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(71) Applicant: **FLUENT BIOSCIENCES INC.** [US/US];
150 Coolidge Ave, Watertown, MA 02472 (US).

(72) Inventors: **KIANI, Sepehr**; 15 Palmer Street, Watertown,
MA 02472 (US). **ALICCHIO, Corey**; 150 Coolidge Ave.,
Watertown, MA 02472 (US).

(74) Agent: **MEYERS, Thomas C.** et al.; Brown Rudnick LLP,
One Financial Center, Boston, MA 02111 (US).

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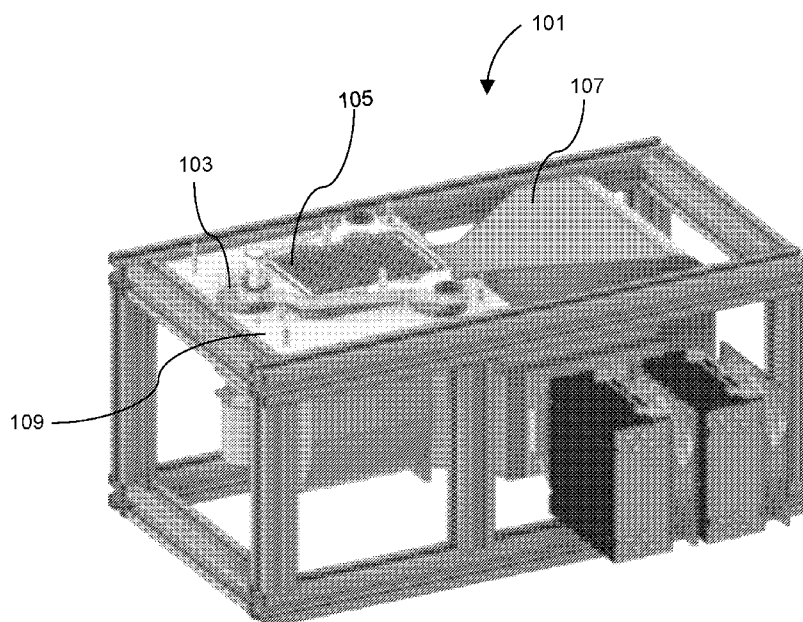


FIG. 1

(57) Abstract: The invention provides devices for generating pre-templated instant partitions. The devices may include a shearing mechanism, such as a vortexer, a holder for holding a vessel containing a liquid onto the vortexer, and a temperature control unit for modulating a temperature of the vessel by convection. The invention also provides methods of using such devices to process analyte inside the pre-templated instant partitions.



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DEVICES FOR GENERATING PRE-TEMPLATED INSTANT PARTITIONS

Technical Field

The invention relates generally to devices and methods for preparing biological samples.

Background

Early detection is a major barrier to successful treatment of many diseases. For example, cancer results from genomic changes in single cells. Those changes allow the cells to rapidly proliferate and invade other tissues. In early stages, however, the genetically altered cells represent only a tiny fraction of the cells in a particular tissue or population and can be more easily eradicated if detected early.

To facilitate early detection of cancer, microfluidic systems that allow isolation and analysis of individual cells have been developed. Unfortunately, the use of microfluidic devices requires specialized hardware and highly technical skills. As such, microfluidic systems are simply not cost-effective for most research or clinical facilities. Consequently, each year millions of cases of early-stage diseases continue to go undetected while the window of opportunity for successfully treating those diseases narrows substantially.

Summary

This invention provides devices for separating and processing cells with pre-templated instant partitions. Consequently, devices of the invention are useful to facilitate clinical diagnostic workflows by detecting aberrant cells or molecules present in low quantities, such as tumorigenic cells at early stages of cancer. Specifically, the invention provides devices and methods for producing numerous pre-templated instant partitions in a single vessel from a bulk sample, such as a sample containing millions of cells. The pre-templated instant partitions are formed by shearing liquids within the vessel causing the near instantaneous self-assembly of uniformly-sized droplets. The droplets are formed around template particles that serve as templates for the droplets and facilitate the segregation of single analytes inside the droplets for processing. Each droplet functions as an individual “reaction chamber” which allows for the simultaneous processing of a large number of cells or cellular material on a massively parallel scale. Cells or cellular material are processed using convective heat transfer, which allows for

very rapid temperature changes. By controlling the temperature of the pre-templated instant partitions, reactions, such as nucleic acid amplification, reverse transcription, and sequencing, can be independently performed on vast numbers of samples simultaneously.

Devices of the invention include a shearing mechanism, such as a vortexer, coupled to a vessel holder and a temperature control unit. When a vessel containing a liquid, i.e., a mixture of an aqueous solution and oil, with analyte is placed in the holder, the device applies a shearing energy to the liquid. By controlled shear force, the device generates an emulsion of essentially monodisperse droplets (pre-templated instant partitions).

By generating pre-templated instant partitions, devices of the invention allow for the isolation of individual targets, such as single cells or molecules, from bulk biological samples. For example, millions of individual target cells can be captured in separate fluid partitions in an emulsion contained in a single reaction vessel. The droplets function as individual micro-reactors for performing sample preparation steps, such as PCR. Thus, the devices can perform large-scale parallel processing of single target cells or molecules in a bulk liquid.

The devices of the invention have numerous advantages. For example, most conventional microfluidic systems require prefabricated microfluidic chips and sophisticated micropneumatic systems. The microfluidic chips are costly to produce and cannot be readily adapted to change production scale. Moreover, the setup and use of microfluidic systems require substantial training. In contrast, devices of the invention can be used with standard microcentrifuge tubes, such as 0.5 milliliter microcentrifuge tubes, or assay plates, and their use does not require extensive setup, maintenance, or technical training. In fact, devices of the invention can accommodate tubes of different shapes or sizes, which is useful for integrating the devices into existing molecular biology workflows.

Because the devices provided herein include an integrated shearing mechanism, e.g., a vortexer, and a rapid temperature control mechanism, they also are easier to use than prior vortexers for generating pre-templated instant partitions and are also useful to automate many library preparation steps. When immiscible liquids are mixed using other commercially available vortexers, the extent to which pre-templated instant partitions are formed is not adequately controlled. Insufficient mixing results in partitions that are heterogeneous in size, while excessive mixing exposes the biological contents of the liquid partitions to unnecessary force that may cause damage. In both instances, downstream library preparation is negatively

impacted. Moreover, because the devices include a temperature control unit (e.g., a convective thermocycler), devices of the invention can perform certain library preparation steps, rapidly, and without any human intervention. This is useful to reduce sample preparation time and minimize opportunities for human error.

Moreover, the temperature control unit operates under principles of convection. As such, the temperature control unit is operable to rapidly change temperatures of sample inside the vessel during sample preparation. In fact, the speed at which sample temperature changes can occur reduces the length of time of certain reactions. This is useful to prepare samples more quickly while minimizing opportunities of sample degradation, such as mRNA decay. Devices of the invention can be used to automate certain library preparation methods, such as lysing single cells segregated inside individual partitions, target capture of analyte (e.g., RNA, DNA, or protein) with capture probes inside the partitions, PCR, cDNA synthesis, etc. Because the library preparations can be automated, chances of human error are substantially reduced.

In preferred embodiments, the temperature control unit operates by forced convection. Forced convection can rapidly change sample temperature and thereby drive enzymatic reactions by quickly supplying, e.g., with a pump or fan, a fluid of a predetermined temperature to a holder securing the vessel. By using forced convection, devices of the invention can process samples more quickly, more precisely, and expend less energy in the process, thereby saving costs. Unlike conventional thermocyclers, which must wait for heating blocks to be heated or cooled to regulate sample temperature, devices of the invention quickly transfer heat to and from the sample by forced fluid movement. By employing forced convection, devices of the invention can process analyte (e.g., capture or amplify nucleic acid), at least 25 percent more quickly.

In one aspect, the invention provides a device for generating pre-templated instant partitions. The device includes a shearing mechanism, i.e., a vortexer, for shearing a liquid contained in at least one vessel. The liquid generally includes a mixture of an aqueous solution comprising analyte (e.g., cells), which is overlaid with oil. Upon shearing the liquid, the liquid divides into a plurality of partitions, near instantaneously, wherein a substantial portion of the plurality of partitions includes one or zero analyte.

Devices of the invention include a holder for securing at least one vessel to the vortexer. Preferably, the holder is designed to accommodate one or more vessels of different shapes and sizes. For example, in preferred embodiments the holder is a clamp. As such, the holder can

secure any number or any type of vessel, such as, one or more tubes (e.g., a 0.5 milliliter microcentrifuge tube), a strip of tubes (e.g., a strip of 2, 3, 4, 6, 8, 10, 12, or more tubes), a 15 milliliter conical tube, or a multiwell plate (e.g., a plate with 2, 4, 6, 8, 12, 24, 48, 96, 192, 384, or more wells), which is useful for high-throughput applications.

Devices of the invention further include a temperature control unit. The temperature control unit preferably operates by forced convection, in which fluid is forced through conduits within the device to transfer and remove heat from a vessel contained in the holder. The temperature control unit can rapidly and precisely change a temperature of a sample inside the vessel by supplying fluid of a predetermined temperature, which is useful for processing analyte within the plurality of partitions. Accordingly, devices of the invention are useful to generate pre-templated instant partitions and process analyte inside those partitions by rapidly vortexing a vessel containing analyte inside a liquid, and rapidly regulating temperatures inside the vessel to orchestrate various reactions, such as, cell lysis and target capture or PCR.

In order to facilitate sample partitioning, devices of the invention are operable to vortex vessels at high speeds. For example, devices of the invention can achieve vortex speeds of up to about 5,000 revolutions per minute. Moreover, devices of the invention allow for vortexing to be carried out while the vessel is held in one or more different positions, which is useful to achieve adequate mixing of samples. For example, devices of the invention may include a holder that is operable to hold at least one vessel in a substantially vertical position when shearing the liquid. The holder can also hold the sample in a substantially horizontal position when shearing the liquid. Or, the holder can alternate between at least two different positions. For example, the holder can hold the vessel in a substantially horizontal position and then in a substantially vertical position, or vice versa, at different timepoints while shearing the liquid.

Standard thermomixers are generally isothermal. They may have a custom machined block with a heater attached to heat and cool through conduction. However, this does not allow for rapid changes in temperature necessary for performing many PCR, or other molecular biology reactions, that are sensitive to temperature transitions. Conversely, devices of the invention include a temperature control unit that can regulate temperatures of partitions, and thus the reactions therein, using forced convection. This dramatically changes the amount of time it takes to transfer heat, especially with the addition of concurrent sample mixing which further improves heat transfer. This process allows for shorter PCR cycles for ePCR (which are 2-6

times longer than standard PCR cycles) since the speeds that devices of the invention are able to heat and cool are an order of magnitude faster than any conventional thermomixer. This rapid and precise temperature cycling enables, among other things, controlled cell lysis and target (e.g., RNA, DNA, protein) capture, for example, with capture probes attached to template particles. In some applications, PCR in pre-templated instant partitions is desirable (dPCR, for example). Rapid thermal cycling may permit integrated emulsion and PCR on the same instrument.

In preferred embodiments, devices of the invention include a temperature control unit having one or more conduits through which fluid is forced. The fluid can be heated or cooled to a preselected temperature within the one or more conduits. For example, the temperature control unit may include a first conduit for heating the fluid to first temperature. The fluid may be selectively heated to the first temperature by passing the fluid through the first conduit. After passing the fluid through the first conduit, the fluid can be flowed, via a pump or fan, towards the holder to thereby heat a sample contained therein. The temperature control unit can also include a second conduit for cooling the fluid to a second temperature. By passing the fluid through the second conduit, the fluid can be selectively cooled to the second temperature. During sample processing, sample temperature can be managed by alternating fluid flow between the first conduit and the second conduit, thereby heating and cooling the fluid, respectively. By alternating fluid flow between the conduits, the temperature of the sample inside the vessel can be cycled.

For example, the first conduit can heat a fluid (e.g., air) to a first temperature (e.g., >90 degrees Celsius). After, or coincident with, shearing a liquid inside a vessel with the device, fluid of the first temperature can be supplied to an area near or in contact with a vessel containing partitions to lyse single cells contained inside those partitions. After cell lysis, the flow of the fluid may be redirected, for example, by changing a position of a valve, to direct the fluid through the second conduit. Flowing the fluid through the second conduit results in the fluid being rapidly cooled to a second temperature (e.g., <80 degrees Celsius), which is subsequently supplied to the area near or in contact with the vessel to thereby cool the sample temperature and cause the capture of analyte released from the single cells.

In some embodiments, devices of the invention further include an optical system to monitor formation of pre-templated instant partitions and/or monitor reactions that are carried

out therein. Accordingly, unlike conventional thermomixers which are a “black box” and do not allow for visual inspection of products during production processes, devices of the invention provide for an optical system to visualize and monitor quality throughout one or more library preparation steps. The optical system may include one or more light sources and one or more photodetectors. Each light source may be positioned to transmit light into the liquid in a vessel. Each light source may be positioned to transmit light to the liquid in a different well of a vessel. Each photodetector may be positioned to sense the transmitted light (e.g., light scatter) from the liquid in a different vessel. Each photodetector may be positioned to sense the transmitted light from the liquid in a different well of a vessel. The light source may be from a laser, a light emitting diode, or from a lamp, such as, mercury arc lamp.

Devices of the invention may further include a control system. The control system may be coupled to the vortexer and the optical system. The control system may control the speed of the vortexer in response to the transmitted light. The control system may increase or decrease the speed of the vortexer. The control system may initiate or stop the vortexer. The control system may direct the vortexer to stop applying a shearing energy to the liquid when the liquid comprises substantially monodisperse droplets.

Moreover, to reduce costs and enhance flexibility for part optimization and manufacturing, devices of the invention include at least one component manufactured by 3D printing. In some instances, the housing or the holder is manufactured by 3D printing. As such, devices of the invention can be quickly and cheaply modified to accommodate different vessel sizes or shapes, which is useful to scale up or scale down library preparation processes. Devices of the invention can use 3D printing to create integrated insulation, i.e., honeycomb infill. Devices of the invention can also use 3D printing to facilitate machine services in the field by quickly and efficiently printing new and replacement parts.

According to another aspect, the invention provides a method for generating pre-templated instant partitions. The method includes contacting at least one vessel containing a liquid with a device comprising a vortexer, a holder to secure at least one vessel to the vortexer, and a temperature control unit in fluidic communication with the holder. The method further includes operating the device to shear the liquid inside at least one vessel into a plurality of pre-templated instant partitions, wherein each of the plurality of pre-templated instant partitions

comprise one or zero analyte. For example, operating the device to shear the liquid may involve vortexing the liquid at a speed of 5,000 revolutions per minute.

Methods of the invention are useful to make pre-templated instant partitions and rapidly process sample material inside those partitions. Advantageously, devices of the invention include modules for regulating temperatures quickly and efficiently by forced heat convection.

Accordingly, some embodiments of the invention include providing a fluid of a predetermined temperature towards the holder via a first conduit. The first conduit may include a solid-state heating mechanism to rapidly heat the fluid to a first predetermined temperature. Additionally, methods of the invention further include directing the flow of fluid through a second conduit. The second conduit comprises a Peltier to rapidly cool the fluid. The cooled fluid is then flowed towards the holder to cool the sample liquid.

Methods may include operating the device for automated production of pre-templated instant partitions or automating reactions carried out within the partitions. For example, methods of the invention may include transmitting a light to the liquid in at least one vessel and sensing the transmitted light from the liquid in at least one vessel. In some embodiments, methods include comparing the transmitted light from the liquid in at least one vessel to a reference and adjusting the speed of the vortexer in response to the comparison. The method can include comparing the transmitted light from the liquid in at least one vessel to a reference. The reference may be transmitted light from a sample that has not been exposed to a shearing force. The reference may be transmitted light from a sample that has pre-templated instant partitions. The pre-templated instant partitions may have a defined size or range of sizes.

Brief Description of the Drawings

FIG. 1 illustrates an exemplary device for making pre-templated instant partitions.

FIG. 2 illustrates an exemplary temperature control unit.

FIG. 3 shows an image of an exemplary holder.

FIG. 4 shows an adjustable latching mechanism.

FIG. 5 shows a device according to one embodiment of the invention.

FIG. 6 shows a device for making pre-templated instant partitions according to a different embodiment.

FIG. 7 shows changes in temperature over time inside a device during convective heating.

FIG. 8 shows changes in temperature over time inside a device during convective cooling.

Detailed Description

This invention provides sample preparation instrumentation for generating pre-templated instant partitions of uniform size and for processing analyte inside those partitions. The formation of the pre-templated instant partitions is useful for a variety of research and diagnostic applications because it allows individual targets, such as single cells or single molecules, to be captured inside separate partitions containing a pre-defined volume of liquid for individual processing. The liquid may include reagents for performing various sample processing reactions inside the partitions. By subsequent manipulation of the partitions, reactions, such as nucleic acid amplification, reverse transcription, and sequencing, can be independently performed on vast numbers of samples simultaneously. Consequently, the devices are useful for detection of aberrant cells or molecules that are present in low quantities, such as tumorigenic cells in an early stage of cancer.

Although the utility of reaction cells that contain individual targets has been recognized for years in molecular biology, prior systems for making emulsions of droplets that contain individual targets are problematic. A predicate to obtaining individualized reaction cells is the production of monodisperse, i.e., uniformly sized droplets. Monodisperse droplets can be generated using microfluidic systems, which typically involve controlled injection of two or more liquids into a microfluidic chip having custom-designed fluid channels to permit proper mixing of the liquids. Because the design of a microfluidic system must be optimized to produce droplets of a particular size based on the input liquids, microfluidic chips generally cannot be adapted to produce droplets of different sizes for different applications. In addition, because the chips must be prefabricated but typically cannot be reused, they are costly. Finally, the setup and maintenance of microfluidic pumping systems is not trivial and requires a level of trained expertise. Devices described herein avoid those pitfalls.

Devices permit manufacture of pre-templated instant partitions from bulk liquid (a liquid containing millions or more analyte, e.g., cells) in simple vessels, such as test tubes or multiwell plates, so they do not require specialized disposable supplies. In addition, because droplet size and contents are determined by the size of particles, discussed in detail below, added to the liquid, the devices can be readily adapted to produce droplets having different properties by altering the content of the input particles. Moreover, the devices are simple to use and do not require extensive cleaning or maintenance between uses.

In addition, devices of the invention are useful to perform certain laboratory preparation steps by rapidly, and precisely, changing sample temperature. To facilitate rapid changes in temperature, devices of the invention generally include a convection-based temperature control unit that is operable to rapidly change temperatures of an environment where the vessel is secured during sample preparation. For example, the temperature control apparatus can be used to raise or lower the temperature by programming the device with instructions via an interface operable to receive user input. Devices of the invention can be used to automate certain library preparation methods, such as lysing single cells segregated inside individual partitions, target capture of analyte (e.g., RNA, DNA, or protein) with capture probes inside the partitions, PCR, qPCR, digital PCR, cDNA synthesis, etc. Because the library preparation steps can be pre-programmed for automated processing, devices of the invention substantially reduce opportunities for human error.

In preferred embodiments, the temperature control unit controls sample temperature by thermal convection. Convection, or convection heat transfer, involves the transfer of heat by the movement of fluid, such as air or water. When fluid is caused to move away from a source of heat, e.g., a heater, thermal energy is carried with it. The convection-based temperature control unit is useful to rapidly adjust a temperature of the vessel by quickly moving fluid of predetermined temperatures to and away from the vessel. Moreover, because portions of the fluid can be maintained at a desired temperature inside one or more conduits that are separate from the sample holder, the temperature of the vessel, and thus the sample therein, can be changed quickly and effectively by forcing (e.g., pumping) the fluid, which can be preheated to the desired temperature, to the holder. Accordingly, unlike conventional thermocyclers, devices of the invention do not need to wait for heating blocks to be heated or cooled to regulate sample temperature.

FIG. 1 illustrates an exemplary device 101 for making pre-templated instant partitions. The device 101 includes a vortexer 103 for shearing a liquid contained in at least one vessel. The device 101 also includes a holder 105 for securing at least one vessel to the vortexer 103. The device further includes a temperature control unit 107 to rapidly change the temperature of a sample in at least one vessel. In particular, the temperature control unit 107 manages a temperature of a fluid, by convection. The temperature control unit further manages a flow path of the fluid through the device to control the transfer of heat to or away from the sample.

The holder 105 is designed to secure at least one vessel to a frame 109 of the device 101. The holder 105 can be any device suitable for holding at least one vessel. The holder 105 may be or include a clamp, a platform, a rack, or a tray. Preferably, the holder includes a clamp for easily accommodating vessels of various sizes and shapes. The clamp (not shown) may be integral with the platform, rack, or tray, or it may be separate from the platform, rack, or tray.

The holder 105 can be mounted to a frame 109 such that the holder 105 can accommodate movement, with respect to the frame 109, while shearing the liquid. For example, the holder 105 can be mounted such that the holder 105 can oscillate or swirl in a circular motion, and/or move along one or more of a horizontal or vertical planes while shearing the liquid for the purpose of applying a shearing force to the liquid inside the vessel held by the holder 105.

The shearing force is applied to the sample to generate pre-templated instant partitions in the liquid, which is typically an aqueous liquid having an oil overlay. Optimal generation of pre-templated instant partitions may be achieved by applying a shearing force within a certain range. For example, when the shearing force is inadequate, large droplets that contain multiple partition-templating particles may not be broken into single-particle droplets. On the other hand, excessive shearing force may damage the particles and/or the targets to be captured by the droplets.

In certain embodiments, the shearing force is applied by vortexing or agitating the sample. The sample, while secured in the holder 105, can be vortexed by the actuation of a motor-driven agitator (vortexer) that drives movement of the holder 105 relative to the frame 109. The sample may be vortexed or agitated for a defined period. For example and without limitation, the sample may be vortexed or agitated for about 1 second, about 2 seconds, about 4 seconds, about 6 seconds, about 8 seconds, about 10 seconds, about 15 seconds, about 20

seconds, about 30 seconds, about 45 seconds, about 1 minute, about 2 minutes, about 3 minutes, about 4 minutes, or about 5 minutes. The sample may be vortexed or agitated at a defined speed. For example and without limitation, the sample may be vortexed or agitated at about 5,000 revolutions per minute (rpm), at about 4,000 rpm, at about 3,000 rpm, at about 1,000 rpm, at about 500 rpm, about 600 rpm, about 700 rpm, about 800 rpm, about 900 rpm. In some instances, a vortexing speed above 5,000 rpm may be desired. For the purposes of the disclosure, about means within 15 percent above or below the identified rpm.

The shearing force applied may be above a lower threshold but below an upper threshold. For example, devices of the invention may achieve an adequate shearing force by vortexing the vessel at speeds of up to 6,000 rpm, and above 2,000 rpm. Or, devices of the invention may achieve an adequate shearing force by vortexing at speeds of up to 5,000 rpm and above 3,000 rpm. Preferably, the shearing force is about 5,000 rpm. In addition, devices of the invention can provide relatively slow vortex speeds, e.g., between about 20-100 rpm, such as, 50 rpm, which is useful for thermomixing.

In some embodiments, devices of the invention include an integrated optical system for visualizing the formation of pre-templated instant partitions. The optical system may operate in conjunction with a control system for regulating an amplitude or duration of vortexing. By controlling the duration and amplitude of the shearing force, the device can reliably generate pre-templated instant partitions of similar construct. Once near-uniformity in droplet size is achieved, the optical system can detect a change in the transmitted light. The device may then cease application of shearing force, notify the user, and/or allow subsequent reactions to be performed. For further discussion of optical systems for use with devices herein, see co-owned Application No. 17/146,768, which is incorporated by reference.

Advantageously, devices 101 of the invention include mechanisms for controlling the temperature of a sample inside the one or more vessels, which is useful to control the speed and/or occurrence of various reactions that are carried out within the pre-templated instant partitions. Specifically, in preferred embodiments the device 101 includes a temperature control unit to modulate the temperature of the sample using thermal convection. By using thermal convection, the amount of time it takes to transfer heat to, or remove heat from, the sample can be substantially reduced, thus increasing the rate and efficiency of reactions inside the sample. This is especially useful for processing sensitive samples of low quantity or low abundance. For

example, to quickly copy unstable RNA of a single cell into cDNA, which is substantially more stable.

FIG. 2 illustrates an exemplary temperature control unit 201. The temperature control unit 201 includes at least two conduits, also referred to as chambers, for heating or cooling a fluid to a predetermined temperature. The temperature control unit 201 includes a first conduit 203 for heating a fluid (e.g., hot air or water) to a first temperature and a second conduit 205 cooling the fluid to a second temperature. The temperature of the fluids within the conduits is adjustable. Preferably the temperature of the fluid is adjustable by solid-state thermoelectric technology, e.g., Peltier cooling.

Solid-state thermoelectric devices use electricity and semiconductors to produce cooling and heating. As opposed to refrigerants used in conventional cooling systems. The magnitude of heat flow is adjustable by varying the amount of electrical current. Solid-state thermoelectric devices are preferable for several reasons. First, there are no moving parts. This means solid-state thermoelectric technology can provide the devices with higher reliability, which leads to substantial cost savings. Also, unlike compressor based conventional cooling systems, solid-state thermoelectric heating and cooling does not require expensive refrigerants. Accordingly, devices of the invention are more environmentally friendly. By virtue of solid-state technology, systems of the invention can be scaled from less than one watt of cooling power up to kilowatts. The ability to scale thermoelectric thermal management systems offers a much wider range of device configurations. Common components can be used for large- and small-scale devices. This results in reduced manufacturing and design costs.

Devices of the invention can regulate temperature by forced convection. Forced convection involves the forced movement of fluid within the system. The movement of fluid can be forced with a fan or a pump 215. To control sample temperature, the temperature control unit forces fluid through one of at least two conduits. The passing of the fluid through the first conduit 203 causes the fluid to increase in temperature. The fluid increases in temperature by virtue of a heater that is associated with a portion of the conduit. The heater transfers thermal energy into the fluid as the fluid passes through the conduit. The heater is operable to heat the fluid up to about 130 degrees Celsius, for example, up to 110 degrees Celsius, or 100 degrees Celsius, or at least 90 degrees Celsius.

The second conduit 205 can be coupled with a Peltier device, which can transfer thermal energy with consumption of electrical energy to achieve reduced fluid temperatures. For example, the second conduit 205 can provide a fluid temperature of about 0 degrees Celsius. For example, of about 5 degrees Celsius, or 10 degrees Celsius, or 15 degrees Celsius. In some embodiments, a third, fourth, or fifth additional conduit may be provided. The additional conduits may be useful to rapidly supply fluids having temperatures between 0 degrees and 130 degrees Celsius.

The first 203 and second conduit 205 provide fluid of a first or second temperature, respectively, to a sample held by the holder 207 by virtue of being in fluidic communication with an area that is near, or in contact with, the holder 207. The first conduit 203 and second conduit 205 provide fluid towards the holder 207, and thus regulate sample temperature, by virtue of being in fluidic communication with a thermal environment associated with the holder 207. For example, a bottom surface of the holder 207 may be disposed substantially within a fluidic channel 211 of the convection heating apparatus. The bottom surface may comprise a material that is of a low specific heat, and thus sensitive to rapid temperature changes of the fluid. Advantageously, the temperature control unit 201 is configured to control fluid flow through the first conduit 203 and second conduit 205 by a single fan or pump. This is useful for minimizing manufacturing costs.

The temperature control unit 201 includes a mechanism to selectively direct forced fluid flow through the first 203 or the second 205 conduit. By controlling which conduit the forced fluid is flowed through, the temperature of the sample can be regulated. For example, preferably the temperature control unit 201 includes a valve 221, such as a sliding valve, for directing forced fluid flow. The valve 221 can be moved into one of at least two positions. In a first position, the fluid is blocked from flowing into the first conduit 203. This forces the fluid to flow through the second conduit 205, as illustrated by the curved arrowhead. Alternatively, the valve can be moved into a second position, in which fluid flow into the second conduit 205 is blocked. Blocking fluid from flowing into the second conduit 205 causes fluid to move through the first conduit 203, thus heating the fluid. The valve can further be positioned into any number of intermediate positions between the first position and second position, thereby blocking various portions of fluid from flowing through the first 203 and second 205 conduits. This is advantageous since it allows the temperature of the fluid to be quickly changed by heating or

cooling various fractions of the total fluid. The temperature control unit 201 further includes self-closing flaps 231 to prevent fluid from flowing back into one of the first or second conduits and also to prevent thermal energy from escaping.

FIG. 3 shows an image of an exemplary holder 301. The holder includes a clamping lid 305 for securing one or more vessels inside the holder. The clamping lid 305 may include a copper spring plate which is useful for heating a top surface of the vessels, thereby ensuring the temperature within the vessel is homogenous. In some embodiments, the clamping lid includes a 30-watt 24 volt membrane heater, which is useful for rapidly changing the temperature of the lid coincident with changes in temperature of the fluid within the temperature control unit 201.

The holder is coupled with a shearing mechanism 309. The shearing mechanism may be any device capable of applying a shearing force to the liquid in the vessel. In some embodiments, the shearing force is applied by moving the holder 301, such as by spinning, rotating, shaking, or rocking the holder 301. In such embodiments, the shearing mechanism may be or include an agitator, shaker, or vortexer. In some embodiments, the shearing force is applied through an electrical force. In such embodiments, the shearing mechanism may be or include a piezoelectric motor. In some embodiments, the shearing force is applied through sound waves. In such embodiments, the shearing mechanism may be or include a sonicator or ultrasonic device. The shearing force may be applied by a combination of means, and the shearing mechanism may be or include any combination of the aforementioned devices.

The holder 301 is designed to hold any vessel or container suitable for holding liquid. For example, and without limitation, the vessel may be a tube or a well in a multiwell plate. The vessel may be or include a set of tubes physically connected to each other. For example, the vessel may be or include a strip of 2, 3, 4, 6, 8, 10, 12, or more tubes. The vessel may be or include a well in plate with 2, 4, 6, 8, 12, 24, 48, 96, 192, 384, or more wells.

To facilitate shearing of the liquid, the holder 301 can hold the least one vessel in either of a substantially vertical position or a substantially horizontal position while shearing the liquid. As such, devices of the invention have the ability to mix horizontally and vertically (asymmetrical mixing), automatically, without physically needing to change the holder or rotate the tubes by hand. This allows for controlled thermo-mixing, which can aid sample distribution in packed templates and accommodate applications where desired thermal incubation is valuable (for example, in emulsion PCR). Shear in samples is enhanced when the axial symmetry of the

containing vessel is broken. This can be enabled by holding standard tubes in a horizontal or angled orientation. For example, the holder 301 may be attached to a moveable arm configured to move axially, in any direction, and/or rotate while shearing the liquid. Shearing can also be evoked by changing the tube shape to a non-circular shape or by using a ribbed tube, all of which can be accommodated by flexible tube holders of the device.

FIG. 4 shows an adjustable latching mechanism 401. The adjustable latching mechanism allows the holder 301 to accommodate vessels of various sizes or shows. By adjusting the latching mechanism, larger or smaller tubes can be accommodated within devices of the invention.

FIG. 5 shows a device 501 according to one embodiment of the invention. The device 501 includes integrated vortexing and thermal controls. To reduce manufacturing costs, at least one component of the device 501 can be manufactured by 3D printing. For example, in some instances the housing 503 is manufactured by 3D printing. The housing 503 can additionally provide insulation for more precise thermo-regulation. By implementing 3D printing, costs of manufacture are substantially reduced and flexibility for part optimization is increased. In some instances, 3D printing is used to create integrated insulation, for example, honeycomb infills. 3D printing also provides for the opportunity to service machines in the field by quickly and efficiently printing new and replacement parts.

FIG. 6 shows a device 601 for making pre-templated instant partitions according to a different embodiment. The device includes a heated lid involving a clamp 603 for accommodating tubes of various shapes and sizes. For example, the clamp 603 is useful to secure vessels having a non-circular shape, or vessels such as a ribbed tube, both of which are incompatible with conventional thermomixers but are useful for efficiently shearing liquids into partitions.

According to another aspect, the invention provides a method for generating pre-templated instant partitions. The method includes contacting at least one vessel containing a liquid with a device comprising: a vortexer, a holder to secure at least one vessel to the vortexer, and a temperature control unit in fluidic communication with the holder. The method further includes operating the device to shear the liquid inside at least one vessel into a plurality of pre-templated instant partitions, wherein each of the plurality of pre-templated instant partitions

comprise one or zero analyte. For example, operating the device to shear the liquid may involve vortexing the liquid at a speed of 5,000 revolutions per minute.

Methods of the invention are useful to make pre-templated instant partitions and rapidly process sample material inside those partitions.

For example, methods of the invention may include performing one or more library steps inside the partitions. For example, in some instances, methods of the invention involve shearing a liquid containing cells, causing single cells to be isolated inside the partitions. The method may further include lysing the single cells inside the partitions. Cell lysis may be induced by heating the holder to a temperature sufficient to cause cell lysis. For example, in some embodiments, lysing involves heating the partitions to a temperature sufficient to release lytic reagents contained inside template particles into the partitions. Advantageously, with devices of the invention, lysis can be rapidly achieved by thermal convection.

In some embodiments, upon lysing the cells inside the partitions, RNA (e.g., mRNA) and/or DNA is released from the cells into the partitions for capture with a capture oligos provided by a template particle, as discussed below. The capture oligo can include unique barcodes specific to each template particle. Accordingly, upon capture, i.e., hybridization, of the RNA and/or DNA and a complementary portion of a capture oligos, all of the RNA and/or DNA of single cells are effectively linked by a common barcode sequence. Since each partition includes only one single cell and one template particle, the unique barcode sequences of any one template particle is useful for associating RNA and/or DNA with single cells from which they are released.

Capture is performed by rapidly cooling the temperature of the partitions to a temperature sufficient for hybridization. Because devices of the invention can manage temperature changes by forced convection, the time between temperature cycles is reduced, thus the time between cell lysis and capture is substantially reduced, compared with conventional methods. This reduction in time between cell lysis and capture is significant since it is during this time window that many nucleic acids are degraded or digested by certain factors, such as nucleases. In some instances, temperature changes are automatic. For example, changes in temperature may occur based on instructions inputted into the device by a user. The instructions may be input into the device via an interface of the device.

After capture, methods of the invention may include reverse transcribing captured RNA into cDNA. Reverse transcription can be carried out to generate a library comprising cDNA with barcode sequences that allows each sequence read of a library to be traced back to the single cell from which the mRNA was derived. Once a library is generated comprising barcoded cDNA, the cDNA can be amplified, by for example, PCR, to generate amplicons for sequencing.

According to some embodiments, methods of the invention include operating a device of the invention to perform PCR on DNA contained inside pre-templated instant partitions. Advantageously, devices of the invention modulate temperature using forced air convection. This dramatically changes the amount of time it takes to transfer heat. This allows for shorter PCR cycles since the speeds of heating and cooling are an order of magnitude faster than most thermomixers. For example, using devices of the invention, DNA amplification can occur in approximately one half or one third the amount of time as would be required using a standard thermocycler.

Methods may include operating the device for automated production of pre-templated instant partitions or automating reactions carried out within the partitions. For example, methods of the invention may include transmitting a light to the liquid in at least one vessel and sensing the transmitted light from the liquid in at least one vessel. In some embodiments, methods include comparing the transmitted light from the liquid in at least one vessel to a reference and adjusting the speed of the vortexer in response to the comparison. The method can include comparing the transmitted light from the liquid in at least one vessel to a reference. The reference may be transmitted light from a sample that has not been exposed to a shearing force. The reference may be transmitted light from a sample that has pre-templated instant partitions. The pre-templated instant partitions may have a defined size or range of sizes.

The invention relates to methods of generating pre-templated instant partitions, for example, as discussed in Hatori, 2018, Particle-templated emulsification for microfluidics-free digital biology, Anal Chem 90:9813-9820, which is incorporated by reference. Briefly, an aqueous mixture is prepared in a reaction tube that includes template particles and analyte (e.g., cells or nucleic acid) in aqueous fluid (e.g., water, saline, buffer, nutrient broth, etc.). An immiscible fluid (e.g., oil) is added to the tube, and the tube is agitated. The particles act to template the formation of partitions that each contain one template particle in an aqueous droplet, surrounded by the oil.

The template particles may provide oligonucleotides for target capture and barcoding of analyte, such as DNA or RNA. Barcodes specific to each template particle may be any group of nucleotides or oligonucleotide sequences that are distinguishable from other barcodes within the group. Accordingly, a partition encapsulating a template particle and a single cell, for example, provides to each nucleic acid molecule released from the single cell the same barcode from the group of barcodes. The barcodes provided by template particles are unique to that template particle and distinguishable from the barcodes provided to nucleic acid molecules by every other template particle. Once sequenced, by using the barcode sequence, the nucleic acid molecules can be traced back to the single cell based on the barcode provided by the template particle that the single cell was partitioned with. Barcodes may be of any suitable length sufficient to distinguish the barcode from other barcodes. For example, a barcode may have a length of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 nucleotides, or more.

In some methods of the invention, an index or barcode sequence may comprise unique molecule identifiers (UMIs). UMIs are advantageous in that they can be used to correct for errors created during amplification, such as amplification bias or incorrect base pairing during amplification. For example, when using UMIs, because every nucleic acid molecule in a sample together with its UMI or UMIs is unique or nearly unique, after amplification and sequencing, molecules with identical sequences may be considered to refer to the same starting nucleic acid molecule, thereby reducing amplification bias.

Template particles may be porous or nonporous. In any suitable embodiment herein, template particles may include microcompartments (also referred to herein as “internal compartment”), which may contain additional components and/or reagents, e.g., additional components and/or reagents that may be releasable into monodisperse droplets as described herein. Template particles may include a polymer, e.g., a hydrogel. Template particles generally range from about 0.1 to about 1000 μm in diameter or larger dimension. In some embodiments, template particles have a diameter or largest dimension of about 1.0 μm to 1000 μm , inclusive, such as 1.0 μm to 750 μm , 1.0 μm to 500 μm , 1.0 μm to 250 μm , 1.0 μm to 200 μm , 1.0 μm to 150 μm , 1.0 μm to 100 μm , 1.0 μm to 10 μm , or 1.0 μm to 5 μm , inclusive. In some embodiments, template particles have a diameter or largest dimension of about 10 μm to about 200 μm , e.g., about 10 μm to about 150 μm , about 10 μm to about 125 μm , or about 10 μm to about 100 μm .

In practicing the methods as described herein, the composition and nature of the template particles may vary. For instance, in certain aspects, the template particles may be microgel particles that are micron-scale spheres of gel matrix. In some embodiments, the microgels are composed of a hydrophilic polymer that is soluble in water, including alginate or agarose. In other embodiments, the microgels are composed of a lipophilic microgel. In other aspects, the template particles may be a hydrogel. In certain embodiments, the hydrogel is selected from naturally derived materials, synthetically derived materials and combinations thereof. Examples of hydrogels include, but are not limited to, collagen, hyaluronan, chitosan, fibrin, gelatin, alginate, agarose, chondroitin sulfate, polyacrylamide, polyethylene glycol (PEG), polyvinyl alcohol (PVA), acrylamide/bisacrylamide copolymer matrix, polyacrylamide /poly(acrylic acid) (PAA), hydroxyethyl methacrylate (HEMA), poly N- isopropylacrylamide (NIPAM), and polyanhydrides, poly(propylene fumarate) (PPF).

In some embodiments, the presently disclosed template particles further comprise materials which provide the template particles with a positive surface charge, or an increased positive surface charge. Such materials may be without limitation poly-lysine or Polyethyleneimine, or combinations thereof. This may increase the chances of association between the template particle and, for example, a cell which generally has a mostly negatively charged membrane.

Methods of the invention include using an optical control system to visualize the formation of pre-templated instant partitions. The optical control system may generally include a light source for transmitting light and a photodetector for detecting scattered light.

Any light source suitable for transmission of light into a liquid may be used for the device. For example and without limitation, the light source may be or include an argon lamp, deuterium lamp, halogen lamp, laser, light emitting diode (LED) mercury lamp, neon lamp, tungsten lamp, xenon arc lamp, xenon flash lamp, or combination of any of the aforementioned light sources.

Similarly, any photodetector suitable for detection of light transmitted from a liquid may be used. For example and without limitation, the photodetector may be or include a camera, charge-coupled device (CCD), complementary metal-oxide-semiconductor (CMOS) sensor, diode array, gaseous ionization detector, photodiode, photomultiplier tube, photoresistor,

phototransistor, phototube, photovoltaic cell, pinned photodiode, quantum dot photoconductor, or quantum dot photodiode.

The holder and optical system may be movable relative to each other so that light can be transmitted to and sensed from multiple vessels or multiple chambers within a vessel. For example, in some embodiments the light source and photodetector is fixed within the device, and the holder is movable in one, two, or three dimensions to adjust the position of the liquid sample in relation to the light source and photodetector. In other embodiments, the holder is fixed within the device, and the light source and photodetector are movable in one, two, or three dimensions to adjust the position of the liquid sample in relation to the light source and photodetector. In other embodiments, both the holder and optical system are movable in one, two, or three dimensions to adjust the position of the liquid sample in relation to the light source and photodetector.

In some embodiments, the device includes a control system coupled to the shearing mechanism and the optical system. The control system directs the shearing mechanism to alter the shearing energy applied to the liquid in response to the transmitted light. The control mechanism may increase or decrease the shearing energy. The control system may direct the shearing mechanism to stop applying shearing energy to the liquid when the liquid comprises an emulsion comprising substantially monodisperse droplets.

In some embodiments, the device includes a user interface that allows interaction between the user and the device. The user interface may provide output about the sample to the user. For example, and without limitation, the user interface may provide information on the optical measurement that indicates whether the sample contains monodisperse droplets or on the duration and/or intensity of shearing forces applied. The user interface may include a display. The user interface may allow the user to provide input, such as information on the desired size or range of sizes of monodispersed droplets to be obtained by shearing or on the duration and/or intensity of shearing forces applied. The user interface may include a button, dial, keyboard, lever, switch, or touchpad.

The optical system may include multiple light sources and photodetectors. For example, when a multitube vessel or multiwell vessel is used, the optical system may have a separate light source and photodetector for each tube or well. Alternatively, or additionally, the optical system may have a separate light source and photodetector for each row of tubes or wells. In some

embodiments, one light source is used in conjunction with multiple photodetectors to allow multiple measurements to be taken from a single liquid sample.

To monitor for the formation of pre-templated instant partitions, a transmitted light signal may be compared to a reference to determine whether additional shearing force should be applied to the sample to achieve monodisperse droplets. The reference may be transmitted light from a sample, e.g., the same sample or a different sample, prior to exposure of the sample to a shearing force. The reference may be transmitted light from a sample that has monodisperse droplets. The monodisperse droplets may have a defined size or range of sizes.

For example, if the first post-shearing optical measurement indicates that the emulsion contains droplets that are heterogeneous in size, the shearing and measurement steps may be repeated as many times as necessary to achieve monodisperse droplets. The decision on whether to repeat the shearing and measuring steps may rely on human input. Alternatively, or additionally, the decision may be made automatically by an algorithm. The algorithm may include predefined maximum and minimum signal intensities. Alternatively, or additionally, the maximum and minimum signal intensities may be determined via a machine-learning process.

The use of partition-templating particles to generate monodisperse droplets allows individual targets to be captured. By adjusting the concentration of targets in the starting sample in combination with the formation of droplets of uniform size, an emulsion can be produced in which all or nearly all droplets contain either zero or one target. See Makiko N. Hatori, Particle-Templated Emulsification for Microfluidics-Free Digital Biology, *Anal. Chem.* 2018, 90, 9813–9820, the content of which are incorporated herein by reference. Therefore, each droplet can serve as a reaction cell for performing a reaction on a single target.

Methods of the invention may include performing reactions in the monodisperse droplets formed by one or more of the steps described above. For example, and without limitation, the methods may include one or more of cell lysis, nucleic acid amplification, reverse transcription, or sequencing. Performing reactions in droplets formed according to one or more of the steps described above may include adjusting the temperature of the emulsions. For example, the methods may include heating and/or cooling the emulsions by convection heat transfer.

The methods and devices described herein are particularly amenable for use with amplification reactions. Any amplification reaction known in the art may be conducted on a released nucleic acid inside a pre-templated instant partition. Exemplary amplification

techniques include polymerase chain reaction (PCR), reverse transcription-PCR, real-time PCR, quantitative real-time PCR, digital PCR (dPCR), digital emulsion PCR (dePCR), clonal PCR, amplified fragment length polymorphism PCR (AFLP PCR), allele specific PCR, assembly PCR, asymmetric PCR (in which a great excess of primers for a chosen strand is used), colony PCR, helicase-dependent amplification (HDA), Hot Start PCR, inverse PCR (IPCR), in situ PCR, long PCR (extension of DNA greater than about 5 kilobases), multiplex PCR, nested PCR (uses more than one pair of primers), single-cell PCR, touchdown PCR, loop-mediated isothermal PCR (LAMP), and nucleic acid sequence based amplification (NASBA). Other amplification schemes include: Ligase Chain Reaction, Branch DNA Amplification, Rolling Circle Amplification, Circle to Circle Amplification, SPIA amplification, Target Amplification by Capture and Ligation (TACL) amplification, and RACE amplification.

In certain embodiments, the reaction is QPCR or digital PCR. Digital PCR is an amplification reaction in which dilute samples are divided into many separate reactions. See for example, Brown et al. (U.S. patent numbers 6,143,496 and 6,391,559), Vogelstein et al. (U.S. patent numbers 6,440,706, 6,753,147, and 7,824,889), as well as Larson et al (U.S Patent Application No. 13/026,120), Link et al. (U.S. Patent Application Nos. 11/803,101, 11/803,104, and 12/087,713), and Anderson et al (U.S. Patent No. 7,041,481, which reissued as RE41,780), the content of each of which is incorporated by reference herein in its entirety. The distribution from background of target DNA molecules among the reactions follows Poisson statistics and at a terminal or limiting dilution, the vast majority of reactions contain either one or zero target DNA molecules.

Example 1

The ability of a device 601 of the invention to rapidly control sample temperature was evaluated.

FIG. 7 shows a graph 701 demonstrating changes in temperature over time inside a device during convection heating. Shown is a graph of temperature changes collected in a tube 705 and a hot chamber 711. The Y axis of the graph corresponds with temperature in degrees Celsius. The X axis shows time in seconds. The smooth, steep slope of lines corresponding to the

tube 705 and the hot chamber 711 show that devices of the invention provide for efficient, rapid changes in temperature using forced convection.

FIG. 8 shows a graph 801 demonstrating changes in temperature over time inside a device during convection cooling.

Incorporation by Reference

References and citations to other documents, such as patents, patent applications, patent publications, journals, books, papers, web contents, have been made throughout this disclosure. All such documents are hereby incorporated herein by reference in their entirety for all purposes.

Equivalents

Various modifications of the invention and many further embodiments thereof, in addition to those shown and described herein, will become apparent to those skilled in the art from the full contents of this document, including references to the scientific and patent literature cited herein. The subject matter herein contains important information, exemplification, and guidance that can be adapted to the practice of this invention in its various embodiments and equivalents thereof.

Claims

What is claimed is:

1. A device for generating pre-templated instant partitions, the device comprising:
a vortexer for shearing a liquid contained in at least one vessel into a plurality of pre-templated instant partitions;
a holder for securing the at least one vessel to the vortexer; and
a temperature control unit operable to adjust a temperature of the liquid by convection.
2. The device of claim 1, wherein the holder comprises a clamp that can accommodate one or more vessels of different sizes or shapes.
3. The device of claim 1, wherein the holder is operable to hold the at least one vessel in a substantially vertical position while the vortexer shears the liquid.
4. The device of claim 1, wherein the holder is operable to move between securing the at least one vessel in a substantially horizontal position and a substantially vertical position while the vortexer shears the liquid.
5. The device of claim 1, wherein the vortexer is operable to shear the liquid by vortexing the at least one vessel at a speed of at least 5,000 revolutions per minute.
6. The device of claim 1, wherein the temperature control unit comprises a first conduit for heating a fluid within the temperature control unit to a first temperature.
7. The device of claim 6, wherein the temperature control unit comprises a second conduit for cooling the fluid to a second temperature.

8. The device of claim 7, wherein the temperature control unit comprises a valve, the valve comprising a first position and a second position, wherein, when the valve is in the first position, fluid is blocked from flowing through the first conduit.
9. The device of claim 1, wherein the device further comprises an optical system, the optical system comprising:
a light source positioned to transmit light into the at least one vessel; and
a photodetector positioned to sense light from the liquid contained in the at least one vessel.
10. The device of claim 9, further comprising a control system coupled to the vortexer and the optical system.
11. The device of claim 10, wherein the control system controls a speed of the vortexer to alter a shearing energy applied to the at least one vessel in response to the transmitted light.
12. The device of claim 11, wherein the control system directs the vortexer to stop applying the shearing energy to the at least one vessel when the liquid is substantially monodisperse.
13. The device of claim 1, wherein the device comprises at least one component manufactured by 3D printing.
14. A method for generating pre-templated instant partitions, the method comprising:
contacting at least one vessel containing a liquid with a device comprising:
a vortexer;
a holder configured to secure the at least one vessel to the vortexer; and
a temperature control unit in fluidic communication with the holder;
operating the device to shear the liquid inside the at least one vessel into a plurality of pre-templated instant partitions, wherein each of the plurality of pre-templated instant partitions comprises one or zero analyte; and
heating, with the temperature control unit, the at least one vessel by convection.

15. The method of claim 14, further comprising vortexing the liquid at a speed of about 5,000 revolutions per minute.
16. The method of claim 14, further comprising providing a fluid of a predetermined temperature to the holder via a first conduit.
17. The method of claim 16, further comprising providing, to the holder, a second fluid of a second predetermined temperature via a second conduit.
18. The method of claim 14, further comprising:
transmitting a light to the liquid in the at least one vessel; and
sensing the transmitted light from the liquid in the at least one vessel.
19. The method of claim 18, further comprising:
comparing the transmitted light from the liquid in the at least one vessel to a reference;
and
adjusting a speed of the vortexer in response to the comparison.
20. The method of claim 17, further comprising processing analyte inside the one or more of the pre-templated instant partitions by providing the first fluid to the holder thereby heating the at least one vessel.

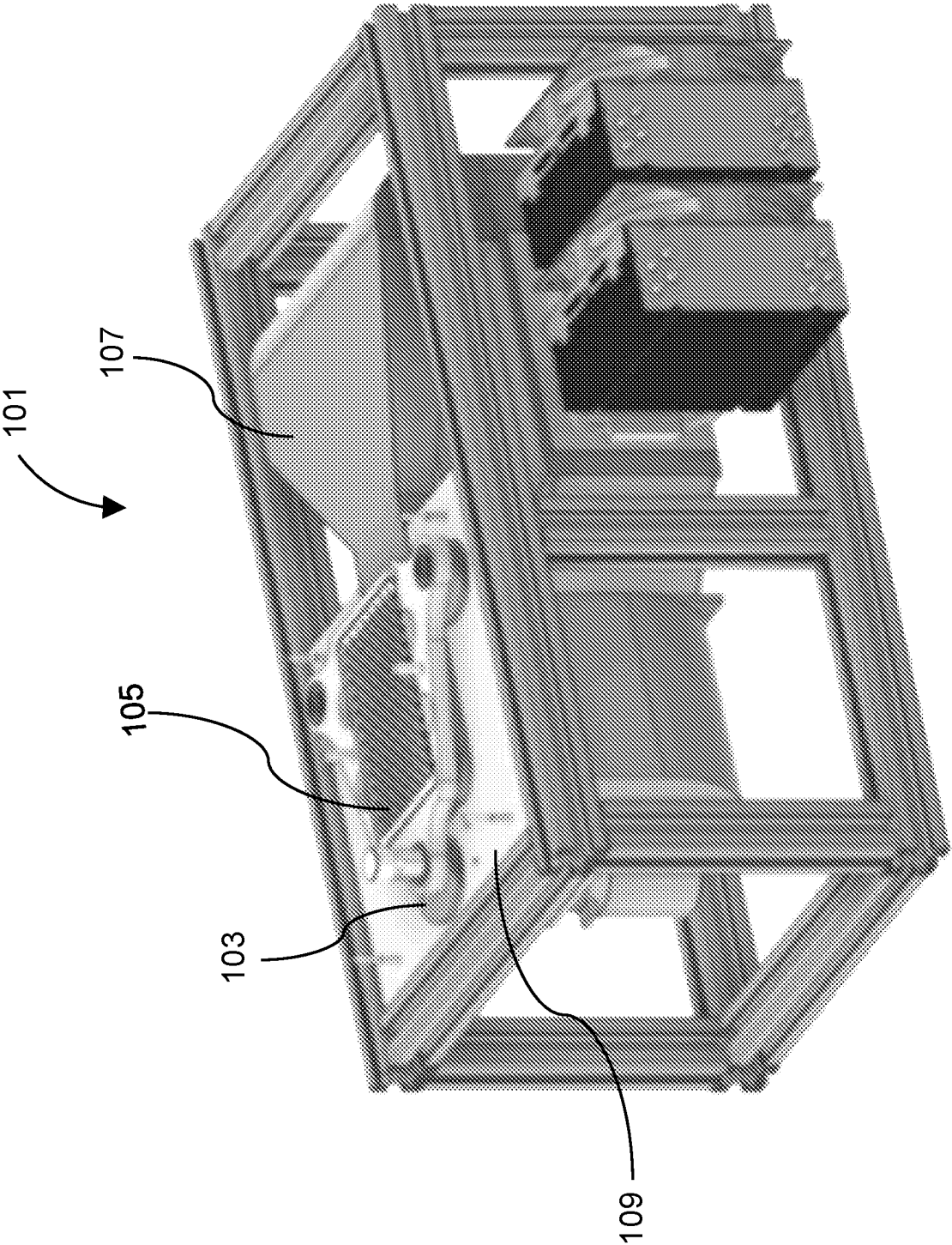


FIG. 1

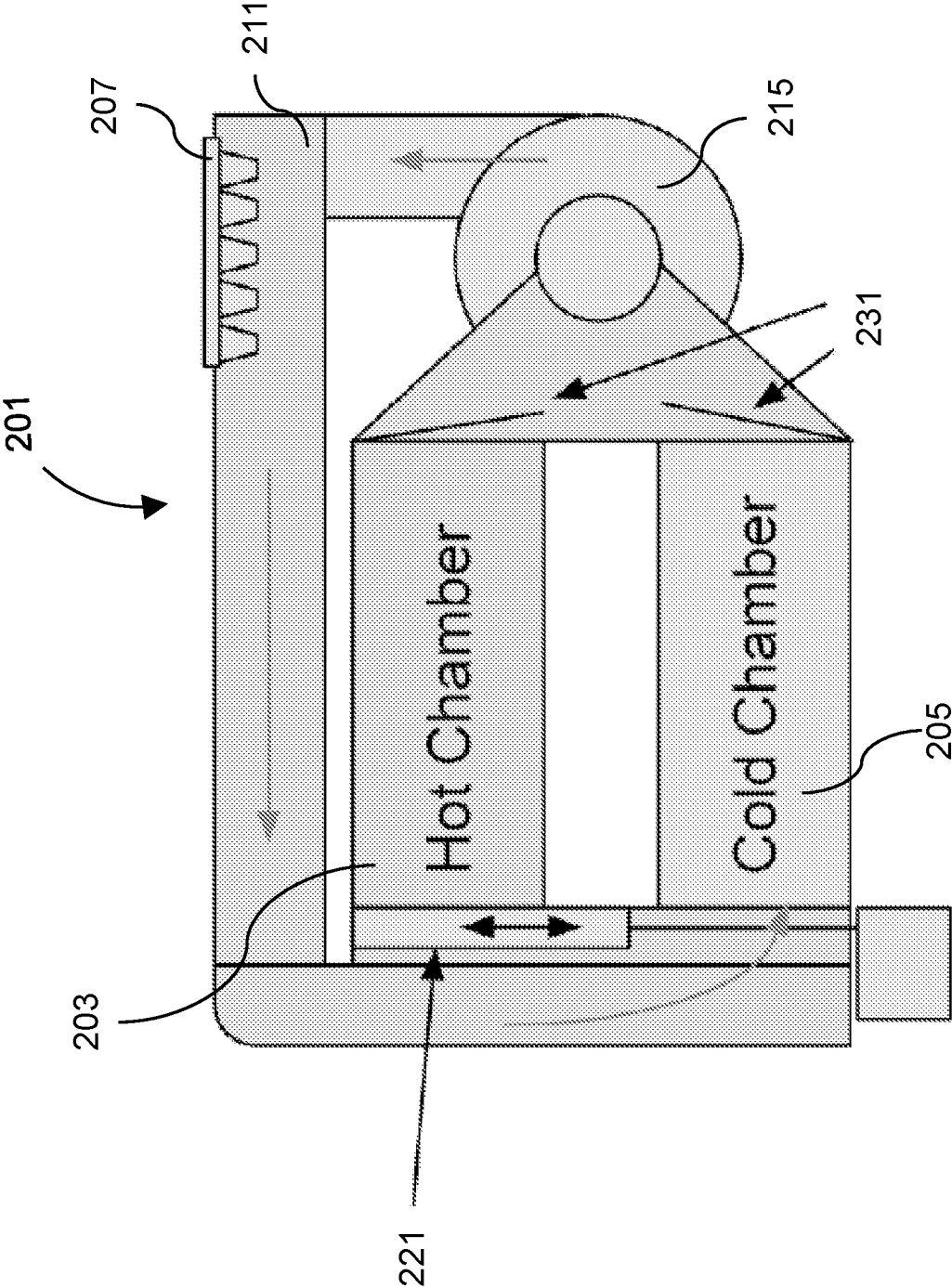


FIG. 2

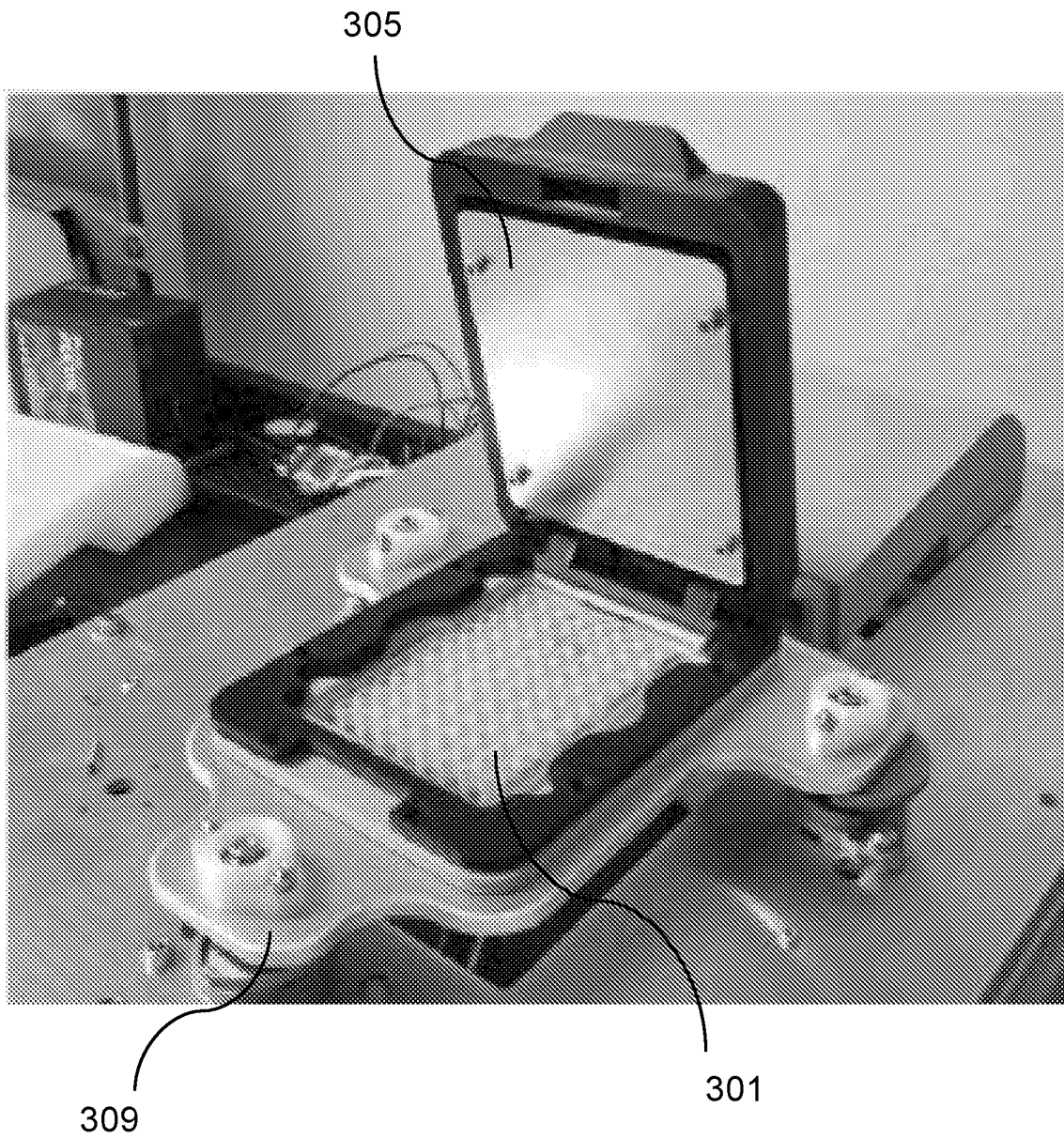


FIG. 3

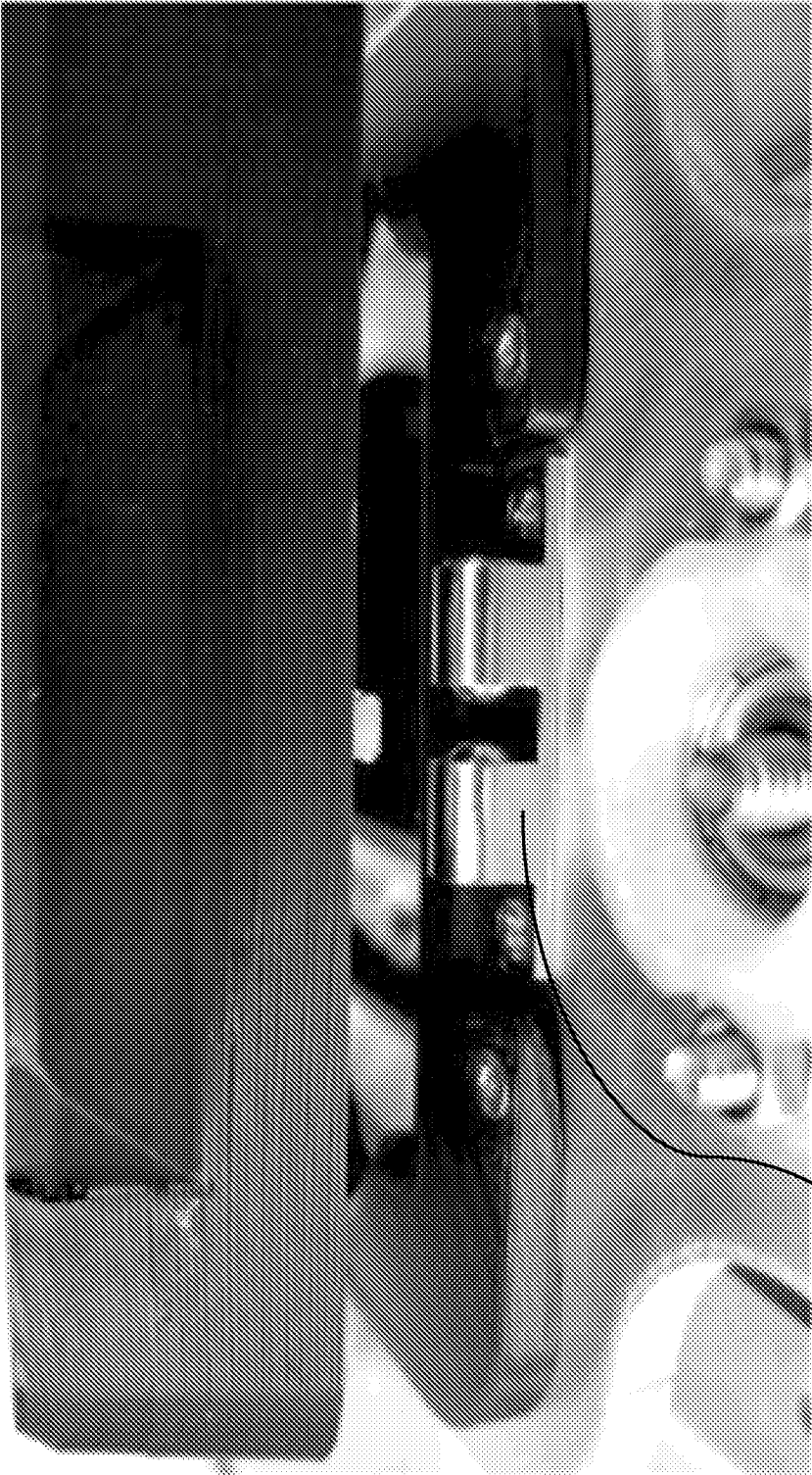


FIG. 4

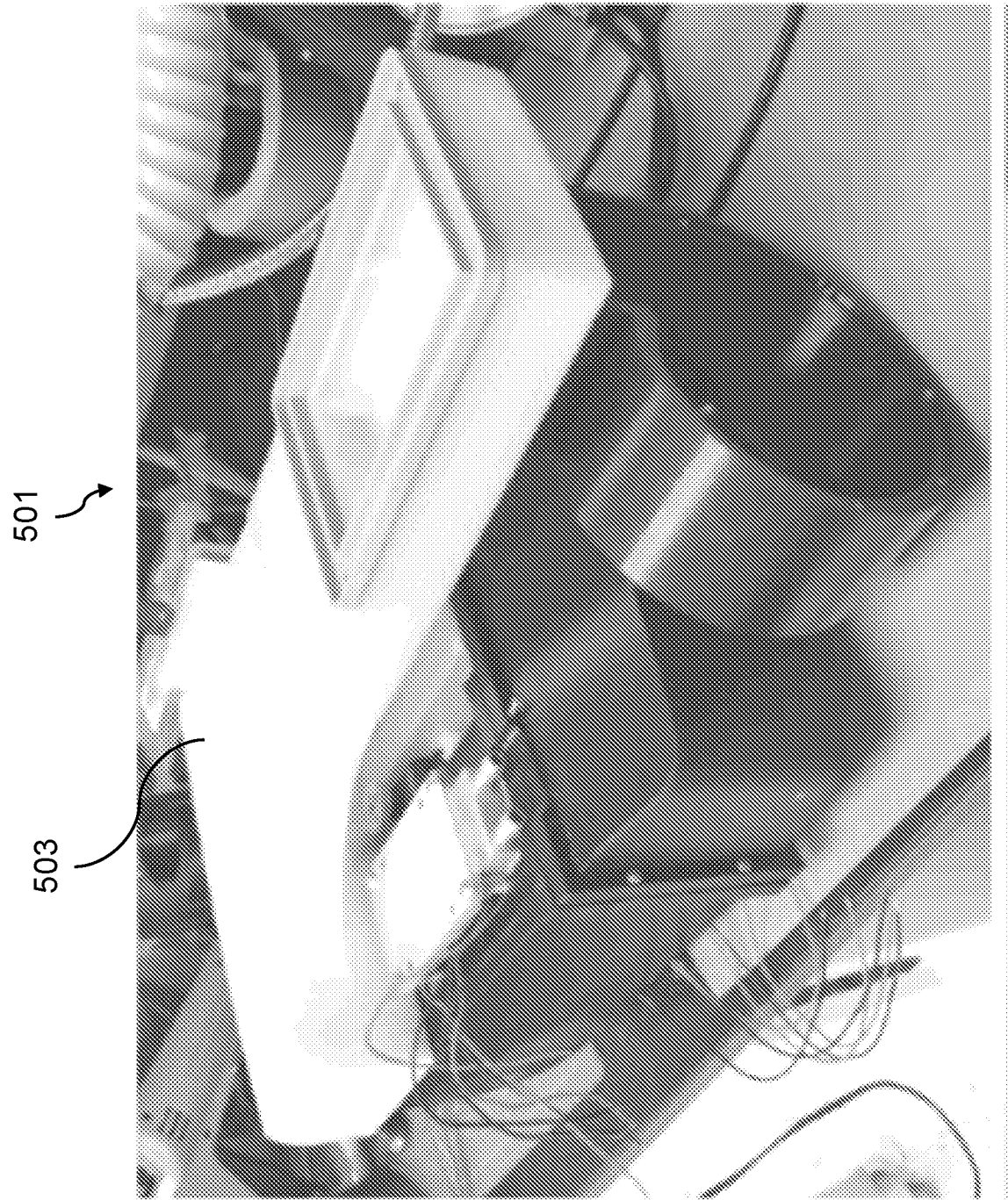


FIG. 5

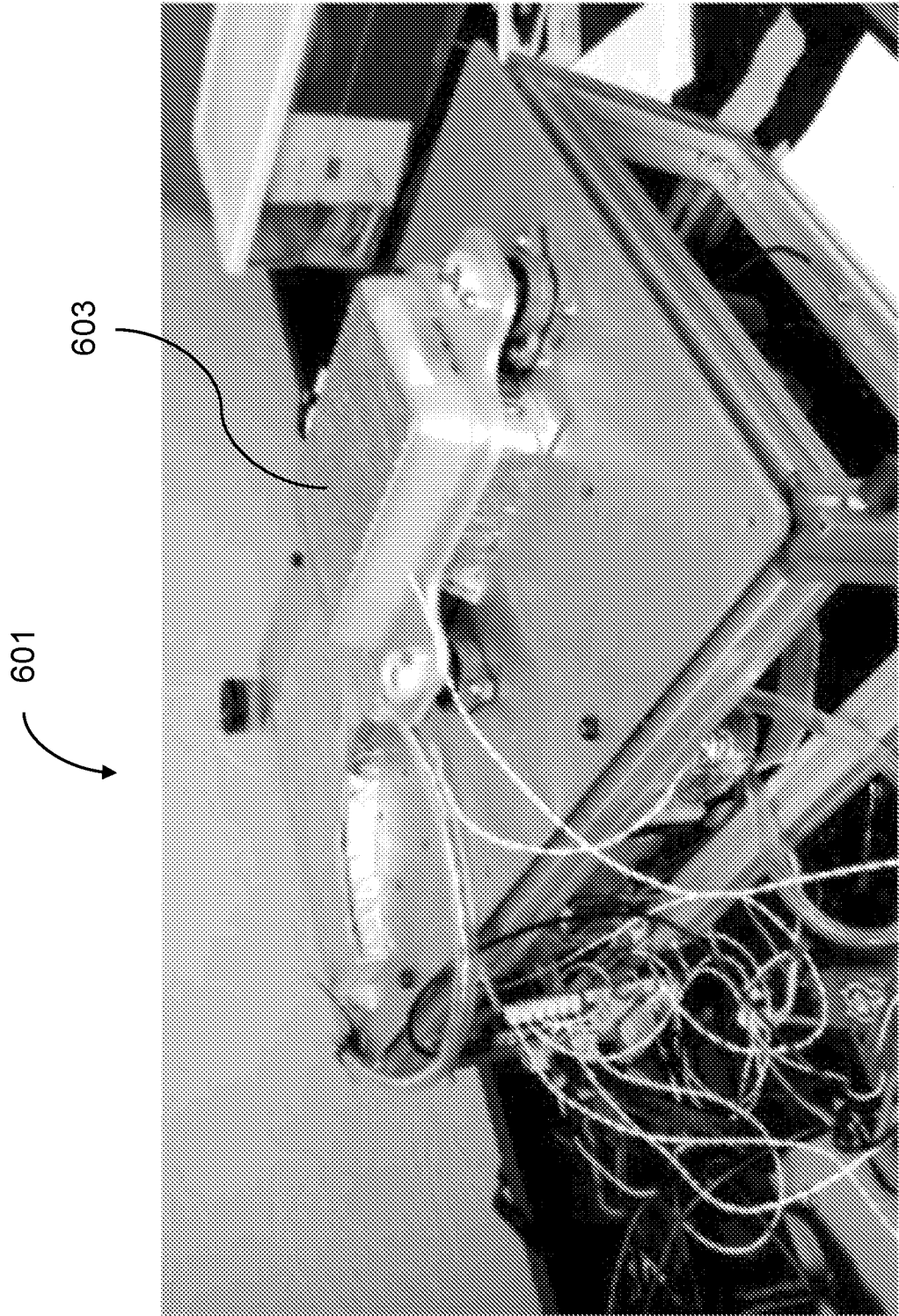
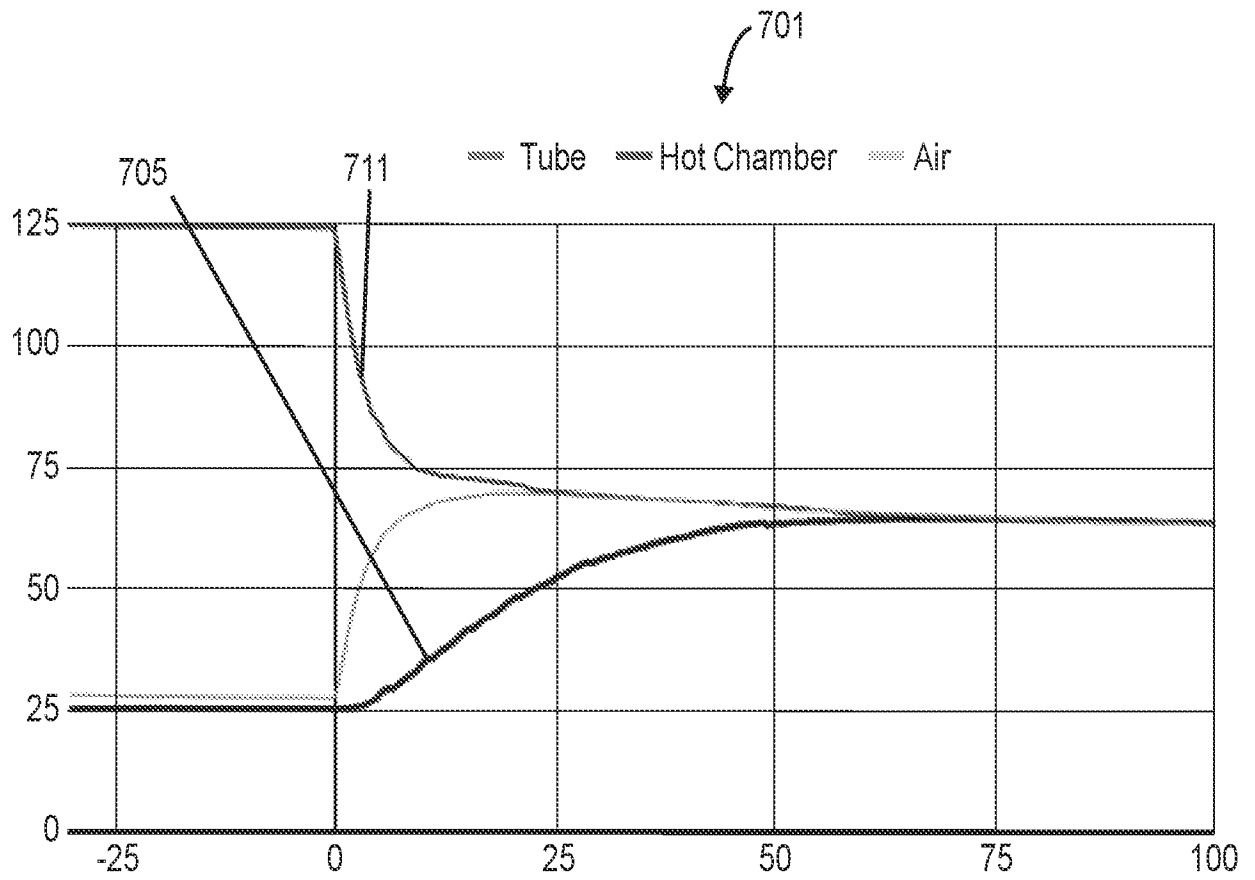
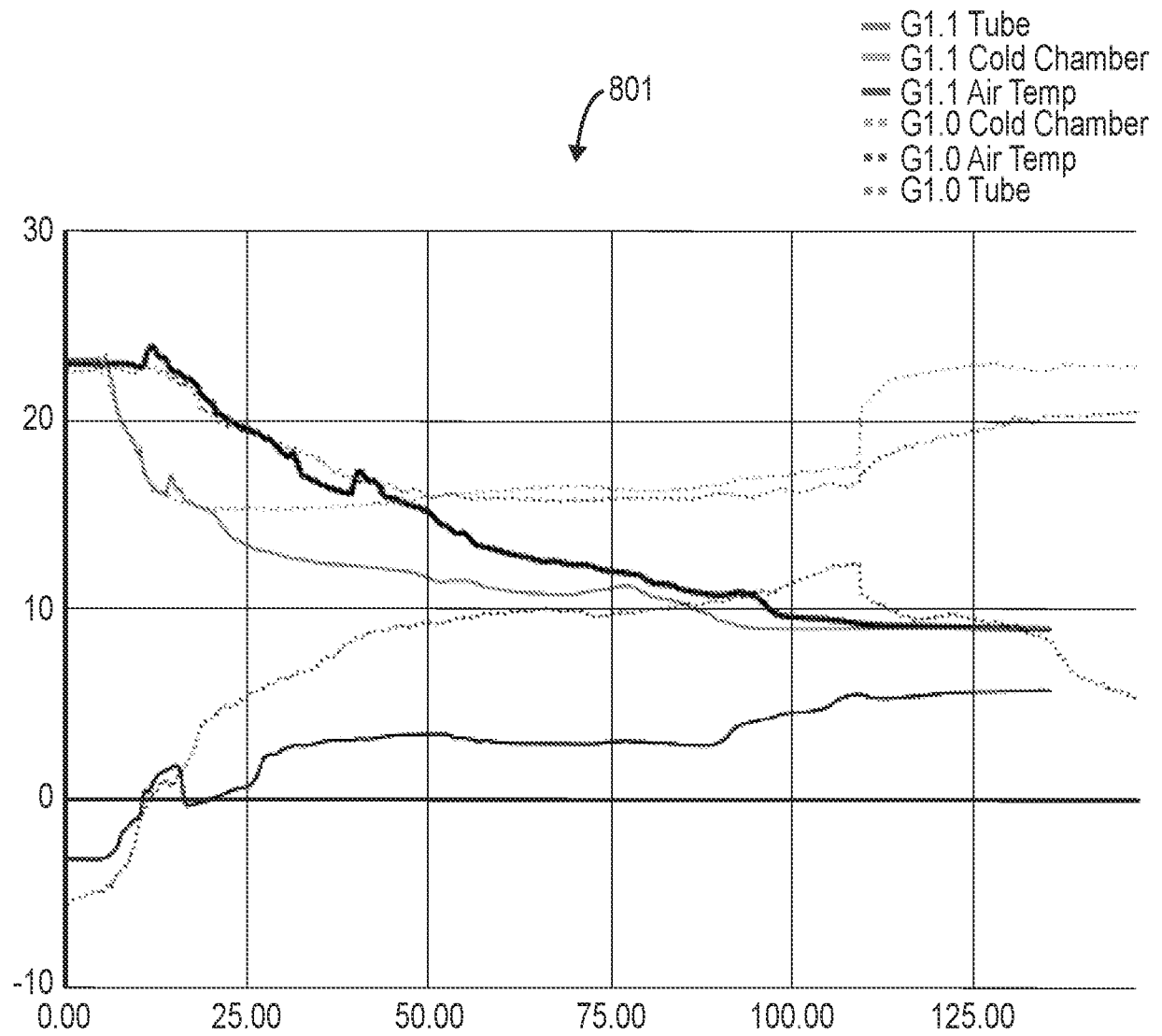


FIG. 6

**FIG. 7**

**FIG. 8**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 22/36464

A. CLASSIFICATION OF SUBJECT MATTER

IPC - INV. B01F 25/10, G01N 1/40, G01N 33/543, B01F 31/20 (2022.01)

ADD. G01N 1/38 (2022.01)

CPC - INV. G01N 1/4077, G01N 2035/00524, B01F 25/10, G01N 1/40, G01N 33/543, B01F 31/20

ADD. B01L 2300/18, G01N 15/1056, B01F 2101/23, G01N 2015/1481, B01F 31/201, G01N 1/38

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	US 2020/0261879 A1 (The Regents of the University of California) 20 August 2020 (20.08.2020), entire document, especially Figs 2-3; para [0028]-[0029], [0044], [0053], [0059], [0069], [0108]-[0109], [0119], [0145], [0187]-[0188], [0328]-[0329]	1, 3 --- 2, 5-7, 13 --- 4, 8-12, 14-20
Y --- A	US 2016/0250608 A1 (Heathrow Scientific LLC) 1 September 2016 (01.09.2016), entire document, especially Figs 1-3, 5; para [0019]-[0020], [0025], [0027]-[0028]	2 --- 4
Y	US 5,813,759 A (Gebrian) 29 September 1998 (29.09.1998), entire document; col 7 ln 42-52	5
Y --- A	US 4,701,415 A (Dutton et al.) 20 October 1987 (20.10.1987), entire document, especially Figs 1-3, 5; col 4 ln 27; col 7 ln 21-29, col 8 ln 3-9, 31-35, 52-58	6-7 --- 8
Y	"Design and Fabrication of Low Cost Vortex Mixer Using Additive Manufacturing" (Patel et al.) December 2019 (12.2018), entire document, especially pg 1 "INTRODUCTION" section para 4	13
A	"How to Maintain a Constant Temp in Your CO2 Incubator" (Biocompare) 17 January 2013 (17.01.2013) [online] retrieved from <URL: ">https://www.biocompare.com/Editorial-Articles/126328-Incubators/#:~:text=A jacket of water circulates,thermal buffer against outside air.> , entire document, especially pg 2 para 2, pg 3 para 1, 6	1, 14

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

20 September 2022

Date of mailing of the international search report

OCT 11 2022

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Authorized officer

Kari Rodriguez

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 22/36464

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	US 2021/0215591 A1 (Fluent Biosciences) 15 July 2021 (15.07.2021), entire document	1-20