **410** and an inboard card **400** are joined at a common pneumatic junction which is sealed using a disposable gasket **405** during operation to the pneumatic control interface in order to pneumatically control and drive the hydraulic workings of the cards. This subassembly **1600** is generally mounted in a cartridge chassis containing reagent reservoirs as described with reference to FIG. 4. The microfluidic works of the cards include the hydraulic works formed of a network or networks of internal channels and chambers that are wetted in the course of the assay and the pneumatic works formed of valve control and pump driving circuits powered by positive and negative gas pressure sources on the host instrument. Diaphragm valves are pneumatically opened and closed to control steps of the assays. Larger diaphragms disposed at the interface between the hydraulic works and the pneumatic works also serve as pneumohydraulic devices for moving fluids and also for converting kinetic motion of fluids into potential energy in the form of elastically distended diaphragm elements of a staging manifold and/or passively driven pumps in the amplification chambers, for example. In this figure, the outboard card **410** is responsible for nucleic acid extraction from a biological sample, and the inboard card **400** is used for amplification and detection. Other combinations are readily conceived within the scope and spirit of the invention, which is not limited by the illustrative examples provided.

FIGS. 17, 18A and 18B are representative of the types of assay results obtained with the microfluidic cartridges of the present invention, while not limiting thereto. FIG. 17 shows scanning data collected for a molecular beacon hybridized to an

amplicon. The scanning axis (x-axis of plot) transects detection wells representing positive and negative test conditions respectively, and it can be seen that signal is limited to the detection wells. In the figure, the sample is scanned repetitively as the temperature in the detection chamber is systematically varied. The scans are overlaid in the plot to illustrate the spatial fidelity of the optical scanning apparatus. Fluorescence scans for 35 °C, 65 °C, 70 °C, 75 °C and 80 °C test conditions are marked. Test scans at 40, 45, 50, 55, and 60 °C, and the 85 and 90 °C plots were not well differentiated, as would be expected, and are not individually marked. It can be seen that fluorescent signal is a function of temperature. Fluorescence quenching in this example is observed to increase as the double stranded probe-target is melted, ie. signal is greatest at 35 °C and is essentially not present at 80 °C. In FIG. 18A, the data is replotted for signal versus temperature for the positive (**2301**, solid line) and negative (no target, **2302**, dotted line) test conditions. In FIG. 18B, a first derivative is plotted, indicating a FRET melt temperature of about 70 °C.

15

Example I

By example, the apparatus of the invention is shown to be useful in diagnosis of infectious disease by detection of the nucleic acids of a pathogen in a human sample such as feces. Of interest by way of illustration were the *rfb* gene useful in genetically detecting Enterobacteriaceae-like O-antigen serotype and the *stx*₁ and *stx*₂ genes (for shigatoxins). These genes are found for example in *Shigella*, *Salmonella*, *Campylobacter*, and *Escherichia coli* serotypes of interest in diarrheal disease.

Negative fecal swabs were diluted in 2.5 mL of PBS and spiked with O157:H7 bacterial culture. Diluted samples 250 uL were loaded for analysis onto a cartridge of the invention. These cartridges contained all required dried and liquid reagents for PCR and molecular beacon amplicon detection. After DNA extraction, target and primers were denatured at 94C for 2 minutes and then cycled for PCR amplification at about 12 sec per thermal cycle. After loading, an instrument having thermal, pneumatic and optical interfaces designed to be compatible with the cartridge was used to run a multiplex nucleic acid assay on the sample. Bacteroides DNA was used as an internal positive control on the amplification; negative controls were also run and produce no false positives.

A FAM-labelled probe for bacteroides is detected by a first fluorescence (excitation 485 nm, emission 535 nm). A CAL fluor Red 610-labelled probe (excitation 590 nm, emission 610 nm) is used to detect the target analyte in this assay. Biplex amplification products were detected at or near a minimum of 80 target copies per extract against an internal control background estimated at 400,000 copies, indicating a high level of sensitivity and specificity. Details of the optics are described in World Patent Application Publication No. PCT/US10/22581, titled PORTABLE HIGH GAIN FLUORESCENCE DETECTION SYSTEM, which is co-pending and is incorporated in full by reference for all purposes herein.

FRET curves for amplicons detected for *stx2* (2411), *stx1* (2412), and *rfb* (2413) genes in fecal samples in a device of the invention are illustrated in FIG. 19. Control data is not shown.

Example II

Using on-board dry and liquid reagents, a blood sample may be processed and RNA associated with Measles virus detected in 30 minutes or less. In a first step, cDNA is formed from the sample at an incubation temperature of about 50 C in one of the devices shown in FIG. 14, and the reverse transcriptase product is then subjected to PCR using two microfluidic chambers (1221, 1222) with dual temperature zone control generally as described in US Patent Nos. 7,544,506, 7,648,835, 7,763,453, and 7,763,453 which are co-assigned, and in pending applications titled, "Integrated Nucleic Acid Assays", which are co-assigned and incorporated in full by reference for all purposes herein. Amplicon is then detected using a FAM fluorescence-tagged molecular beacon directed at the amplified target. Optionally, a control consisting of a California Red-tagged RNAase P leukocyte exon sequence (which is generally characteristic of any genuine human blood sample) with multiplex amplification in a one-pot reaction mixture, is used to validate the assay.

Other examples illustrating various combinations of inventive elements and features are readily demonstrated. Devices configured per the teachings of the invention may be used in molecular assays for a target nucleic acid (either DNA or RNA) associated with, for example, an infectious agent selected from a bacterium (including *Acinetobacter baumannii*, *Actinobacillus equuli*, *Bacillus anthracis*, *Brucella melitensis*, *Brucella abortus*, *Bordatella pertussis*, *Bordatella bronchioseptica*,

Burkholderia pseudomallei, *Corynebacterium diphtheriae*, *Coxiella burnetii*, *Eikenella*
corrodens, *Escherichia coli*, *Francisella tularensis*, *Francisella novicida*,
Fusobacterium necrophorum, *Haemophilus influenzae*, *Klebsiella oxytoca*, *Klebsiella*
pneumoniae, *Kingella denitrificans*, *Legionella pneumophila*, *Leishmania* ssp, *Listeria*
5 *monocytogenes*, *Moraxella catarrhalis*, *Mycobacterium tuberculosis*, *Mycoplasma*
pneumoniae, *Neisseria gonorrhoeae*, *Neisseria meningitides*, *Pasteurella multocida*,
Proteus vulgaris, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Pseudomonas*
putrefaciens, *Pseudomonas cepacia*, *Salmonella typhi*, *Shigella dysenteriae*,
Staphylococcus aureus, *Streptococcus pyogenes*, *Streptococcus pneumoniae*,
10 *Treponema pallidum*, *Yersinia pestis*, or *Vibrio cholera*), a Rickettsial agent (including
Chlamydia pneumoniae, *Chlamydia trachomatis*, *Rickettsia prowazekii*, or *Rickettsia*
typhi), a viral agent (including Measles virus, HIV virus, Hepatitis C virus, Hepatitis B
virus, Dengue Virus, Western Equine Encephalitis virus, Eastern Equine Encephalitis
virus, Venezuelan Equine Encephalitis virus, Enteroviruses, Influenza virus, bird flu,
15 Coronavirus, SARS Coronavirus, Polio virus, Adenovirus, Parainfluenza virus, Hanta
virus, Rabies virus, Argentine Hemorrhagic Fever virus, Machupo virus, Sabia virus,
Guanarito virus, Congo-Crimean Hemorrhagic Fever virus, Lassa Hemorrhagic Fever
virus, Marburg virus, Ebola virus, Rift Valley Fever virus, Kyasanur Forest Disease
virus, Omsk Hemorrhagic Fever, Yellow Fever virus, Smallpox virus, a retrovirus,
20 Monkeypox virus, and foot and mouth disease virus), a fungal agent (including
Coccidioides immitis, *Candida albicans*, *Cryptococcus neoformans*, *Histoplasma*
capsulatum, *Blastomyces dermatitidis*, *Sporothrix schenki*, or *Aspergillus fumigates*), a
parasitic agent (including *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium*
ovale, *Plasmodium malariae*, *Toxoplasma gondii*, *Plasmodium bergeri*, *Schistosoma*
25 *mansoni*, *Schistosoma hematobium*, *Schistosoma japonicum*, *Entamoeba histolytica*,
Babesia, *Toxoplasma gondii*, *Trypanosoma cruzi*, *Leishmania* ssp, *Trypanosoma*
brucei, *Trichinella spiralis*, *Toxocara canis*, *Necator americanus*, *Trichuris trichura*,
Enterobius vermicularis, *Dipylidium caninum*, *Entamoeba histolytica*, *Dracunculus*
medinensis, *Wuchereria bancrofti*, *Brugia malayi*, *Brugia timori*, *Strongyloides*
30 *stercoralis*, *Ascaris lumbricoides*, *Onchocerca volvulus*, *Naegleria fowleri*, *Clonorchis*
sinensis, *Cryptosporidium parvum*, *Leishmania* spp), or also a gene or a sequence
including an antibiotic resistance gene, a gene associated with virulence or toxigenicity,
a molecular marker, a single-nucleotide polymorphism, an insect gene, a bee disease

agent gene, a plant gene, a plant disease agent, a molecular marker associated with a cell having a pathogenic or carcinogenic condition, a mitochondrial nucleotide sequence, a plasmid sequence, a messenger RNA, a ribosomal RNA, or a panel of target nucleic acids, and the like, as may be interesting or useful. And may be used in
5 molecular diagnosis of infectious agents in a mammal or vertebrate, including livestock, veterinary and aquaculture applications broadly. And also diagnostic applications in plants, animals or insects suffering more generally from a pathogenic condition, for example, infectious or otherwise. Immunological and biochemical assays employing cartridge devices having the features of the invention are also conceived and claimed
10 for diagnostic use.

While the above is a description of the preferred embodiments of the present invention, it is possible to use various alternatives, modifications, combinations, and equivalents. Therefore, the scope of the present invention should be determined not
15 with reference to the above description but should, instead, be determined with reference to the appended claims, along with their full scope of equivalents. The appended claims are not to be interpreted as including means-plus-function limitations, unless such a limitation is explicitly recited in a given claim using the phrase "means for."

20 All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent literature and publications referred to in this specification and/or cited in accompanying submissions, are incorporated herein by reference, in their entirety. Aspects of the embodiments may be modified, if necessary to employ concepts of the various patents, applications and
25 publications to provide yet further embodiments. These and other changes may be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents
30 to which such claims are entitled. Accordingly, the claims are not limited by the specifics of the disclosure.

CLAIMS

What is claimed is:

1. A microfluidic cartridge for assay of a target analyte or analytes in a biological sample, which comprises:

- a) a plastic, thermally insulative cartridge body enclosing:
 - i) a hydraulic works comprising a sample inlet, one or more on-board liquid or dry reagents for an assay, and a wettable downstream microfluidic subcircuit with channels and chambers fluidly connected to said upstream inlet and vented at a downstream vent; and
 - ii) a pneumatic works comprising an inlet or inlets for receiving pneumatic pressure and a pneumatic subcircuit with channels and chambers for conveying said pneumatic pressure therefrom; and
- b) at least one pneumohydraulic diaphragm configured for interfacing a hydraulic chamber of said wettable microfluidic subcircuit with a pneumatic chamber of said pneumatic subcircuit, said pneumohydraulic diaphragm having a first side facing said hydraulic chamber and a second side facing said pneumatic chamber;

characterized in that

said pneumohydraulic diaphragm is selected from:

- A) an elastic, energy-storing pneumohydraulic diaphragm configured for passively storing a liquid volume under a hydraulic pressure and releasing said liquid volume during wetout of a downstream channel or chamber of said wettable microfluidic subcircuit;
- B) a duplexedly layered pneumohydraulic diaphragm having a liquid center for storing and releasing a liquid reagent;
- C) a pneumohydraulic diaphragm configured for eliminating headspace from a hydraulic chamber during wetout; or
- D) a pair of pneumohydraulic diaphragms comprising a first pneumohydraulic diaphragm interfacing a first hydraulic chamber with valved inlet and

a second pneumohydraulic diaphragm interfacing a second hydraulic chamber with valved outlet, and an elevated intercommunicating channel between said first and second hydraulic chambers, wherein said pair is configured for reciprocally exchanging fluid through said channel by applying opposing pressure differentials across said first and second pneumohydraulic diaphragms; and

wherein said hydraulic chambers and diaphragms are configured for preventing or reducing bubble entrainment or reagent washout during wetout, fill, pumping or rehydration steps of an assay.

2. The microfluidic cartridge of claim 1, wherein said hydraulic works is configured for operation when mounted at a tilt angle θ of 10 – 35 degrees relative to the ground plane on a tilted stage of a host instrument and at least one hydraulic chamber is configured with an outlet and intercommunicating channel positioned superiorly relative to said chamber for venting a gas or discharging a bubble from said chamber.

3. The microfluidic cartridge of claim 1 or claim 2, wherein said hydraulic works comprises a plurality of wettable downstream microfluidic subcircuits with channels and chambers fluidly connected to said upstream inlet and vented at one or more downstream vents, wherein said plurality of wettable microfluidic subcircuits are configured for performing each an assay in parallel, and each said wettable microfluidic subcircuit is provided with a separate detection chamber.

4. The microfluidic cartridge of claim 1, wherein a hydraulic pressure and liquid volume of said elastic, pneumohydraulic diaphragm in a distended state is passively converted by opening a downstream valve to a work of advancing a meniscus in a wettable downstream microfluidic subcircuit fluidly connected thereto and thereby displacing any gas to said downstream vent.

5. The microfluidic cartridge of claim 4, wherein said liquid volume and hydraulic pressure of said elastic, passively-operated pneumohydraulic diaphragm is calibrated to fill said downstream microfluidic subcircuit to a mark.

6. The microfluidic cartridge of claim 5, wherein a plurality of chambers of downstream wettable microfluidic subcircuits are configured each with an elastic, passively-operated pneumohydraulic diaphragm for filling a downstream wettable microfluidic subcircuit equally to a mark, wherein each said subcircuit is configured for performing each an assay in parallel, and each said subcircuit is provided with a separate detection chamber.

7. The microfluidic cartridge of claim 1, wherein said pneumohydraulic diaphragm is a polyurethane diaphragm, a polyvinylidene chloride diaphragm, a polyolefin diaphragm, a polyester diaphragm, a polyethylene diaphragm, a polyethylene terephthalate diaphragm, a nylon diaphragm, or a laminated or co-extruded combination thereof, and optionally includes a metallized film layer.

8. The microfluidic cartridge of the preceding claims,
further characterized by

a) a staging manifold having a plurality of chambers, wherein each said chamber of said plurality of chambers is separated into a hydraulic chamber and a pneumatic chamber by an elastic, energy-storing pneumohydraulic diaphragm sealedly mounted therebetween, such that a liquid volume admitted through an inlet into each said hydraulic chamber in series or in parallel distends each said energy-storing pneumohydraulic diaphragm according to an isobaric pressure proportionate to the displacement volume thereof;

b) said inlet is valvedly closeable for equilibrating said hydraulic pressure throughout said staging manifold after filling is complete; and

c) a plurality of vented downstream channels in parallel, wherein one said channel of said plurality of channels is in fluidic communication with each said hydraulic chamber of said staging manifold, each said vented downstream

channel having a valve for closing during filling and pressurization and for opening during draining and depressurization, whereby said hydraulic pressure of said elastic, pneumohydraulic diaphragm in a distended state is passively converted to the work of advancing a meniscus during initial wetout of said plurality of vented downstream channels in parallel.

9. The microfluidic cartridge of claim 8, wherein each said downstream vented channel is fluidly configured as an inlet to a microfluidic subcircuit, and wherein each said elastic, energy-storing pneumohydraulic diaphragm is adapted for splitting a liquid volume equally between each said downstream microfluidic subcircuit.

10. The microfluidic cartridge of claim 9, wherein said each microfluidic subcircuit comprises at least one reaction chamber with provision for mixing a liquid reagent, a dry reagent, or a combination thereof, with a liquid sample, and at least one detection chamber configured for interfacing with a detection means for detecting a target analyte or analytes.

11. The microfluidic cartridge of the preceding claims, wherein said pneumatic chamber is vented to atmosphere.

12. The microfluidic cartridge of the preceding claims, which comprises an on-board reagent reservoir for dispensing a liquid volume into a microfluidic subcircuit of said hydraulic works; wherein said reagent reservoir is

characterized by

a) a duplexedly layered diaphragm sealedly separating a pneumatic chamber of said pneumatic works and a hydraulic chamber of said hydraulic works, said duplexedly layered diaphragm having a first side facing said pneumatic works and a second side facing said hydraulic works, a first layer forming said first side thereof, and a second layer forming said second side thereof, said first and second layers enclosing said liquid volume as a liquid center therebetween;

b) a fluid outlet for receiving and conveying said liquid volume to said downstream microfluidic subcircuit; and

c) a sharp disposed in said hydraulic chamber, said sharp for rupturing said second layer and for releasing said liquid volume into said hydraulic works when said duplexedly layered diaphragm is piercingly urged into contact with sharp by application of a pressure differential across said diaphragm.

13. The microfluidic cartridge of claim 12, wherein said first layer of said duplexedly layered diaphragm is rupture resistant and said second layer is rupture sensitive.

14. The microfluidic cartridge of claim 13, wherein said first layer is a laminated polymer with outer nylon base layer configured to be puncture resistant and said second layer is a laminated polymer with outer polyethylene terephthalate member configured to be puncture susceptible.

15. The microfluidic cartridge of claim 12, wherein said on-board reagent reservoir is configured for releasing serial liquid volumes by the action of serial pulses of pneumatic pressure applied thereto.

16. The microfluidic cartridge of claims 12 through 15, wherein said liquid volume comprises a liquid reactant, a buffer, a rehydrating fluid, or a diluent, said liquid volume for an assay step selected from rehydrating a dry reagent disposed in a downstream chamber or channel, for rinsing a solid phase, for eluting a target analyte or analytes from a solid phase substrate, for making a dilution, for performing a chromatographic separation, for actuating or stopping a reaction, or for detecting said target analyte or analytes and optionally wherein said liquid volume is degassed and said duplexedly layered diaphragm is gas impervious.

17. The microfluidic cartridge of the preceding claims, which comprises at least one microfluidic subcircuit with hydraulic chamber formed as a

downstream reaction chamber with upstream inlet and downstream vent, said downstream reaction chamber containing a dried reagent spot or spots,

further characterized in that

said pneumohydraulic diaphragm is configured to operate with a first position wherein the diaphragm is distended against the floor of the chamber so as to displace headspace air and form a protective temporary tent around and over the reagent spot or spots during wetout, and a second position wherein the diaphragm is relaxedly positioned or aspirated against the roof of the chamber so as to fill the chamber with the liquid volume and uncover and dissolve the reagent spot at full strength without bubble entrainment or reagent washout.

18. The method of claim 17, wherein said dried reagent spot is a buffer, an enzyme, a co-enzyme, a co-factor, a polymerase, a primer, a molecular beacon, a probe, a fluorophore, a dehydrogenase, an oxidase, a reactant, a chromogen, a substrate, an antibody, an antigen, or a control.

19. The microfluidic cartridge of the preceding claims for performing PCR, wherein said microfluidic cartridge further comprises:

a first pneumohydraulic diaphragm overlying a first hydraulic chamber and a second pneumohydraulic diaphragm overlying a second hydraulic chamber, said first and second hydraulic chambers having a fluidically interconnecting channel;

a thermal interface for two-zone PCR thermocycling, with first thermal interface of said first hydraulic chamber configured for apposing a first heating element and second thermal interface of said second hydraulic chamber configured for apposing a second heating element;

wherein said first pneumohydraulic diaphragm is configured with a pneumatic means for driving reciprocal fluid flow between said first and second hydraulic chambers during PCR amplification,

characterized in that

said interconnecting channel is configured to be operated at a tilt angle θ of 10 – 35 degrees so as to reduce interference from bubbles.

20. The microfluidic cartridge of claim 19, wherein said second pneumohydraulic diaphragms is an elastomeric diaphragm and is worked passively by the urging of said first pneumohydraulic diaphragm.

21. The microfluidic cartridge of the preceding claims, further comprising a detection chamber enclosed on a first opposite side by an optical window and on a second opposite side by a thermo-optical window;

characterized in that

said detection chamber is configured to be operated at a tilt angle θ of 10 – 35 degrees so as to flush air and bubbles to a vented port superiorly disposed thereon.

22. A kit including one or more disposable microfluidic cartridges of the preceding claims for use as a consumable in a host instrument, said microfluidic cartridge with on-board reagents for performing at least one assay for a nucleic acid, a protein, an antigen, an antibody, a metabolite, or an enzyme, optionally in a gas-tight package with inert atmosphere therein.

23. A kit including one or more microfluidic cartridges of the preceding claims for use as a consumable in a host instrument, said microfluidic cartridge with on-board reagents for performing at least one nucleic acid assay, each cartridge packaged in a gas tight sealed pouch, with instructions for use, wherein the user need only place a biological liquid sample to be assayed in a sample inlet port and insert said microfluidic cartridge into a said host instrument, said cartridge having all primers, enzyme-cofactors, salts, buffers, polymerase, and detection chemistries for testing said liquid sample for a target nucleic acid (either DNA or RNA) associated with a bacterium (including *Acinetobacter baumannii*, *Actinobacillus equuli*, *Bacillus anthracis*, *Brucella melitensis*, *Brucella abortus*, *Bordetella pertussis*, *Bordetella bronchioseptica*, *Burkholderia pseudomallei*, *Corynebacterium diphtheriae*, *Coxiella burnetii*, *Eikenella corrodens*, *Escherichia coli*, *Francisella tularensis*, *Francisella*

novicida, *Fusobacterium necrophorum*, *Haemophilus influenzae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Kingella denitrificans*, *Legionella pneumophila*, *Listeria monocytogenes*, *Moraxella catarrhalis*, *Mycobacterium tuberculosis*, *Mycoplasma pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pasteurella multocida*, *Proteus vulgaris*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Pseudomonas putrefaciens*, *Pseudomonas cepacia*, *Salmonella typhi*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Treponema pallidum*, *Yersinia pestis*, or *Vibrio cholera*), a *Rickettsia* (including *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Rickettsia prowazekii*, or *Rickettsia typhi*), a virus (including Measles virus, HIV virus, Hepatitis C virus, Hepatitis B virus, Dengue Virus, Western Equine Encephalitis virus, Eastern Equine Encephalitis virus, Venezuelan Equine Encephalitis virus, Enteroviruses, Influenza virus, bird flu, Coronavirus, SARS Coronavirus, Polio virus, Adenovirus, Parainfluenza virus, Hanta virus, Rabies virus, Argentine Hemorrhagic Fever virus, Machupo virus, Sabia virus, Guanarito virus, Congo-Crimean Hemorrhagic Fever virus, Lassa Hemorrhagic Fever virus, Marburg virus, Ebola virus, Rift Valley Fever virus, Kyasanur Forest Disease virus, Omsk Hemorrhagic Fever, Yellow Fever virus, Smallpox virus, a retrovirus, Monkeypox virus, and foot and mouth disease virus), a fungal agent (including *Coccidioides immitis*, *Candida albicans*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Sporothrix schenki*, or *Aspergillus fumigates*), a parasitic agent (including *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *Toxoplasma gondii*, *Plasmodium bergeri*, *Schistosoma mansoni*, *Schistosoma hematobium*, *Schistosoma japonicum*, *Entamoeba histolytica*, *Babesia*, *Toxoplasma gondii*, *Trypanosoma cruzi*, *Leishmania* spp, *Trypanosoma brucei*, *Trichinella spiralis*, *Toxocara canis*, *Necator americanus*, *Trichuris trichura*, *Enterobius vermicularis*, *Dipylidium caninum*, *Entamoeba histolytica*, *Dracunculus medinensis*, *Wuchereria bancrofti*, *Brugia malai*, *Brugia timori*, *Strongyloides stercoralis*, *Ascaris lumbricoides*, *Onchocerca volvulus*, *Naegleria fowleri*, *Clonorchis sinensis*, *Cryptosporidium parvum*, *Leishmania* spp), an antibiotic resistance gene, a gene associated with virulence or toxigenicity, a molecular marker, a single-nucleotide polymorphism, an insect gene, a bee disease agent gene, a plant gene, a plant disease

agent, a molecular marker associated with a cell having a pathogenic or carcinogenic condition, a mitochondrial nucleotide sequence, a plasmid sequence, a messenger RNA, a ribosomal RNA, or a panel of target nucleic acids, and so forth.

24. A method for wetout of a microfluidic cartridge while limiting bubble entrainment therein, which comprises:

- a) pumping a liquid volume through an inlet and into a plurality of hydraulic chambers of a microfluidic card so that an elastic pneumohydraulic diaphragm overlying said liquid volume in each said hydraulic chamber is stretchedly distended, thereby isobarically pressurizing said liquid volume in said plurality of hydraulic chambers;
- b) valvedly opening an outlet from each said hydraulic chamber, each said outlet with fluidic connection to a vented downstream microfluidic subcircuit; and
- c) splitting said liquid volume substantially in equal measure into each said wettable downstream microfluidic subcircuit.

25. A method for wetout of a microfluidic cartridge which contains dried reagent spots, while limiting bubble entrainment therein, which comprises:

- a) pumping a liquid volume through an inlet and into a plurality of hydraulic chambers of a microfluidic card so that an elastic pneumohydraulic diaphragm overlying the liquid volume in each said hydraulic chamber is distended, thereby isobarically pressurizing said liquid volume;
- b) pressurizing a second diaphragm in a plurality of downstream reaction chambers, each said downstream reaction chamber containing a dried reagent spot, the second diaphragm forming a protective temporary tent for sealing around and over the reagent spot and for displacing headspace air from the downstream reaction chamber;
- c) valvedly opening an outlet from each said hydraulic chamber, each said outlet with fluidic connection to one of said plurality of downstream reaction chambers;

d) wetting the downstream reaction chamber around said temporary tent and displacing any residual air from the reaction chamber by allowing the distended elastic pneumohydraulic diaphragm to relax, the liquid volume forming an advancing meniscus;

e) optionally closing a valve downstream from the downstream reaction chamber; and

f) lifting said temporary tent and conveying a remaining part of the liquid volume into each reaction chamber, thereby dissolving the reagent spot at full strength without bubble entrainment or reagent washout.

26. The method of claim 25, wherein said dried reagent spot is a buffer, an enzyme, a co-enzyme, a co-factor, a polymerase, a primer, a molecular beacon, a probe, a fluorophore, a dehydrogenase, a reactant, a chromogen, a substrate, an antibody, an antigen, or a control.

27. A method for assaying a target analyte or analytes, which comprises supplying a microfluidic cartridge having an on-board liquid reagent sealedly enclosed in a compartment that is substantially gas impervious, wherein said liquid reagent is substantially degassed, said cartridge having means for dispensing said liquid reagent into a microfluidic works therein, said degassed liquid reagent preventing or reducing bubble formation associated with mixing, pumping or heating.

28. The devices, cards, cartridges, steps, features, elements, integers, compositions and/or methods disclosed herein or indicated in the specification of this application individually or collectively, and any and all combinations of two or more features of said devices, cards, cartridges, steps, features, elements, integers, compositions and/or methods.

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Fig. 1A

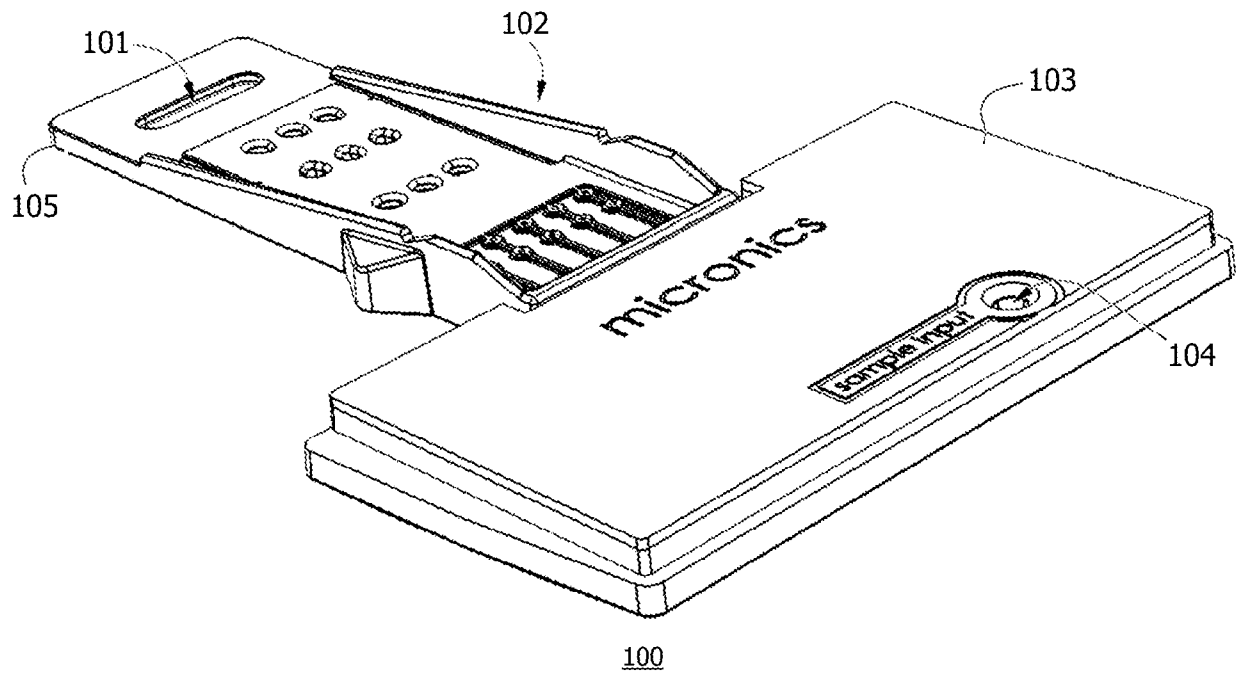


Fig. 1B

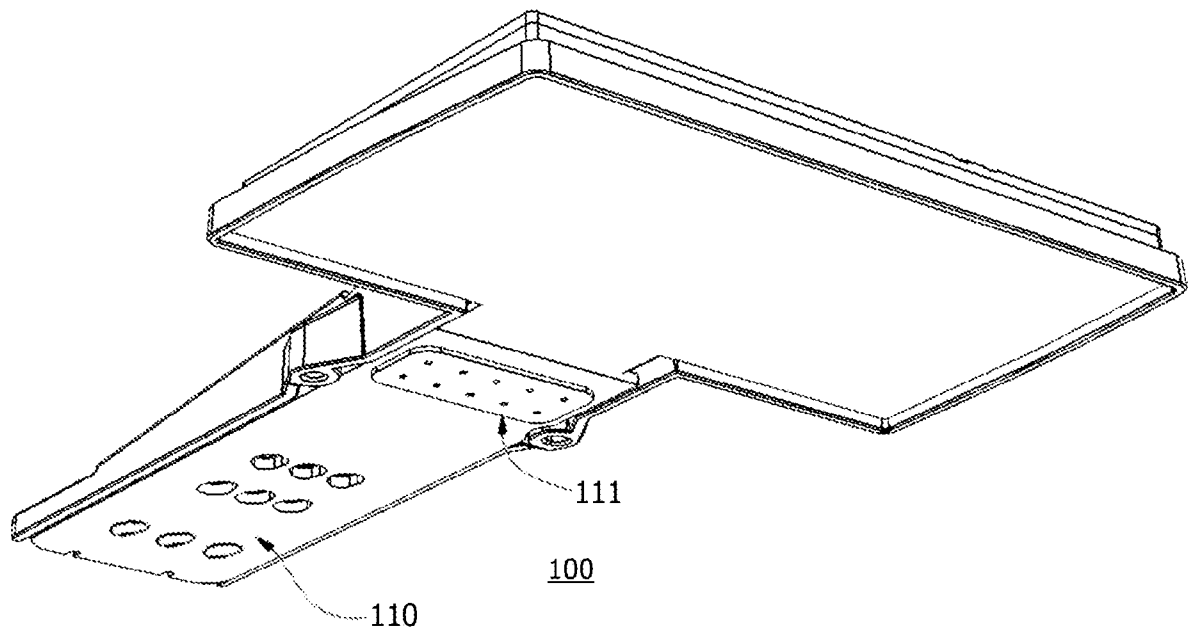
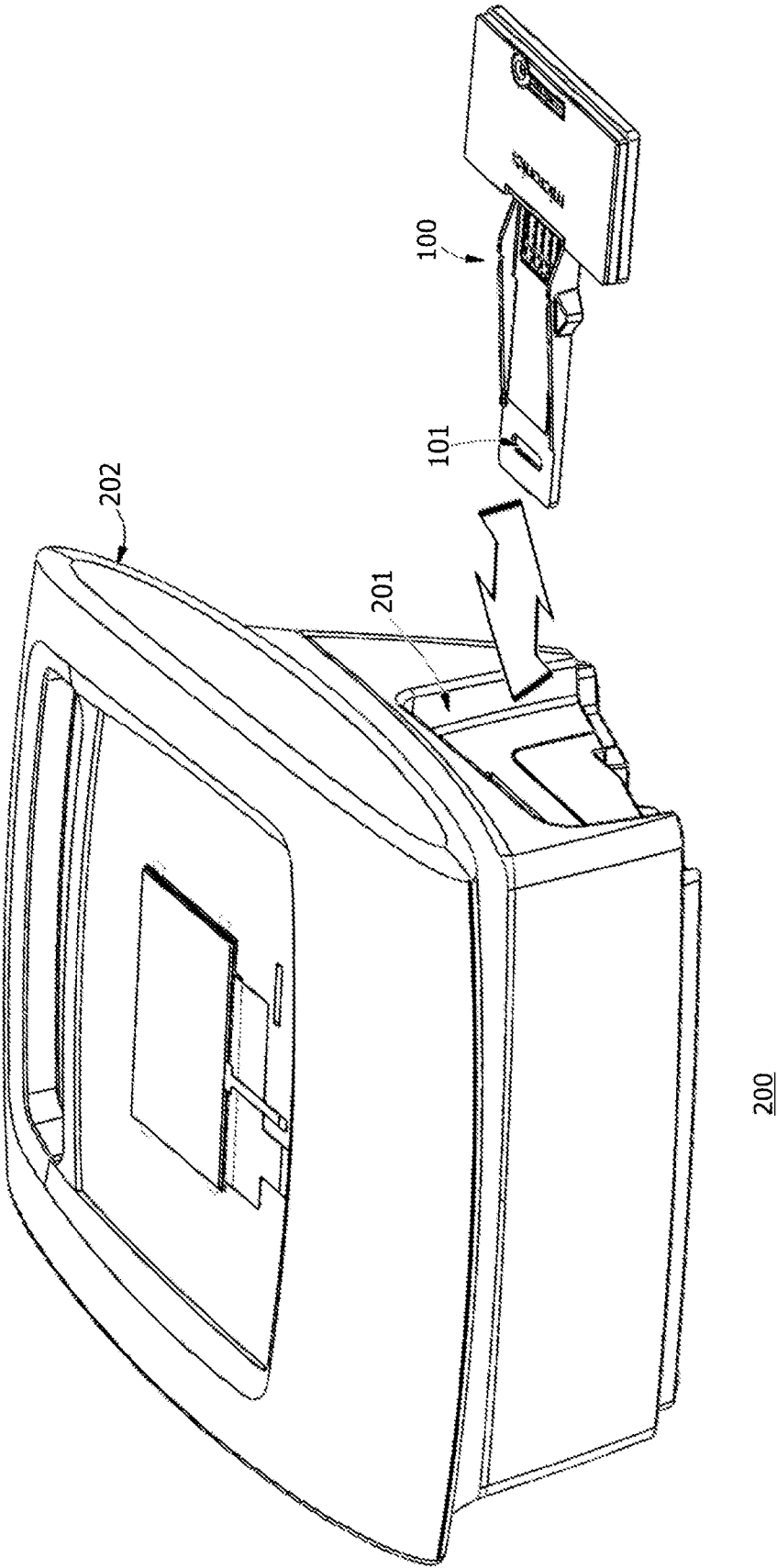
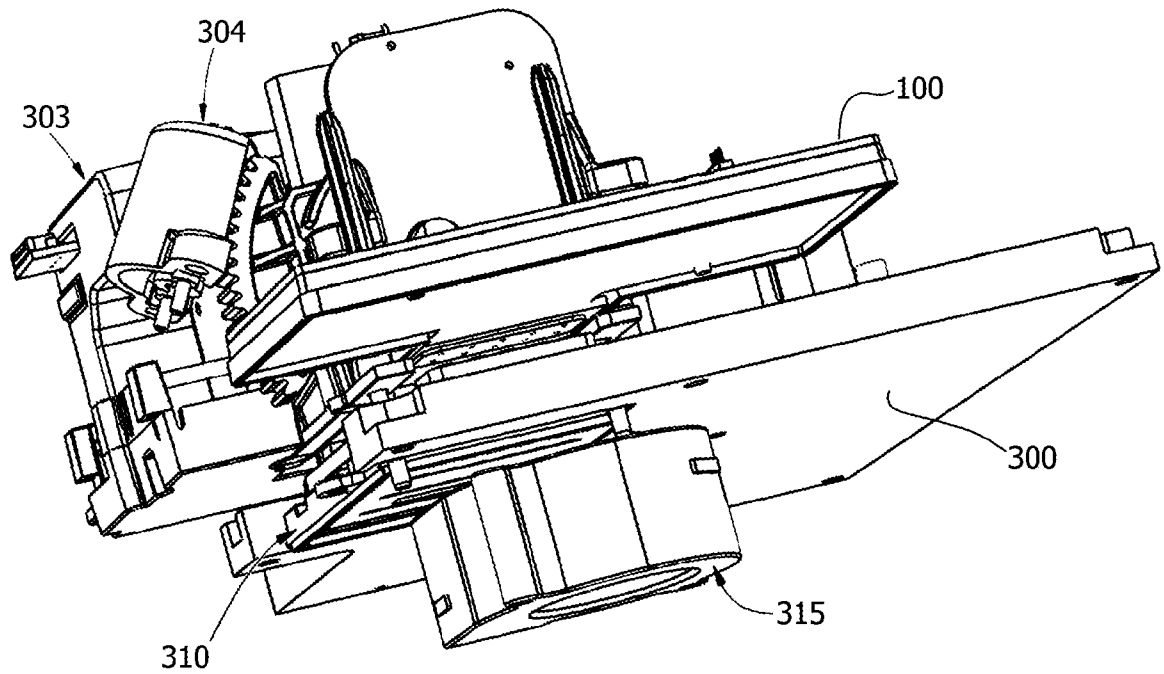
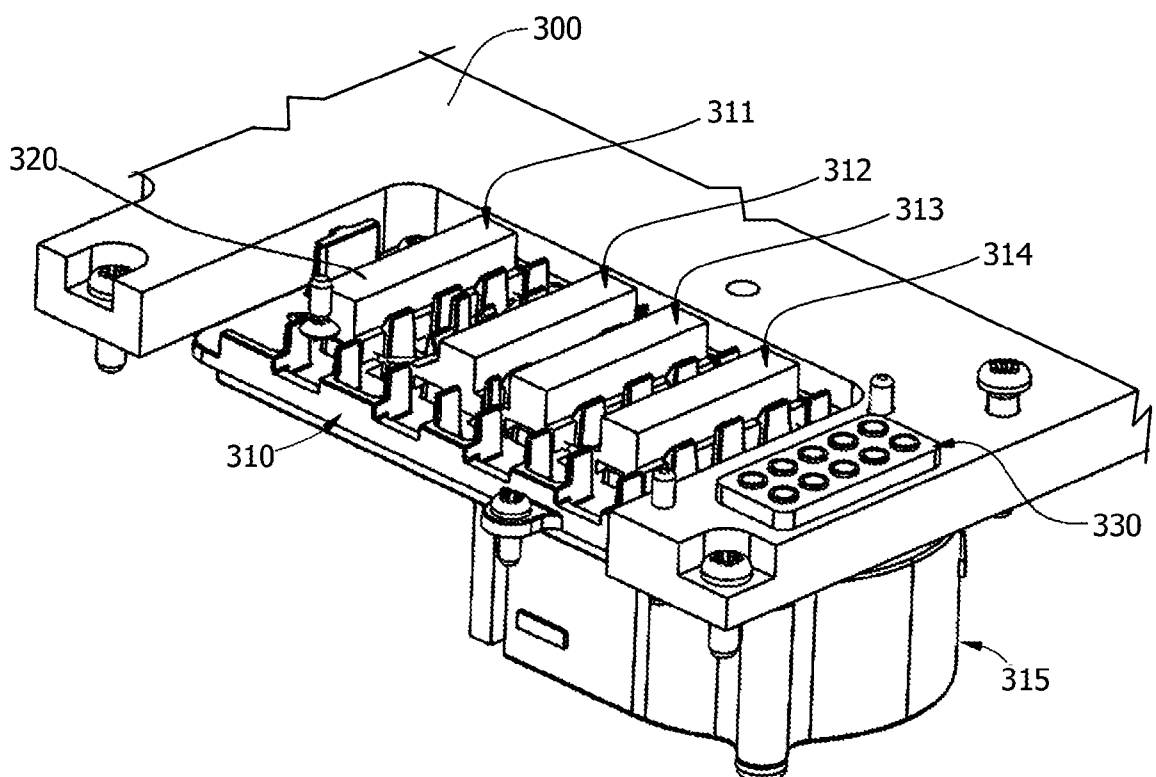


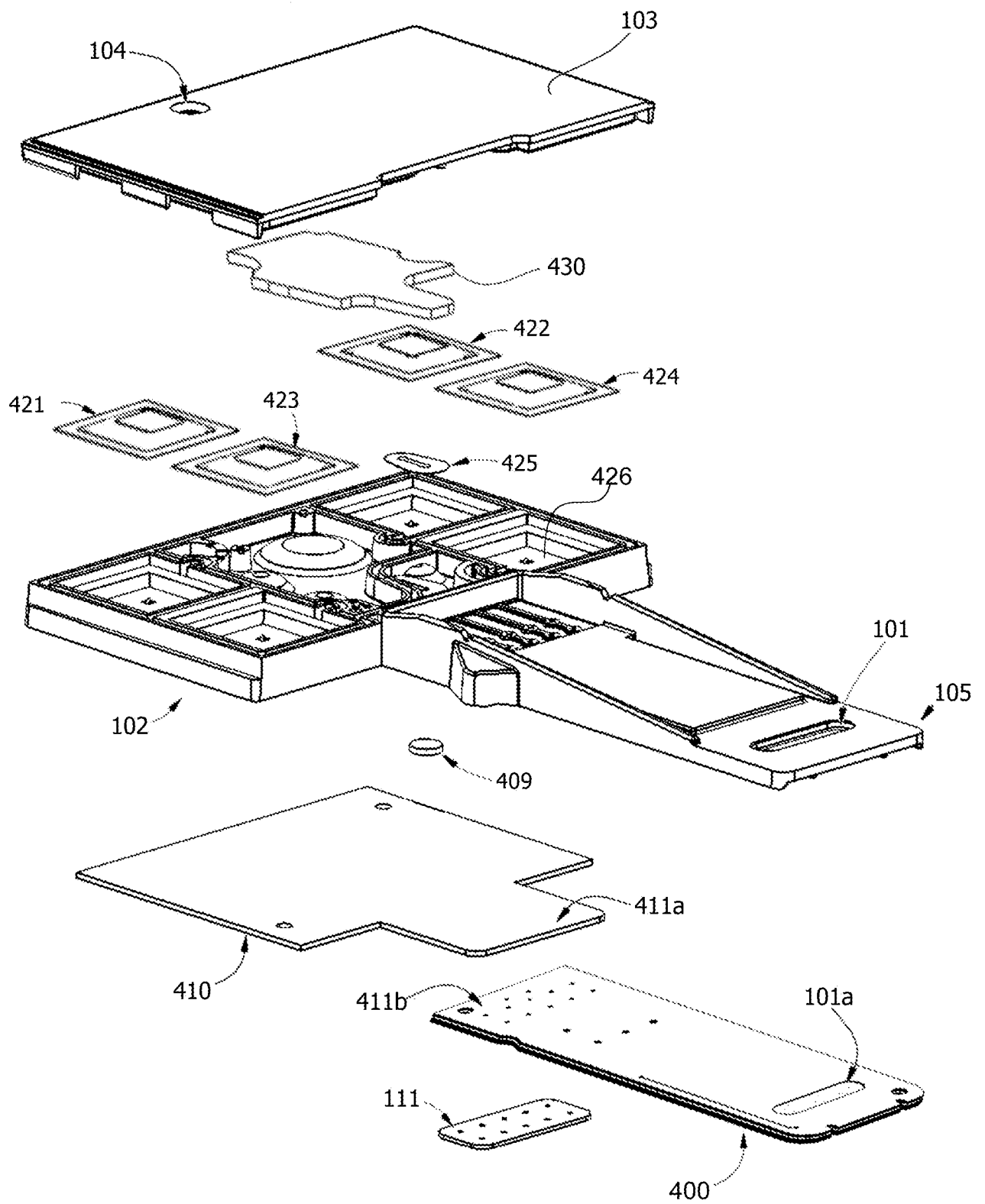
Fig. 2



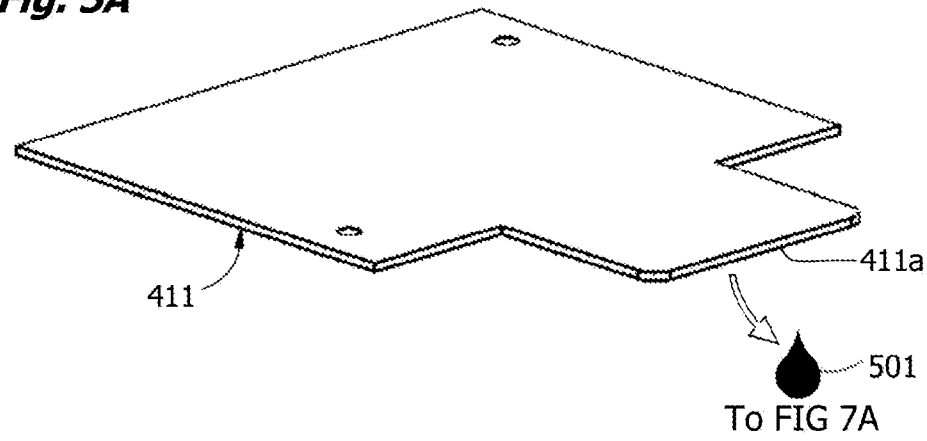
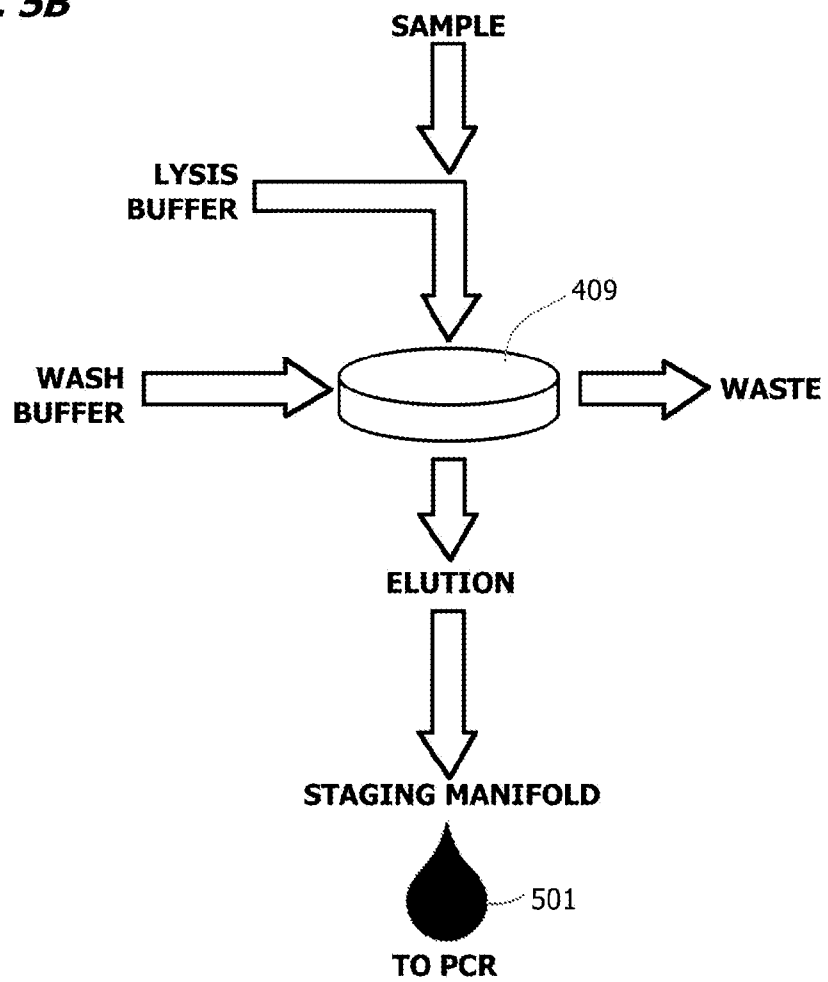
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Fig. 3A**Fig. 3B**

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Fig. 4100

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Fig. 5A**Fig. 5B**

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Fig. 6A

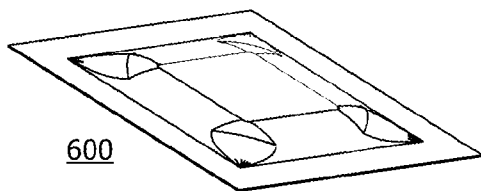


Fig. 6B

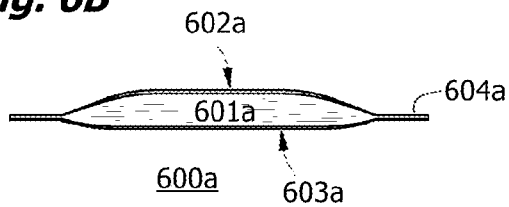


Fig. 6C

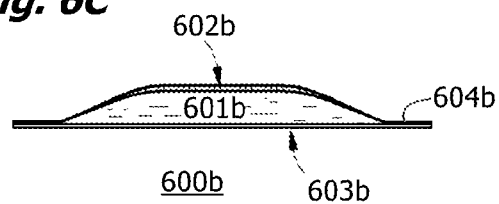


Fig. 7A

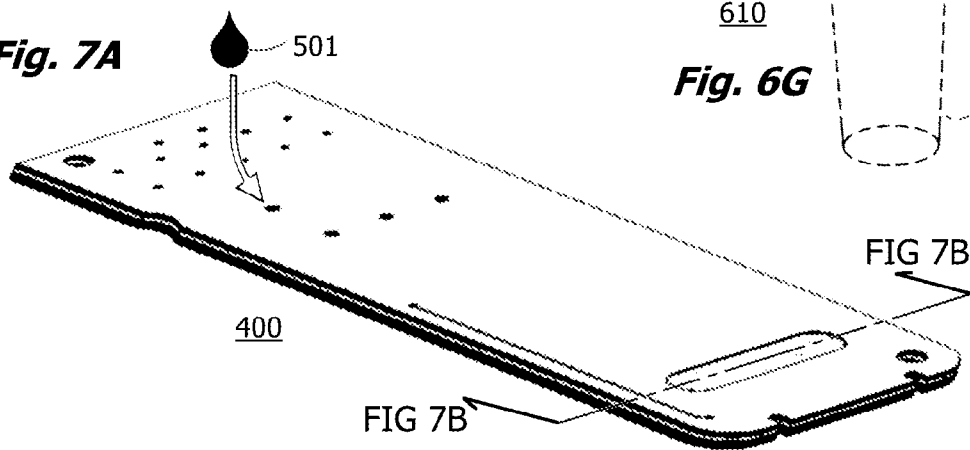


Fig. 7B

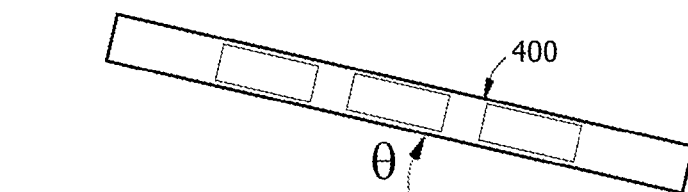


Fig. 6D

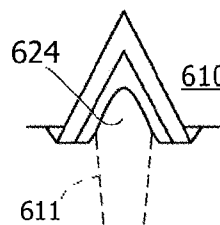
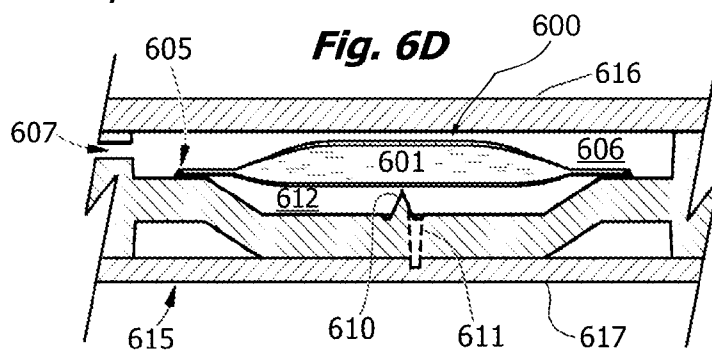


Fig. 6E

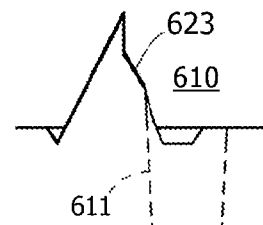


Fig. 6F

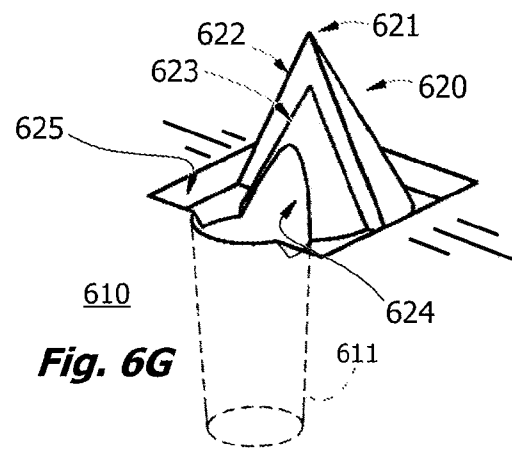
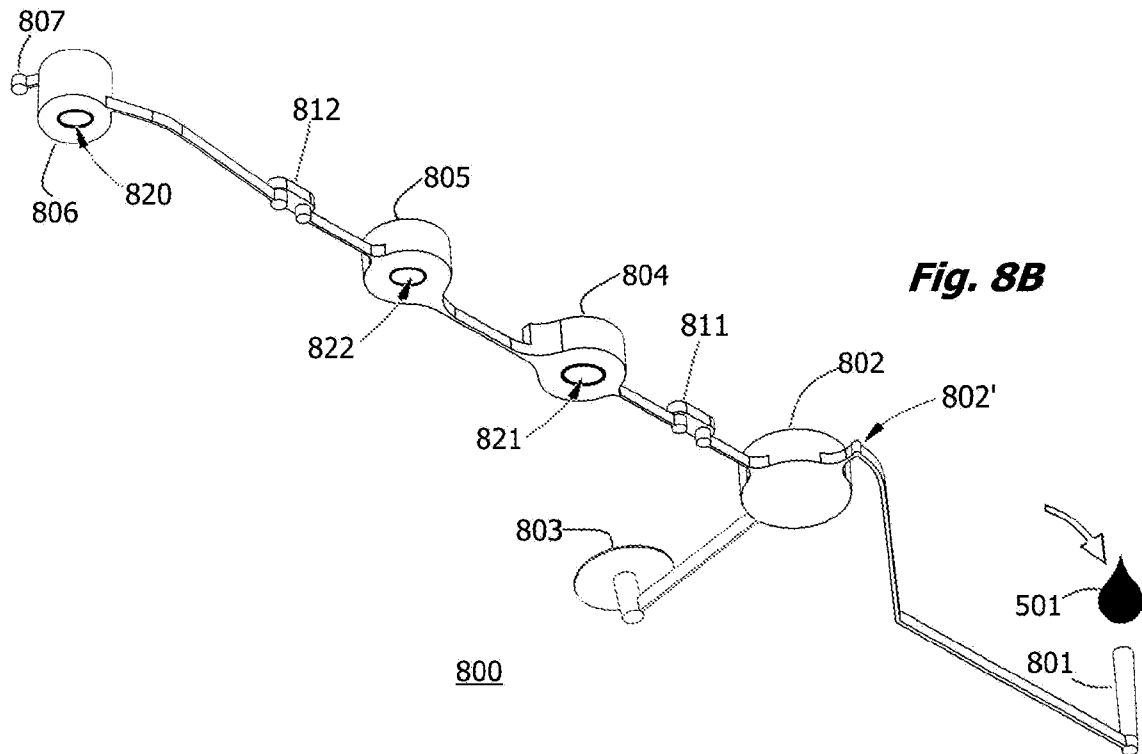
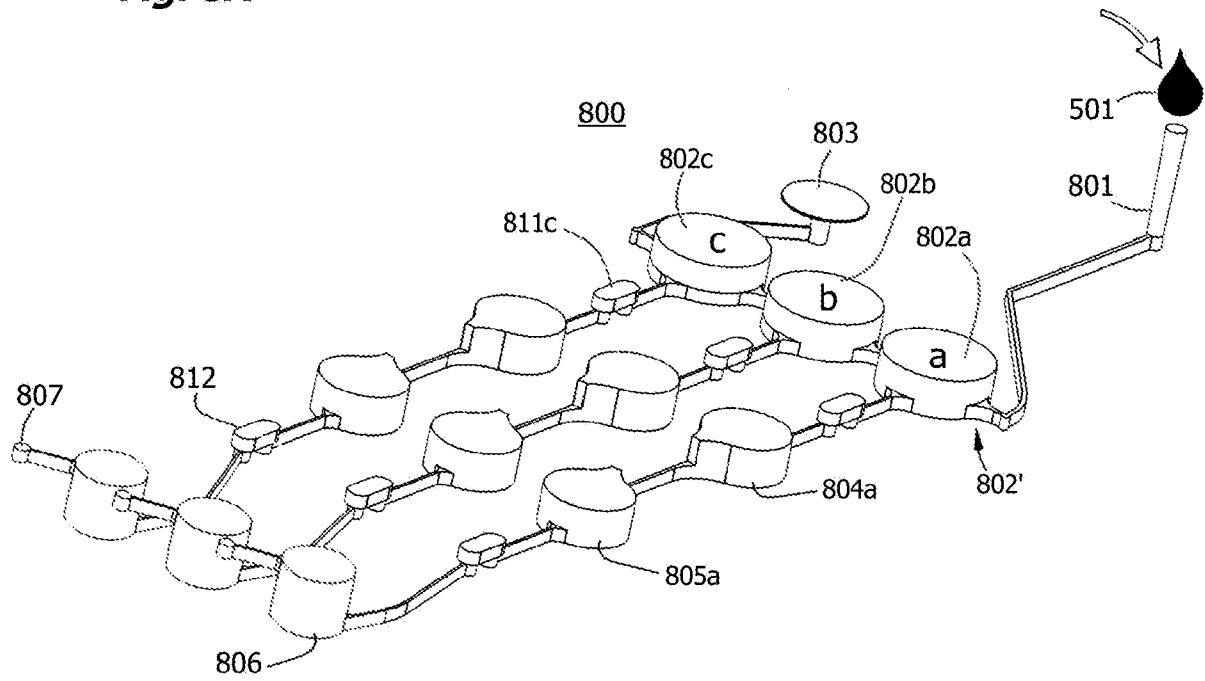


Fig. 6G

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Fig. 8A



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Fig. 8C

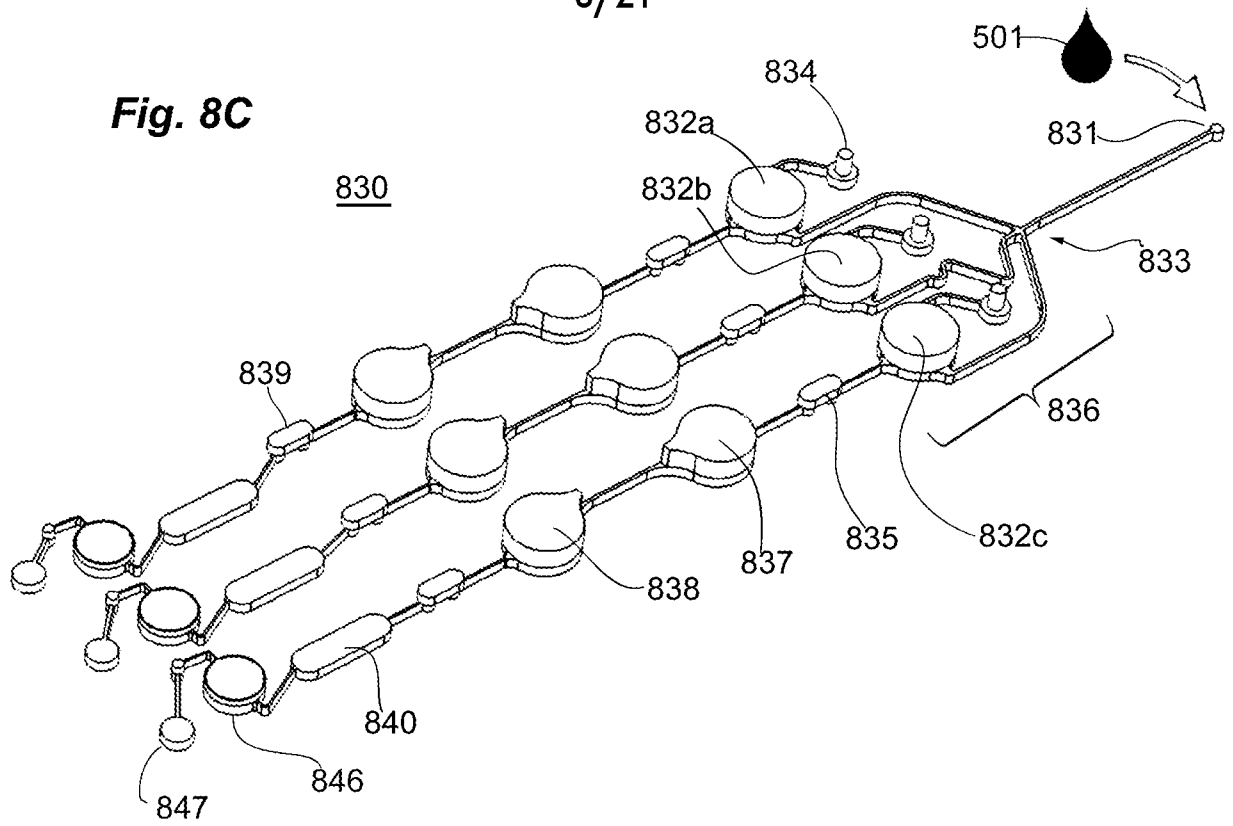
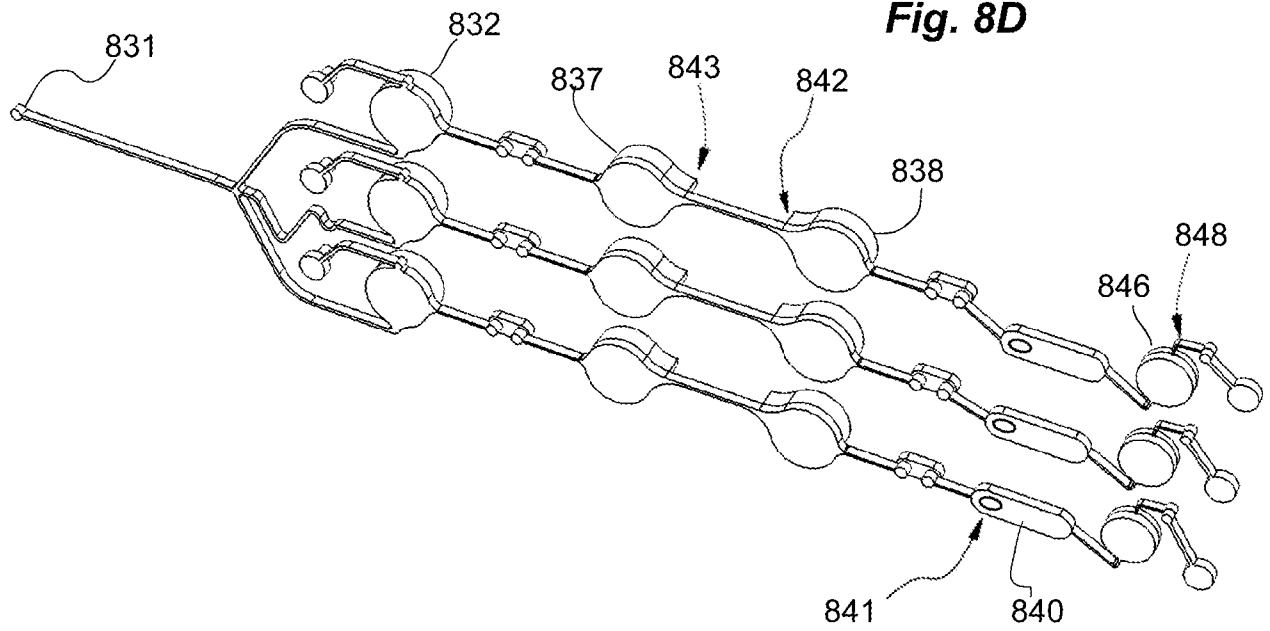


Fig. 8D



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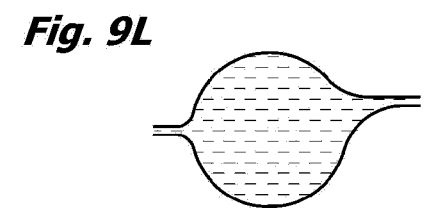
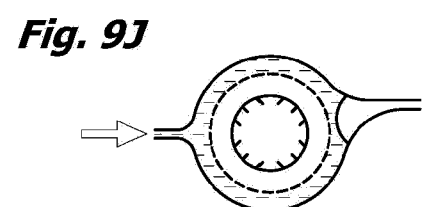
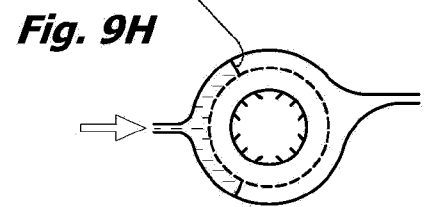
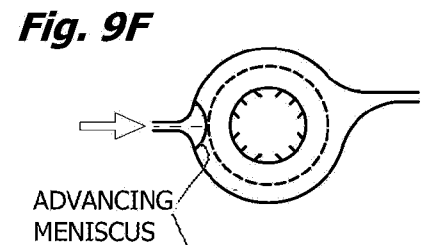
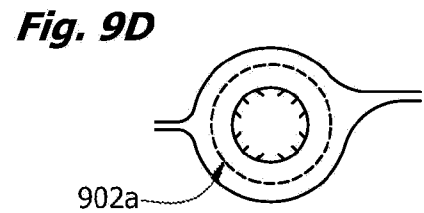
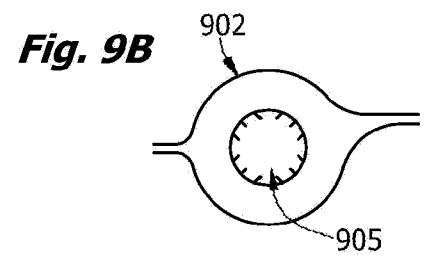
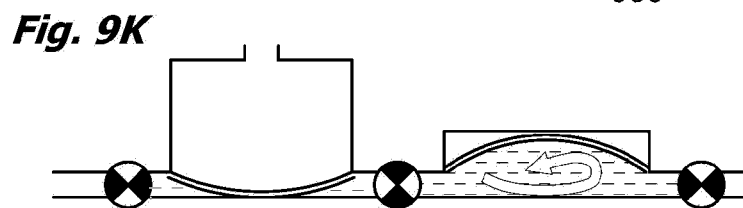
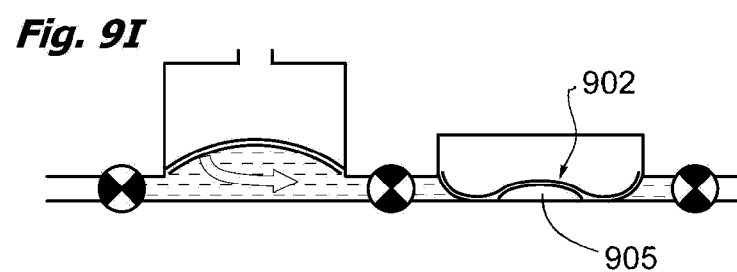
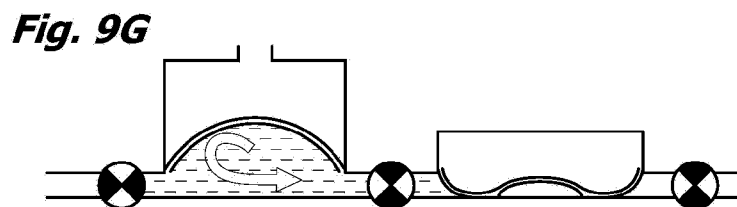
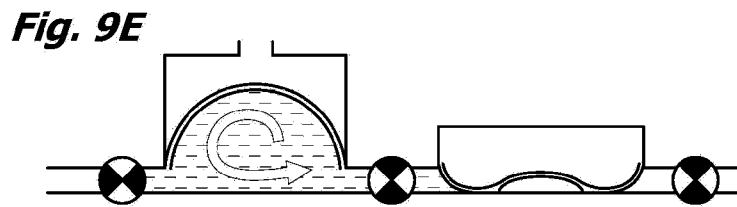
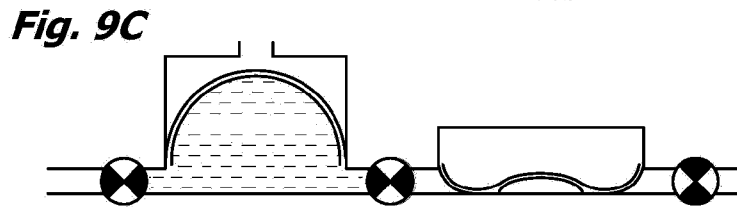
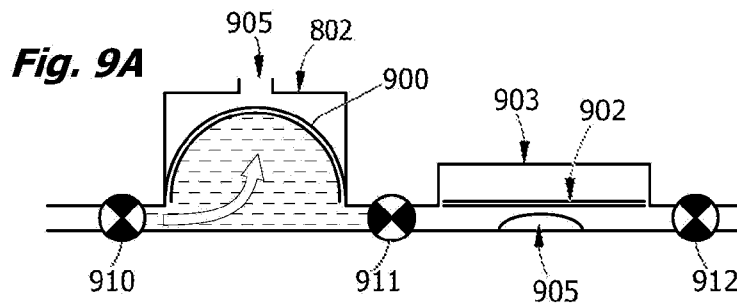


Fig. 10A

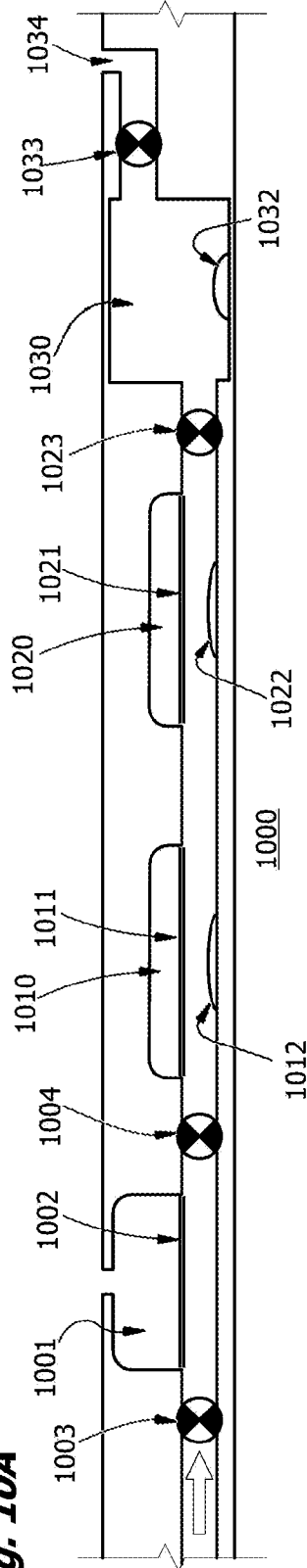


Fig. 10B

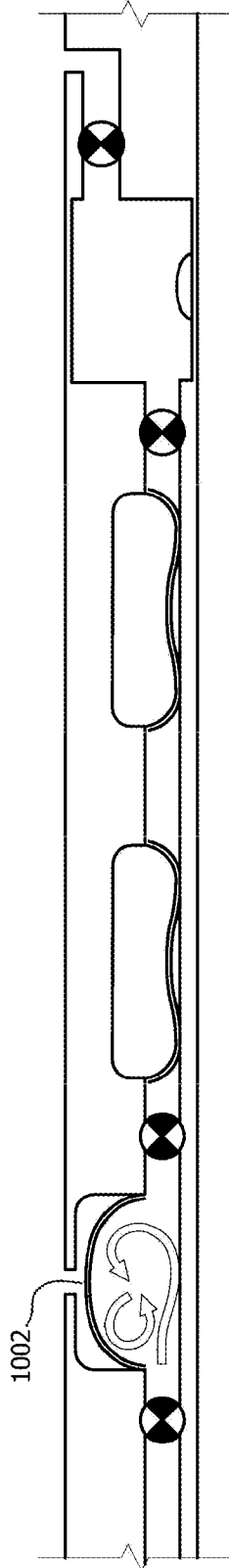
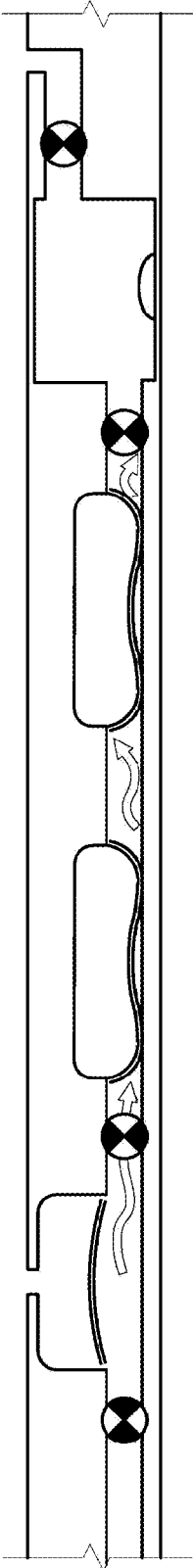
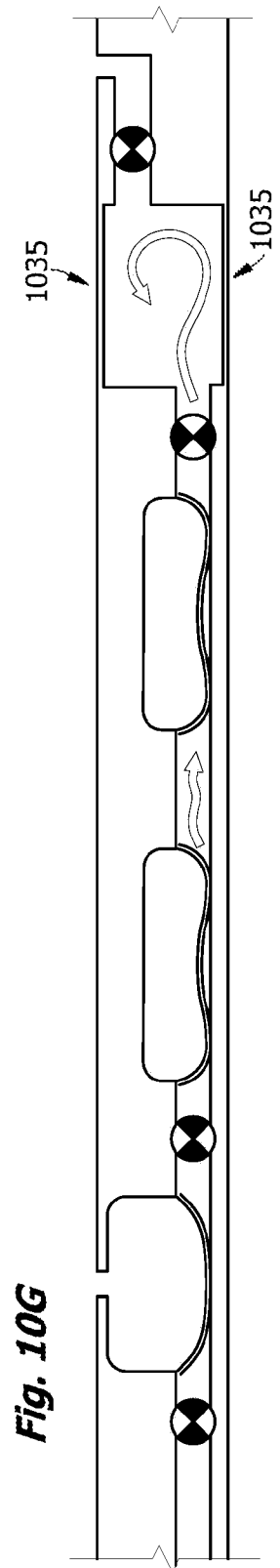
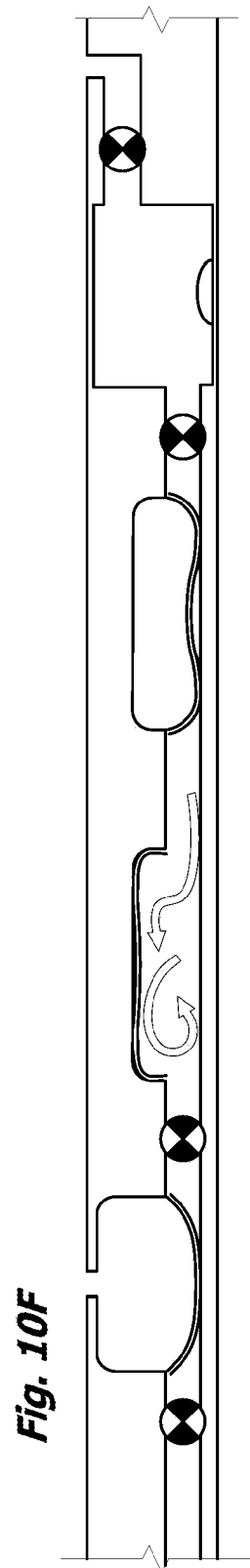
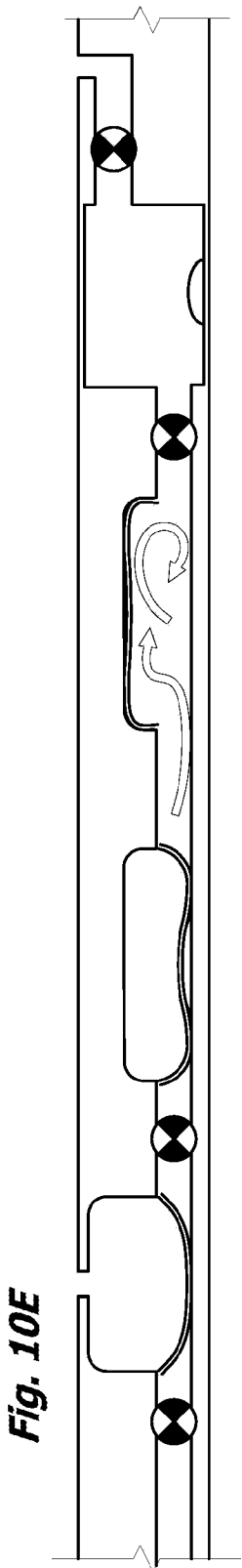
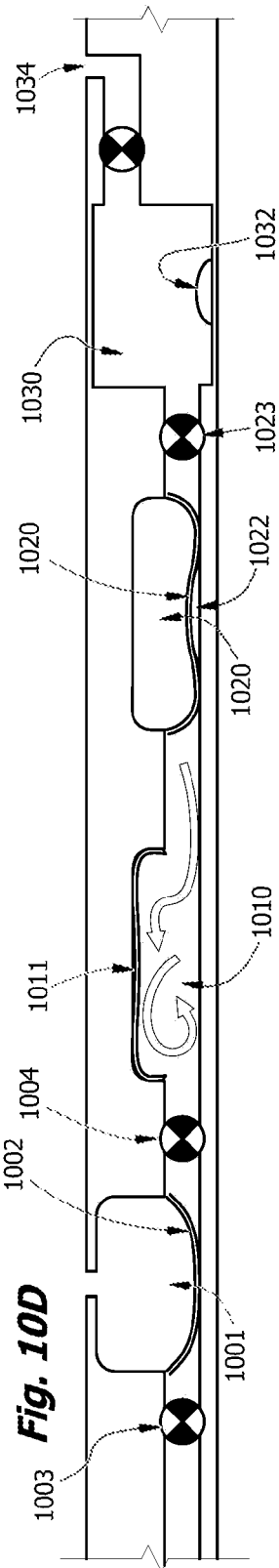


Fig. 10C

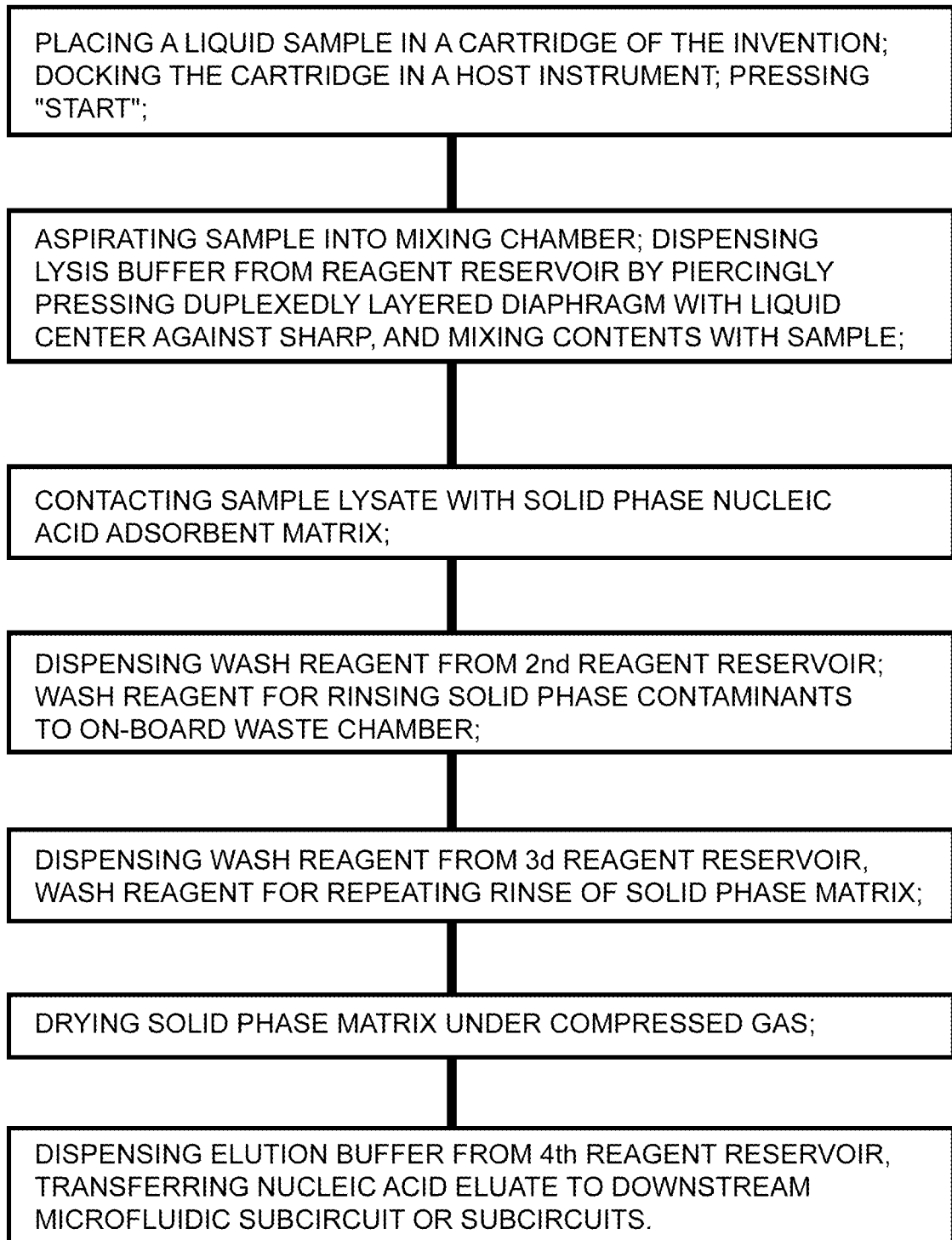




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Fig. 11

METHOD FOR EXTRACTING NUCLEIC ACIDS FROM A BIOSAMPLE

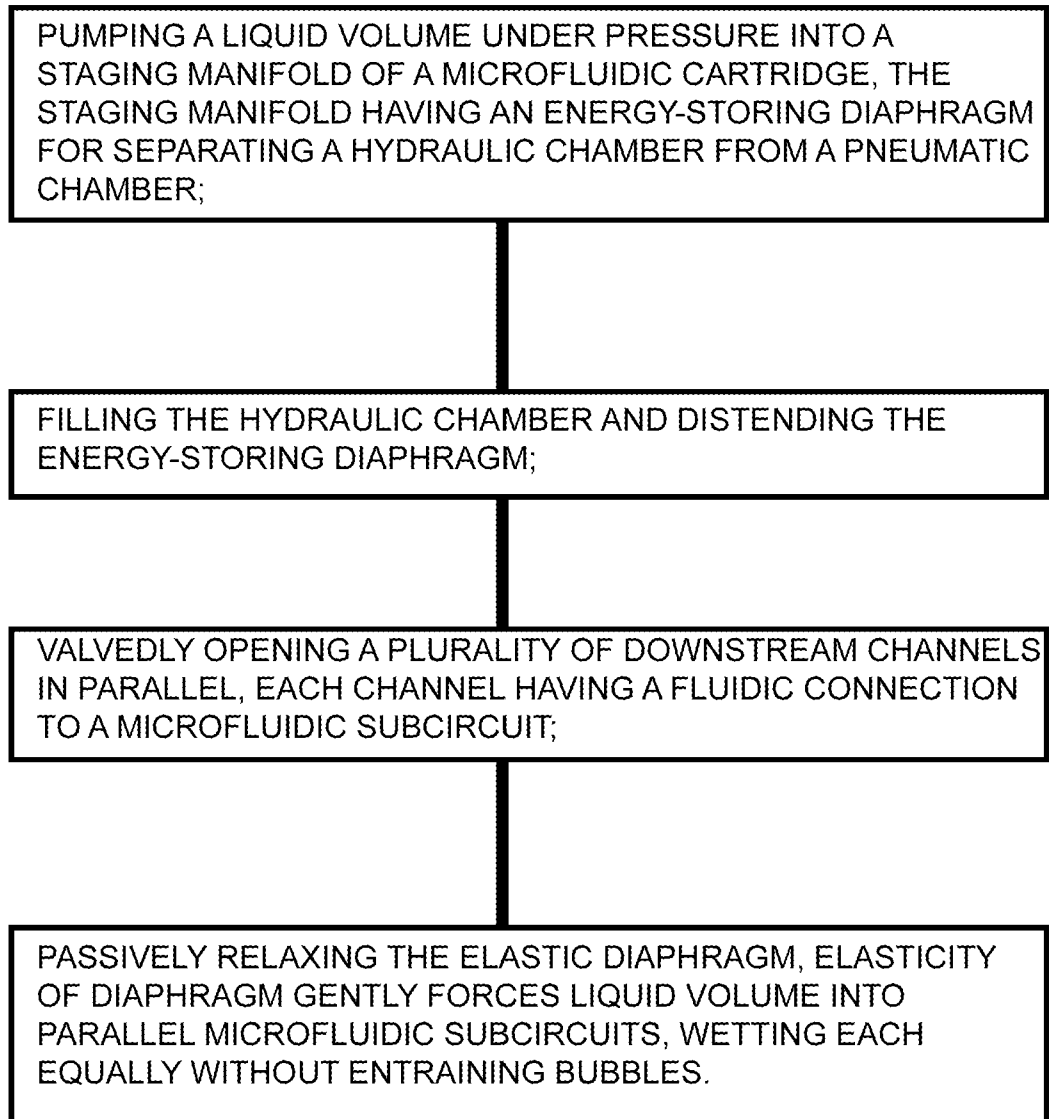


NUCLEIC ACID ASSAY IS AUTOMATICALLY CONTINUED ...

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Fig. 12

FIRST METHOD FOR PRIMING MICROFLUIDIC CHANNELS AND
CHAMBERS WITHOUT BUBBLE ENTRAINMENT



ASSAY CONTINUES

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Fig. 13METHOD FOR REHYDRATING DRIED REAGENTS
WITHOUT BUBBLE ENTRAINMENT

APPLYING A PNEUMATIC PRESSURE ON THE PRESSURIZABLE DIAPHRAGM OF A REAGENT CHAMBER, THEREBY INFLATINGLY TENTING THE PRESSURIZABLE DIAPHRAGM ON TOP OF A DRIED REAGENT SPOT IN THE CHAMBER;

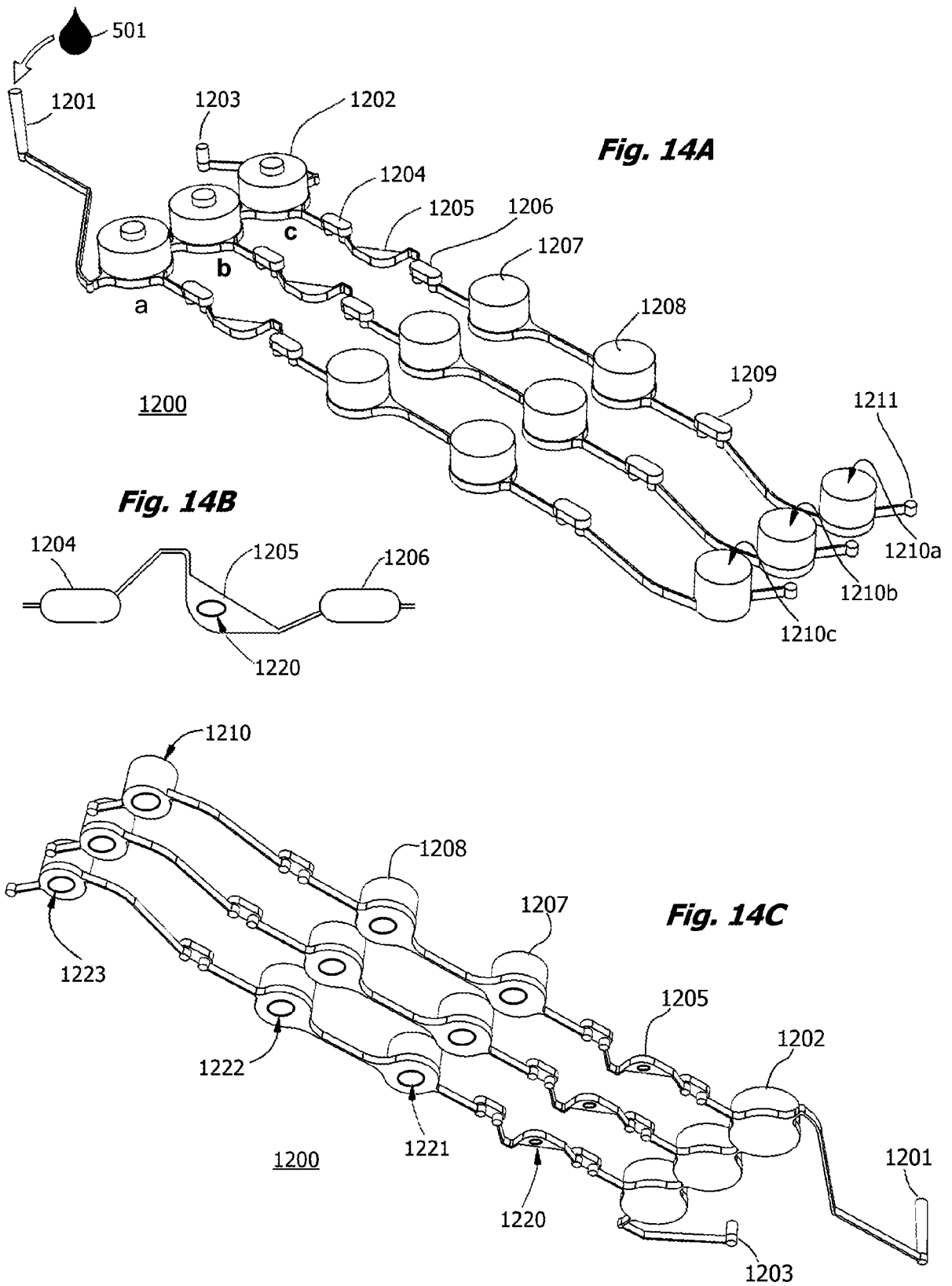
PUMPING A LIQUID VOLUME UNDER PRESSURE INTO A STAGING MANIFOLD, THE STAGING MANIFOLD HAVING AN ENERGY STORING DIAPHRAGM FOR SEPARATING A HYDRAULIC CHAMBER FROM A PNEUMATIC CHAMBER;

FILLING THE HYDRAULIC CHAMBER WITH THE LIQUID VOLUME AND DISTENDING THE ENERGY STORING DIAPHRAGM;

VALVEDLY OPENING A DOWNSTREAM CHANNEL FLUIDLY INTERCONNECTING THE HYDRAULIC CHAMBER AND THE REAGENT CHAMBER; SO THAT AN ADVANCING MENISCUS WETS THE REAGENT CHAMBER PERIPHERALLY WHILE DISPLACING RESIDUAL AIR TO A DOWNSTREAM VENT;

ASPIRATING LIQUID INTO THE REAGENT CHAMBER BY APPLYING A SUCTION PRESSURE TO THE PRESSURIZABLE DIAPHRAGM, THEREBY FILLING TO A CONSTANT VOLUME WHILE AVOIDING BUBBLE ENTRAINMENT OR REAGENT WASHOUT.

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Fig. 14D

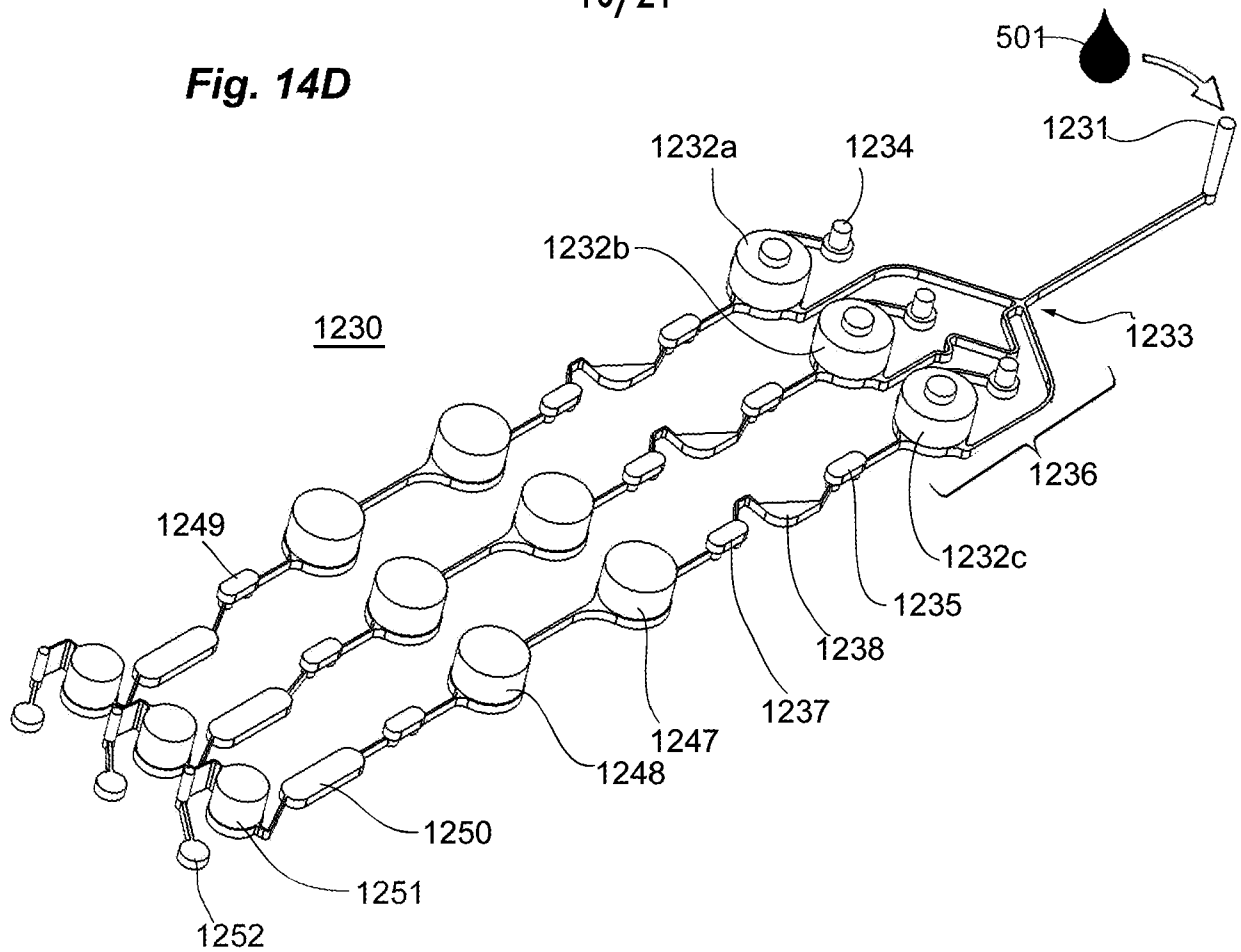
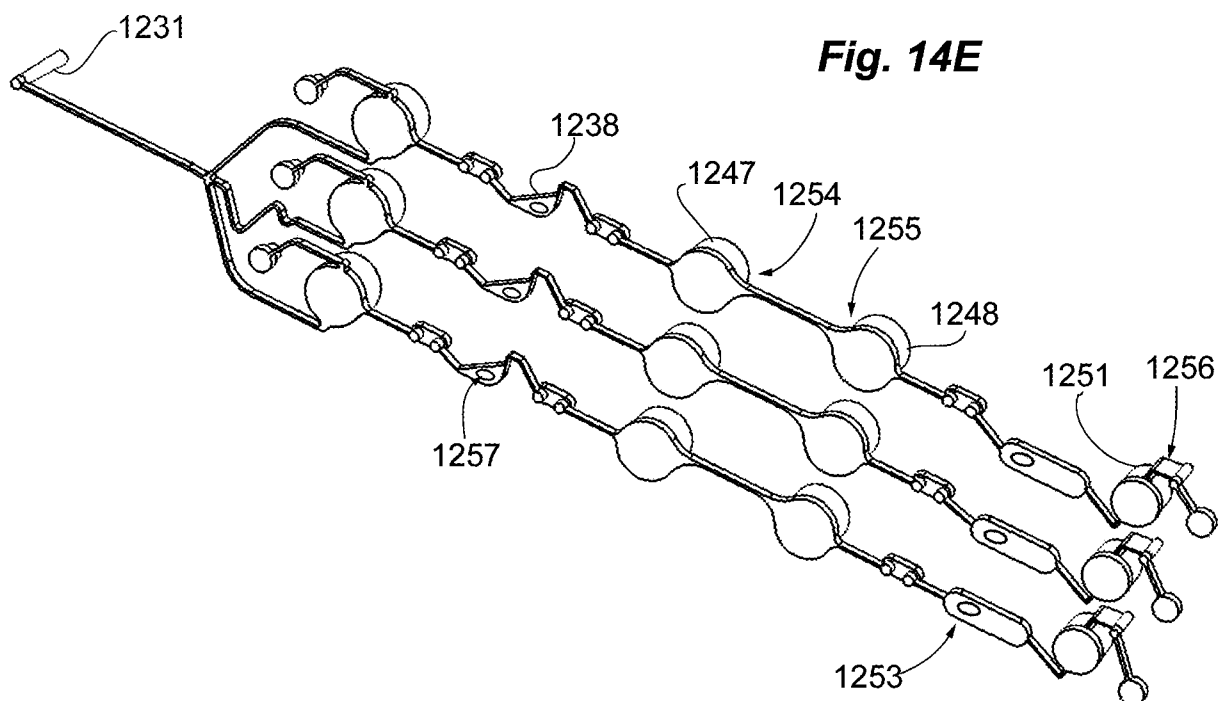
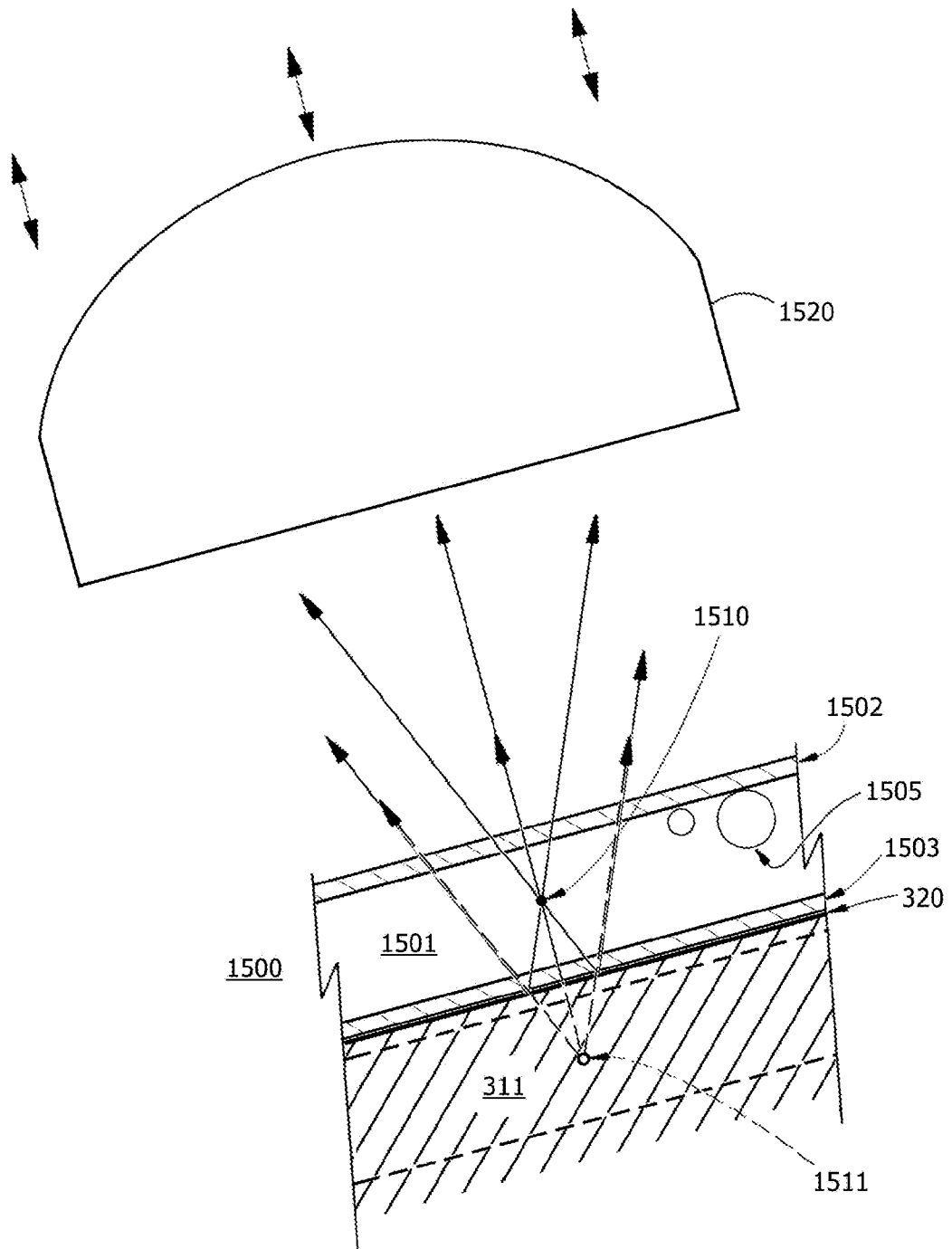


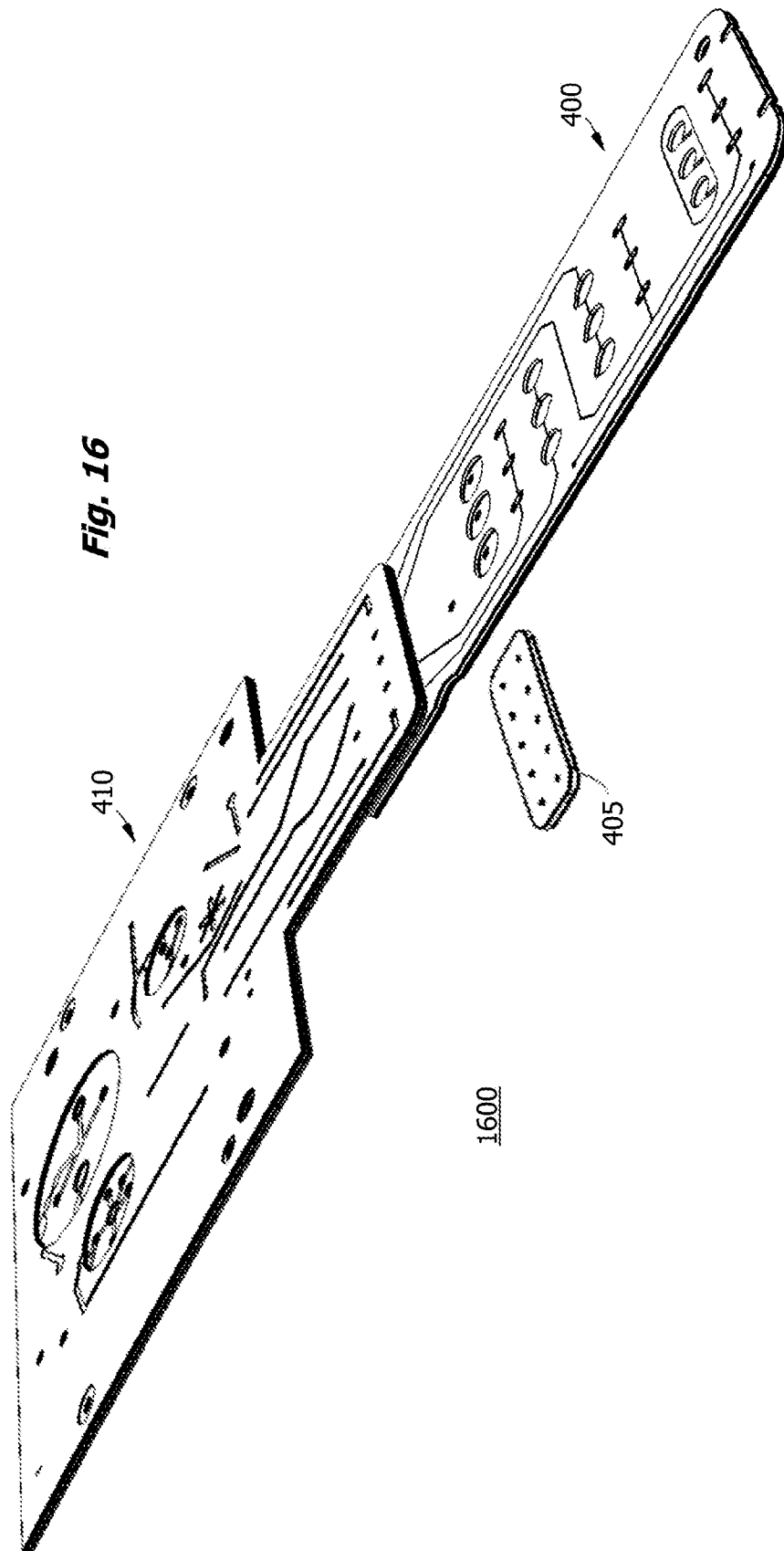
Fig. 14E



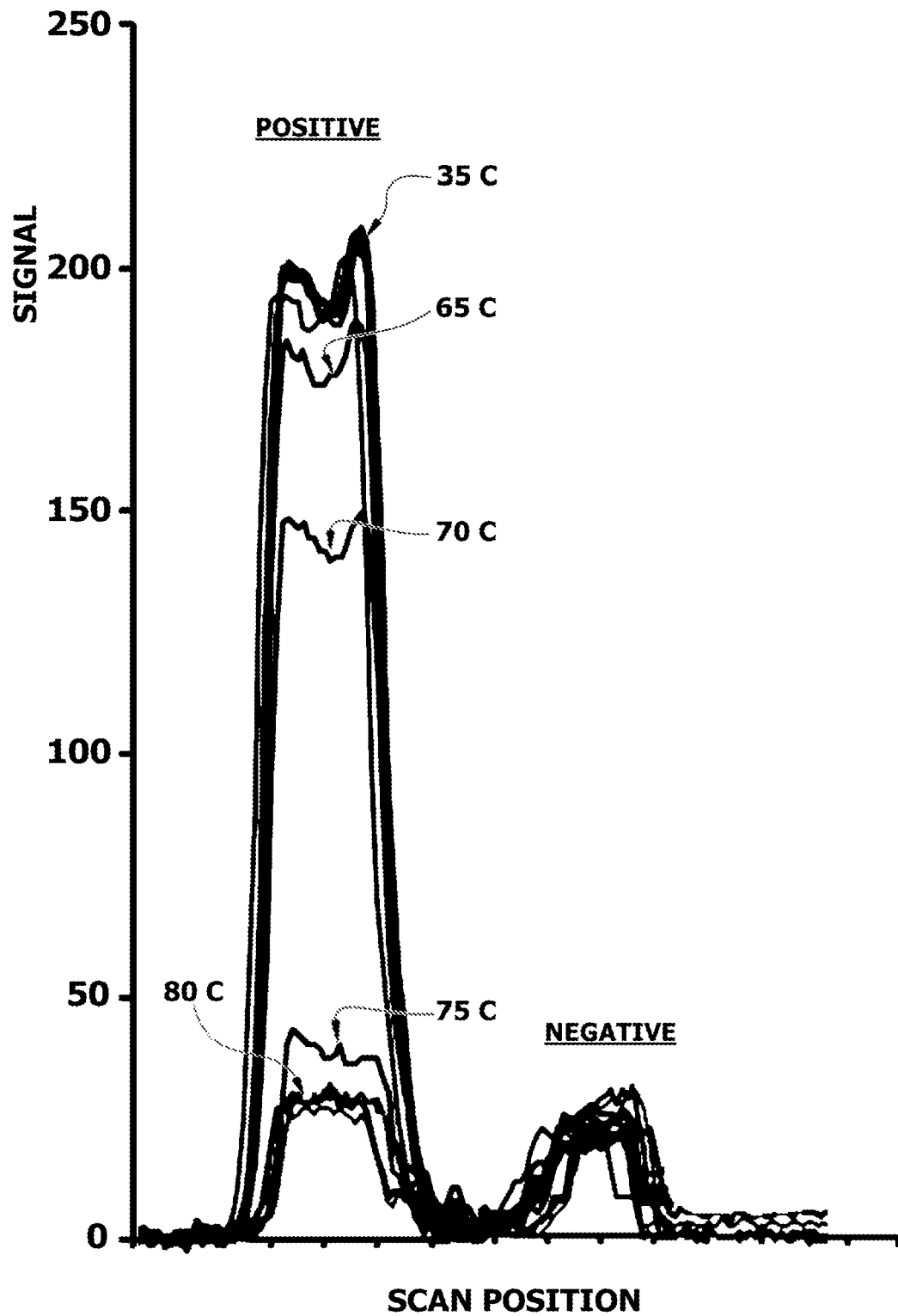
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Fig. 15

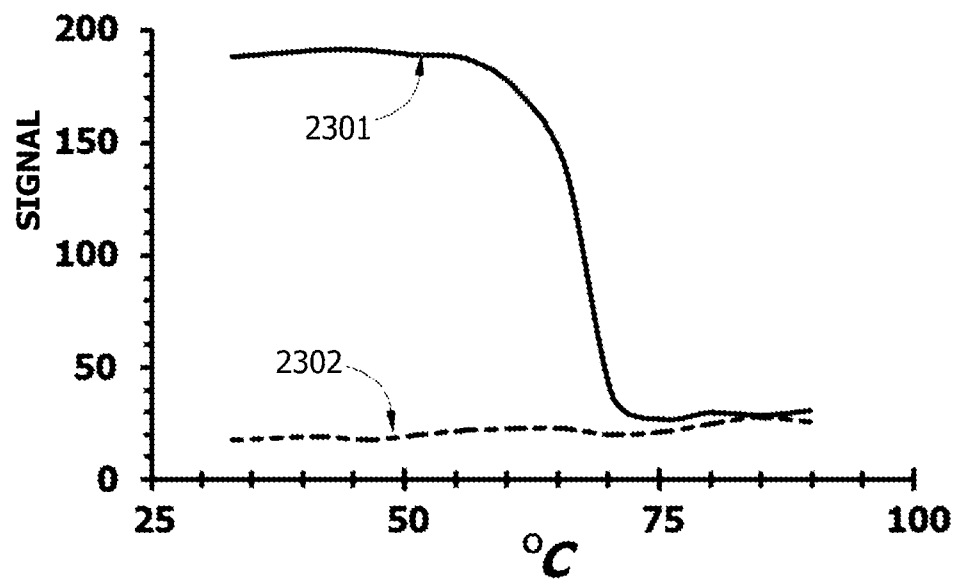
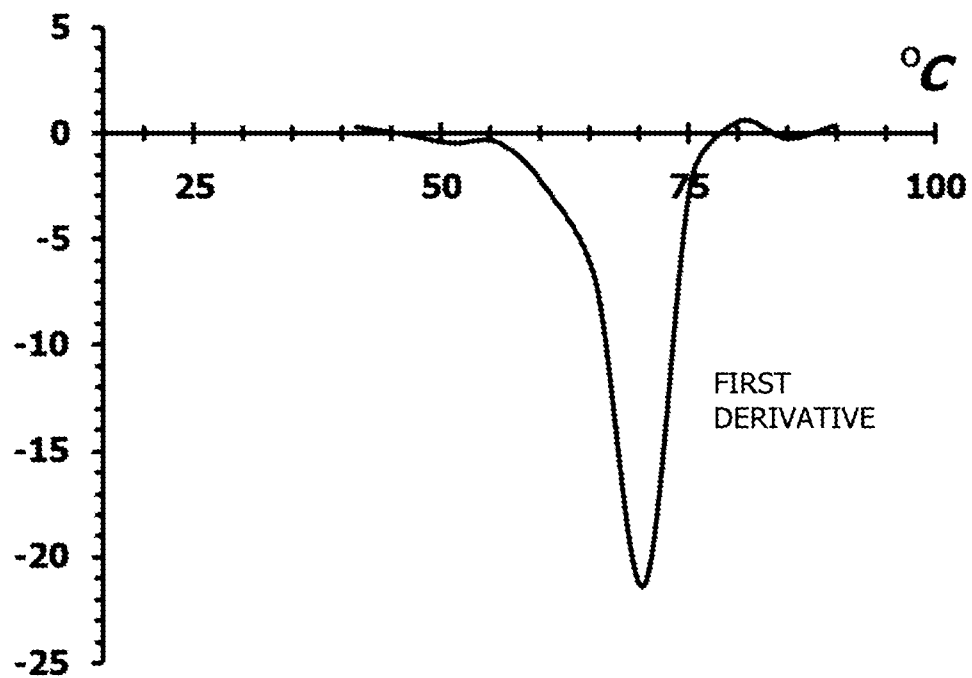
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Fig. 17

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Fig. 18A**Fig. 18B**

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Fig. 19