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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 October 2012 (18.10.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/142192 A2

(51) International Patent Classification:
B05C 5/02 (2006.01) *C12Q 1/68* (2006.01)
B05B 9/03 (2006.01)

(21) International Application Number:
PCT/US2012/033165

(22) International Filing Date:
11 April 2012 (11.04.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/474,076 11 April 2011 (11.04.2011) US

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

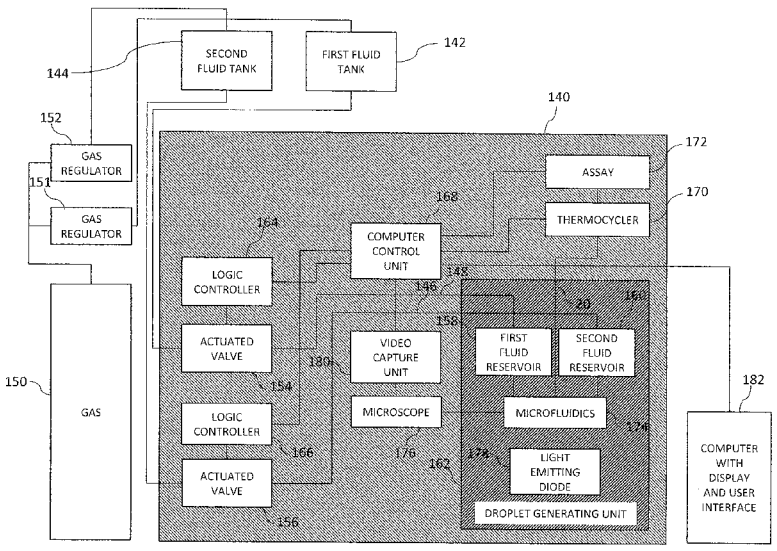
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

(54) Title: APPARATUS AND METHOD FOR MAKING UNIFORM EMULSIONS

FIG. 10



(57) Abstract: An apparatus and method are provided for the controlled production of a known number of small droplets having a narrow size distribution. The present invention provide a method for the merging of droplets using an apparatus that controls the droplet formation.

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APPARATUS AND METHOD FOR MAKING UNIFORM EMULSIONS

CROSS-REFERENCE TO RELATED APPLICATION(S)

The present application claims priority to and the benefit of U.S. Provisional Application Serial No. 61/474,076 filed on April 11, 2011, the entire contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0001] The present invention teaches improved systems and processes for emulsion preparation and microscale mixing. Many embodiments of the present invention are directed toward the amplification of genetic material as an improved means for rapid and uniform sample preparation for emulsion PCR.

INTRODUCTION

[0002] PCR is well known to the biological arts as an important means of amplifying nucleic acids, usually in preparation for sample sequencing. Emulsion PCR is a variation of traditional PCR in which the reagents comprising a reaction are isolated in aqueous droplets within an oil phase.

[0003] As sequencing devices are handling ever increasing sample numbers, current techniques and systems for preparing emulsions of nucleic acid samples for PCR amplification prior to sequencing are reaching their performance limits. Emulsion PCR is gaining importance as a method of rapidly amplifying genetic material prior to performing sequencing analysis.

[0004] For a given PCR reaction protocol, a droplet will contain all of the reagents necessary to carry out the PCR reaction, and so may contain linkers, beads, primers, DNA templates, and other chemistries required for the PCR reaction or for downstream assays.

[0005] In some instances, droplets may first be prepared containing some, but not all, of the reagents comprising a PCR reaction, so that they may undergo separate processing steps in isolation of the other reagents, or so that they may be set aside for later use. Collections of droplets containing nucleic acid samples, primers, or other elements of a PCR reaction are sometimes called droplet libraries.

[0006] In a non-limiting example PCR protocol, droplets containing a partial mixture of PCR reagents are combined with droplets from a different collection of droplets containing a complementary mixture of chemistries capable of supporting a PCR reaction. Thus, PCR

reagents may be divided into multiple droplet collections as emulsions, and those separate emulsions may be subjected to additional processing steps, which may include mixing chemistries by merging droplets together.

[0007] It will be appreciated by those familiar with PCR and in the arts of laboratory sample preparation and assays, protocols, and reagent kits, that every laboratory exercise is directed toward a particular objective, and that the contents and use of droplets within the scope of a particular laboratory exercise will depend upon, and vary according to its objective. Chemistries within droplets will depend upon their application, and the present invention addresses the need to prepare, and in some cases mix, droplets containing different chemistries.

[0008] In many sample preparation applications, and particularly in emulsion PCR, droplet size is a critical parameter to optimize the reaction. In order for droplets to function efficiently, as micro reactors for a given PCR protocol, they need to be as monodisperse as possible, i.e., have as narrow a size distribution as possible. Droplet uniformity is essential to optimizing the physio/chemical parameters of a PCR reaction and is useful for the prevention of the common failure modes associated with PCR such as primer dimers.

[0009] As with other volumetric means of dispensing reagents, it is the aim of such tools as measuring cups, pipettes or graduated syringes, and the invention, to deliver uniform and repeatable volumes of reagent. For any reagent supplied at a given concentration, the amount of the reagent contained within a droplet is directly proportional to its volume. It follows that reducing dimensional variation of droplets is equivalent to reducing the variation of reagent dispensing.

[0010] It follows that droplet size uniformity allows for the most efficient use of reagents. In one embodiment of the invention, droplet size uniformity allows for optimized Poisson distributions. For many applications, it is desirable to have only one, or just a few, specific elements within the one or more droplets comprising an emulsion.

[0011] For example, several embodiments of the present invention are as an emulsion PCR sample preparation platform. In some applications it is ideal to have one capture bead contained within each droplet comprising an emulsion. Poisson distributions may be used to determine the precise volume of a droplet at which an inclusion event is most likely to occur. Control over droplet size and uniformity is thus essential to optimizing Poisson distributions for a given event.

[0012] Another benefit of uniform emulsions is that they are more stable than polydisperse emulsions. Large or inconsistently sized droplets comprising an emulsion will tend to agglomerate into larger units as a result of secondary instabilities in solution.

[0013] Droplet size distribution is a critical parameter with respect to maintaining uniform conditions within the plurality of microreactors comprising an emulsion. Although various methods of making small droplet emulsions are available, from a process perspective, the formation of stable emulsions with a narrow and predictable droplet size distribution remains a challenge.

[0014] A first approach to commercial emulsion production utilizes shear force and/or ultrasonic energy dispersion to reduce the size of the droplets to an approximated average. Shear forces may be applied to a mixture of fluids by a variety of methods. For example, one commercial device for preparing PCR emulsions employs a rotating paddle immersed in a reagent mixture. Another means of generating shear force is by repeatedly passing the emulsion through a membrane or by directing the flow of fluids against a surface at high velocity or at high pressure. Advantageously, the approach produces droplets rapidly and in high concentration. A significant disadvantage is that methods employing shear force typically result in emulsions with a wide range of droplet sizes.

[0015] A second common approach to generating more uniform droplets for PCR is the use of microfluidic T-junction chips. Such chips operate by directing two or more fluids into microfluidic channels in such a way that at least one of the fluids emerges as droplets. T-junction chips can produce substantially uniform droplets, but they do not operate quickly enough to support the most common applications of PCR and have been thus limited to smaller throughput processes, such as re-sequencing. To put this in perspective, the present invention can generate 20 million uniform droplets in about 3 minutes whereas a RainDance™ system would require 2 hours. As next-gen sequencing platforms like Ion Torrent™ move toward higher sample densities, sample droplet production rate becomes increasingly important.

[0016] The production of large emulsion batches with very true homodispersity (i.e., substantially monodisperse), has not been reported, and there is no protocol available in the literature for the production of such droplets, let alone a protocol for achieving narrow size distributions under the demanding conditions and in the large volumes required for emulsion PCR sample preparation.

[0017] Another limitation of using emulsions is that there are limited means of mixing and merging droplets.

[0018] The inability to create droplets quickly and uniformly and the limited means of mixing and merging droplets has limited the commercial utility of emulsion PCR and other biological assays. Polydisperse emulsions translate into wasted biological material and reagents. Accordingly, there exists a need for a robust means of emulsion manufacturing, particularly emulsions of PCR reagents with diameters ranging from 1-50 micrometers, and the corresponding means to mix and merge droplets.

[0019] Another limitation of current emulsion production systems and droplet manufacturing techniques is that it is exceedingly difficult if not impossible to know exactly how many droplets are in a given emulsion. This is because available manufacturing processes are batch processes that form emulsions by mechanical agitation that results in the wide range of droplet sizes. Batch yield and droplet characterization must be performed after emulsion production. A linear digital droplet manufacturing is a significant improvement over the art, as it would enable PCR reagent and other emulsions to be produced with great accuracy and precision.

[0001] The above introduction is intended to improve understanding of the invention described in this application, and may contain information and descriptions that do not constitute prior art, but rather form a part of the described invention. Accordingly, this introduction should not be read as a "Background" of the invention in that the introduction is not intended to solely provide an overview of the related art and the state of the art, but rather should be read in conjunction with the following detailed description as an explanation and description of embodiments of the present invention.

SUMMARY OF THE INVENTION

[0020] The present invention provides a method and apparatus for the controlled production of a known number of small droplets having a narrow size distribution. The present invention also teaches the merging of droplets by timing the production of droplets, using emulsion PCR as an example application.

[0021] According to a first aspect of the invention, the method comprises (a) forming droplets of a first liquid in a laminar flow of a second liquid, each droplet having a volume of between 1 fL and 1nL, and wherein the first and second liquids are, at most, sparingly soluble (more preferably, substantially immiscible) in one another, and the first liquid contains PCR reagents dissolved, dispersed, or suspended therein. Laminar flow is defined as a non-turbulent fluid flow.

[0022] The method and apparatus are particularly well-suited for making a substantially monodisperse collection of droplets having a mean volume of from 1 fL to 1 pL, for example,

a collection of droplets having a normal distribution of diameters, with a mean diameter D of from 1 micrometer to 100 micrometers, and a coefficient of variation of 3%.

[0023] Droplet size control is fundamental to optimizing an emulsion PCR reaction. For next-generation sequencing sample preparation, aqueous droplets contain all the reagents necessary for PCR; bases, polymerase, a bead with at least one primer bound, and generally a single initial template DNA molecule, and sometimes primer pairs. Obtaining the correct quantity of each reagent in the micro reactor droplet is essential. In one embodiment of emulsion PCR, Poisson statistics describe the probability P of a single bead to be captured within a single droplet:

$$P(x;\lambda) = (\lambda^x e^{-\lambda})/x!$$

[0024] Where bead reagent concentration is matched perfectly to droplet volume, there is about a 40% chance of a single bead present in a given droplet. In other words, Poisson distributions show us that in an idealized standard reaction that requires only a single bead per droplet, 60% of PCR reagents do not contribute to the reaction. Importantly, even a small variation in droplet diameter translates into substantial reagent waste. For example, if the target droplet diameter is 10 μm, but the device is producing 15 μm droplets, then the volume of those larger droplets is 3x the anticipated volume and only 15% of the droplets will contain a single bead; 80% of droplets will contain two or more beads. Moreover, even those droplets that hold a single bead will still contain 3x the calculated volume of the other PCR reagents. It is significant that wasted reagents are not removed from the sample prep stream at this point but instead contribute to sample bulk and ultimately to reduced data quality. While this may seem wasteful, it is important to note that emulsion PCR reactions usually operate at less than 1% efficiency owing to sample material and reagents being processed into polydisperse droplets. Emulsion PCR is a robust process, however, and overcomes poor sample preparation by processing even larger numbers of droplets and using software algorithms to cull bad data points.

[0025] Accurately dimensioned droplets minimize reagent consumption, enable smaller total emulsion sample volumes, fewer purification steps, and cleaner data.

[0026] The ideal solution to emulsion PCR (emPCR) sample prep is to routinely produce nearly perfectly uniform droplets. The uniformity of a droplet population is described by the Coefficient of Variation (CV), which is the ratio of the standard deviation, σ, to mean, μ:

$$C_v = \sigma/|\mu|$$

[0027] In one embodiment of the invention, substantially uniformly sized droplets are generated using nozzles, actuators, software, and electronics. By controlling the electric impulses to the actuator(s) via a computer control system, very precisely sized volumes of fluid are ejected as droplets into a laminar flow of a substantially immiscible, or at least no more than sparingly soluble, liquid. For example, droplets for PCR are made by ejecting volumes of aqueous solvent containing PCR reagents dissolved, dispersed or suspended therein through the nozzles of the apparatus into a laminar flow of water or other non-aqueous medium in the transverse liquid channels, and collecting the resulting droplets for further processing. Advantageously, the droplets' narrow size distribution makes each droplet functionally interchangeable, and the number or concentration of reagents within the droplets can be described by a Poisson distribution.

[0028] In another embodiment of the invention, library droplets are formed at high speed for later use.

[0029] In another embodiment of the invention, droplets containing all of the reagents needed by a PCR reaction are produced.

[0030] In another embodiment of the invention, the PCR reagents are formed in two or more complementary droplet emulsions and the reaction does not take place until the different droplet types are combined by timing of droplet formation.

[0031] In yet another embodiment of the invention, the protocol requires that a single bead is present in a droplet.

[0032] In another embodiment of the invention, the protocol does not include a bead.

[0033] In another embodiment of the invention, the droplets are driven straight into a theromcycling chip.

[0034] In another embodiment of the invention, a droplet library is created. The droplet library can contain any mixture of reagents, but that does not contain all of the reagents necessary to initiate or complete a PCR reaction.

[0035] In another embodiment of the invention the system is built into the front end of a PCR unit.

[0036] In another embodiment of the invention, the system is a standalone bench top unit for the production of emulsions.

[0037] In another embodiment, the device is paired with an automated fluid handling system. An automated fluid handling system may be used to either load fluid into the device, or offload fluids from the unit.

[0038] In another embodiment, the droplet generating unit is built into a microfluidics chip. Alternatively, the microfluidics unit is made into a disposable microfluidics chip.

[0039] In another embodiment, the droplet generating unit is built into a disposable microfluidics chip with a disposable component. In yet another embodiment of the invention, the disposable component may contain any one or all of the reagent fluids comprising a PCR reaction. In another embodiment of the invention, the disposable component comprises both the microfluidics chip necessary to create the droplet emulsion as well as fluid reservoirs containing all of the fluid reagent fluids comprising a PCR reaction. The disposable microfluidics chip may include all the components of the droplet generating unit including the first and second reservoirs 158,160, the microfluidics unit174 and the light emitting diode (LED) 178, or the microfluidics unit alone, or in combination with one of the LED or the two reservoirs.

BRIEF DESCRIPTION OF THE DRAWINGS

[0040] Various aspects, embodiments, and advantages of the invention will become better understood when reference is made to the accompanying drawings, wherein:

[0041] FIG. 1 is a schematic diagram of a nozzle ejecting a droplet of liquid into a laminar flow of a second liquid, according to embodiments of the present invention;

[0042] FIG. 2 is a schematic, partially exploded view of one embodiment of an apparatus for making substantially uniformly sized droplets in accordance with the present invention;

[0043] FIG. 3 is a plan view of a microfluidics channel plate, a component of embodiments of the present invention;

[0044] FIG. 4 is a close-up of a portion of the place of FIG. 3 denoted by circle A;

[0045] FIG. 5 is a more highly magnified close-up of a portion of the plate shown in FIG. 4 taken along the line B-B, according to embodiments of the present invention;

[0046] FIG. 6 is an exaggerated schematic view of a nozzle ejecting droplets into a microfluidics channel according to embodiments of the present invention;

[0047] FIG. 7 is a sectional view of a portion of FIG. 6, taken along line C-C;

[0048] FIG. 8 is a schematic diagram of one embodiment of a pervaporation unit used with in the practice of the invention;

[0049] FIG. 9 is a schematic diagram of a fluid flow plate according to one embodiment of the invention;

[0050] FIG. 10 is a schematic diagram illustrating other components (reservoirs, pumps, logic controls, etc.) of one embodiment of an apparatus according to the present invention;

[0051] FIG. 11 is a schematic diagram illustrating a PCR reaction protocol involving the production and merging of reagent droplets in accordance with the practice of the present invention; and

[0052] FIG. 12 shows several exemplary sketches of fluid pathways for droplet merging, according to embodiments of the present invention.

DETAILED DESCRIPTION

[0053] According to the various embodiments of the invention, a PCR emulsion is created when aqueous droplets are formed in a non-aqueous carrier fluid. The emulsion may contain any number of droplets, depending on the application. Moreover, the droplets will contain the active reagents necessary for the PCR reaction, and the composition of active reagents will depend on the application.

[0054] In some embodiments, the aqueous droplets of a first emulsion preparation will contain any one or a mixture of the following standard emulsion PCR reagents: template beads, polymerase, nucleic acids, primers, salts, and reporters. In some embodiments a second and third aqueous emulsion preparation will contain one or a mixture of the same reagents. In some embodiments, droplets from the first, second or third emulsions are mixed together.

[0055] In one embodiment, the emulsification process takes place in a standalone emulsifier. In another embodiment the emulsifier unit is in fluid communication with an adjacent sample preparation system. In another embodiment it is a part of a continuous flow PCR system.

[0056] The combination of materials and stepwise sample preparation operations required by any given PCR are specific to that assay and will be known to the system operator. The

assay design will include, for example, the appropriate chemistries and concentrations for each reagent used in the reaction.

[0057] The present invention teaches emulsion steps that comprise the workflow required to complete the various PCR applications. Rapid and uniform microdroplet production using an actuated nozzle to eject a first fluid into the laminar flow of a second fluid. The monodisperse droplet emulsions exhibit greater stability than polydisperse emulsions and are particularly useful for PCR reactions. Extreme monodispersity creates the ideal circumstances for emPCR reagent stability.

[0058] Within the device a first fluid enters a chamber in proximity to an actuator element and in fluid communication with a nozzle such that an electrically induced displacement of the actuator will apply a force on the first fluid with respect to the chamber surface and proximal end of the nozzle feature. An electrical potential is applied to an actuator element which causes the first fluid to be ejected into the laminar flow stream of a second fluid.

[0059] EXAMPLES

[0060] Example 1: One embodiment of the invention is an emulsifier unit. The device is used to combine two or more fluids into an emulsion. The device is comprised of a first fluid inlet port in fluid communication with a pressure chamber in fluid communication with the inlet side of one or more nozzles, a second fluid inlet port in fluid communication with the outlet side of the nozzle or nozzles, and each nozzle is connected to one or more nozzle actuators connected to an actuator control system, the computer control system. The device is divided into one or more temperature zones in order to maintain uniform viscosity and fluid flow. A product outlet port allows an emulsion stream to exit the unit.

[0061] Example 2: A device as in Example 1 that also contains a charged-couple device (CCD) camera directed at the emerging droplet stream. By illuminating the droplet stream with a light emitting diode (LED) strobing at the same rate as droplet production, a stable image of the droplet stream is displayed in real time, which will appear as a series of static spheres at fixed positions within the channel space. Capturing motion of droplets within a channel can be achieved by strobing an LED that it is slightly out of phase with droplet production. A CCD camera can further be linked to an video capture device integrated into the assembly.

[0062] Example 3: A device as in Example 1 including a fluid control unit. The fluid control unit is comprised of one or more valves, valve actuators, and a fluid distribution system. The fluid control unit is used to selectively direct fluids within the emulsifier unit. In

one embodiment, the fluid control unit is actuated by the emulsifier unit operator or by an automation device controller. In another embodiment, a pressurization system delivers a regular pressure to fluid reservoirs coupled to the fluid inlet channels by supplying pressurized gas to the headspace thereof.

[0063] Example 4: A device as in Example 1 wherein it includes a waste outlet port that directs waste materials from the emulsifier unit to an external collection reservoir. Waste materials include any reagents introduced to the device through its inlet ports but that are not directed toward the product exit port.

[0064] Example 5: A device as in Example 1 wherein it includes a vacuum port. The vacuum port provides a mean of connecting the emulsifier unit to a vacuum supply line. Fluids and gases may be directed to the vacuum port during operation and maintenance of the emulsifier unit via the fluid control unit.

[0065] Example 6: A device as in Example 1 wherein it contains a pressurized gas port. The pressurized gas port provides a means of connecting the emulsifier unit to a pressurized gas supply line. Pressurized gas may be directed to specific portions of the emulsifier unit during operation and maintenance of the emulsifier unit during operation and maintenance via the fluid control unit.

[0066] Example 7: A device as in Example 1 wherein the singular or plurality of nozzle actuators is capable of translating an electric signal into motive force by an electronic control device capable of providing an electronic function with a user-defined amplitude, waveform, and frequency.

[0067] Example 8: A device as in Example 1 wherein a transverse fluid channel is capable of supporting laminar fluid flow.

[0068] Example 9: A device as in Example 1 wherein the nozzle or multitude of nozzles is capable of producing an emulsion wherein the droplets have a coefficient of variation of less than 10%.

[0069] Example 10: A device as in Example 1 wherein the nozzle or multitude of nozzles is capable of producing an emulsion wherein the droplets have a coefficient of variation of less than 7%.

[0070] Example 11: A device as in Example 1 wherein the nozzle or multitude of nozzles is capable of producing an emulsion wherein the droplets have a coefficient of variation of less than 5%.

[0071] Example12: A device as in Example 1 wherein the product outlet port is in fluid communication with the next step in the workflow process, such as in a continuous flow emulsion PCR system. In one embodiment of such a system, droplets flow directly from the emulsion generating unit into a thermocycling platform. In another embodiment the emulsifier unit is a modular component of a sequencing platform. In a continuous flow system the microfluidic elements of a complex device may be in close physical proximity to one another and may even occupy the same microfluidic chip.

[0072] Example 13: A device as in Example 1 wherein the outlet port is directed into one or more capture containers or wells on a multi-well plate. In one embodiment of the invention, droplets emerging from the product exit port may be directed dropwise into one or more liquid containers, such as a microtube. In another embodiment of the invention the emulsifier unit is integrated with a fluid handling station such that liquid emerging from the product exit port is distributed among one or more liquid containers, such as multi-well plates.

[0073] Example 14: A method for sample preparation for emulsion PCR wherein the sample format is an oil-in-water emulsion with uniform droplet diameters.

[0074] Example 15: A method as in Example 14 wherein the droplet or multitude of droplets comprising an emulsion contains at least one solid bead. In one embodiment, the bead may be of any type as designated by an emulsion PCR reagent kit. In another embodiment, the droplet or multitude of droplets does not contain a solid bead.

[0075] Example 16: A method as in Example 14 wherein the droplet or multitude of droplets comprising an emulsion contains at least one of the reagents necessary to perform a PCR reaction. Such PCR reagents may include nucleic acids, polymerase, beads, templates, primers and salts.

[0076] Example 17: A method for combining mixing reagents wherein two or more droplets are merged by virtue of timing their formation.

[0077] Example 18: A commercially available emulsion PCR reagent kit is supplied with a protocol that is optimized for about 10 micrometer diameter (523 femtoliter) droplets. The kit contains all the reagents necessary to perform an emulsion PCR reaction and the protocol is includes a process flow chart that describes each sample processing step. One step in the protocol calls for an aqueous mixture of reagents to be emulsified in a carrier fluid. Since the protocol is optimized for 10 micrometer droplets, an emulsifier unit comprising one embodiment of the present invention is selected for use. The unit is loaded with the reagents

as specified in the PCR protocol and is set up to produce 10 micrometer droplets. The unit produces the emulsion.

[0078] Example 19: An emulsion PCR kit protocol requires that two types of microdroplet emulsions are formed separately and then combined. Three fluid reagents are loaded into an emulsifier unit comprising one embodiment of the present invention. The fluids are a first aqueous fluid, a second aqueous fluid, and a third non-aqueous carrier fluid. Each of the aqueous fluids are directed through one or more separate nozzles and combined with the carrier fluid to create two separate emulsion droplet streams. The two droplet streams are directed toward each other such that the droplets are caused to merge before exiting the emulsifier unit.

[0079] Example 20: An emulsion PCR kit protocol requires that three types of microdroplet emulsions are formed separately and then combined. Four fluid reagents are loaded into an emulsifier unit comprising one embodiment of the present invention. The fluids are a first aqueous fluid, a second aqueous fluid, a third aqueous fluid, and a fourth non-aqueous carrier fluid. Each of the aqueous fluids are directed through one or more separate nozzles and combined with the carrier fluid to create two separate emulsion droplet streams. The three droplet streams are directed toward each other such that the droplets are caused to merge before exiting the emulsifier unit.

[0080] Example 21: An emulsion PCR kit protocol requires that two types of microdroplet emulsions are formed separately and then combined after one of the emulsions has been exposed to a UV light. Three fluid reagents are loaded into an emulsifier unit comprising one embodiment of the present invention. The fluids are a first aqueous fluid, a second aqueous fluid, and a third non-aqueous carrier fluid. Each of the aqueous fluids are directed through one or more separate nozzles and combined with the carrier fluid to create two separate emulsion droplet streams. One droplet stream is directed under the UV light source while the other droplet stream is protected from UV exposure. The two droplet streams are directed toward each other such that the droplets are caused to merge before exiting the emulsifier unit.

[0081] Example 22: An emulsion PCR kit protocol requires that two types of microdroplet emulsions are formed separately and then combined. Three fluid reagents are loaded into an emulsifier unit comprising one embodiment of the present invention. The fluids are a first aqueous fluid, a second aqueous fluid, and a third non-aqueous carrier fluid. Each of the aqueous fluids are directed through one or more separate nozzles and combined with the carrier fluid to create two separate emulsion droplet streams. Those droplet streams

are directed toward each other such that the droplets are caused to merge before exiting the emulsifier unit.

[0082] Example 23: An emulsion PCR kit protocol requires that two types of microdroplet emulsions are formed separately and then combined. The combination of droplets is such that only alternating droplets are to be combined. Three fluid reagents are loaded into an emulsifier unit comprising one embodiment of the present invention. The fluids are a first aqueous fluid, a second aqueous fluid, and a third non-aqueous carrier fluid. Each of the aqueous fluids are directed through one or more separate nozzles and combined with the carrier fluid to create two separate emulsion droplet streams. The nozzle actuating computer control unit is configured to produce droplets from the first aqueous liquid at twice the frequency of droplet production from the second aqueous fluid reagent. Those droplet streams are directed toward each other such that alternating droplets of the first reagent emulsion are caused to merge a droplet of the second reagent emulsion before exiting the emulsifier unit.

[0083] Example 24: In one embodiment of the method in Example 23, the droplets comprising the first emulsion stream are equally sized to the droplets in the second emulsion stream. In another embodiment of the method in Example 23, the droplets comprising the first emulsion stream are differently sized to the droplets in the second emulsion stream.

[0084] Example 25: In one embodiment of a device as in Example 1, the nozzles are sized such that they are capable of producing droplets with a mean volume between about 1 to about 100 picoliters. In another embodiment of the device as in Example 1, the nozzles are sized such that they are capable of producing droplets with a mean volume between about 50 and about 5000 femtoliters. In yet another embodiment of the device the nozzles are sized such that they are capable of producing droplets with a mean diameter between about 300 and about 700 femtoliters.

[0085] Example 26: In one embodiment of a device as in Example 1, the nozzles actuated such that they are capable of producing droplets at a rate of greater than 5,000 per second. In another embodiment of the device as in Example 1, the nozzles are actuated such that they are capable of producing droplets at a rate of greater than 15,000 per second. In yet another embodiment of the device the nozzles are sized such that they are capable of producing droplets at a rate of greater than 50,000 per second. In yet another embodiment of the device as in Example 1, the nozzles are actuated such that they are capable of producing droplets at a rate of greater than 80,000 per second.

[0086] Example 27: In one embodiment of a device as in Example 1, the emulsifier unit is capable of producing at least one emulsion of greater than 2,000,000 droplets from at least

one nozzle, at least one actuator, and at least one microfluidic channel. In one embodiment of the example the droplets are formed in support of an emulsion PCR reaction, where many droplets are needed, and more than 2,000,000 droplets are created to support such a reaction.

[0087] Example 28: In one embodiment of a device as in Example 1, one or more droplet detectors (31) (Fig. 12)are integrated into the device such that when a passing droplet is detected, an electronic signal is generated. The droplet detector may either detect only the presence of a passing droplet, or it may measure specific characteristics of the passing droplet. For example, the detector may measure the impedance of a droplet passing through an electric field, and in so doing, measure the diameter of the passing droplet. Likewise, the sensor may measure and transmit other aspects of the passing droplet.

[0088] Example 29: The detection of a droplet in one part of the system is used to create two droplets in another part of the system, and the timing of the second and third droplets are such that the first droplet will combine with the second and third droplets.

[0089] Example 29: In one embodiment of a device in Example 28, one or more droplet detectors (31) are linked to at least one nozzle actuator (27). In another embodiment of the device as in example 28, the signals from one or more detectors are transmitted to an automated system control module for additional processing.

[0090] Example 30: In one embodiment of a device as in Example 29, the detection of one or more droplets in one part of the system is used to create one or more droplets in another part of the system.

[0091] Example 31: In one embodiment of a device as in Example 1, two or more droplets are ejected from one or more nozzles with temporal synchronicity such that the droplets merge together. Synchronizing the droplet production sequence between two or more nozzles is essential to ensure that the two or more nozzles arrive at a merging location at the same time. That point of merging can be in the immediate proximity of one or more nozzles, or it can be located distally.

[0092] Example 32: In another embodiment of a device as in Example 31, two or more droplets are caused to merge in the presence of an electromagnetic field.

[0093] Example 33: One example of a useful material combination is an emulsion of aqueous material in a hydrophobic medium. This is achieved by ejecting droplets of aqueous material into a laminar flow of oil, lipid, or other hydrophobic, liquid material or other non-aqueous media, thereby forming an emulsion or dispersion of fine droplets. The preparation of substantially uniformly sized small droplets should make it possible to achieve emulsions

that are far more stable than emulsions produced by other means because the uniformly sized droplets tend to distribute the forces of agglomeration. The aqueous droplets are suspended in a non-aqueous fluid as, or as part of, the final product. Such a material is in demand by the sequencing industry and is useful as an emPCR sample preparation and processing. Droplets are produced at elevated or lowered temperatures depending on the viscosity of the first and second fluid, with the optimum operating temperature determined empirically. A variation on this example utilizes oil or another non-aqueous medium as the first fluid, while the second fluid is a hydrophilic material with or without a solvent component.

[0094] Example 34: Droplets formed from a first aqueous fluid containing a compound of interest are produced by ejecting the impregnated fluid into oil or another non-aqueous second fluid. Upon droplet formation the temperature of the emulsion is reduced to below freezing temperature as a means of increasing the stability of droplet library storage. In this example, temperature of the complete emulsion may also be reduced such that the non-aqueous component becomes a solid.

[0095] Example 35: Solid droplet filtration Droplets formed from a first aqueous fluid containing a compound of interest are produced by ejecting the impregnated fluid into oil or another non-aqueous second fluid. Upon droplet formation the temperature of the emulsion is reduced to below freezing temperature as a means of increasing the stability of droplet library storage. Temperature reduction takes place immediately after droplet formation while the emulsion is flowing. In this example the aqueous droplets within an emulsion are in a solid, frozen state while the oil component remains liquid. They can then be applied to a filter or spun to remove the oil component and then, optionally, resuspended in another liquid media.

[0096] According to one aspect of the invention, a method of making substantially uniformly sized droplets comprises: (a) forming droplets of a first liquid in a laminar flow of a second liquid, each droplet having a diameter of from 0 – 100 micrometers (preferably between 0.5 – 5,000 fL, more preferably between 0.5 - 500 fL) wherein the first and second liquids are, at most, sparingly soluble in one another, and the first liquid contains a solute dissolved, dispersed or suspended therein; and (b) removing the first liquid to form a plurality of substantially uniformly sized droplets or (c) reacting . As used herein, the term "sparingly soluble" refers to a solubility of 10% or less, under the conditions of temperature and pressure encountered during droplet formation. In some embodiments, the first and second liquids are substantially immiscible in one another.

[0097] In the case where the uniformly sized droplets are particles, the method comprises forming droplets of a first liquid containing one or more molecular species dissolved,

suspended, or dispersed therein by ejecting the first liquid into a laminar flow of a second liquid, i.e., oil or other non-aqueous medium, wherein the first liquid is no more than sparingly soluble in the second liquid, and wherein the population of droplets has mean droplet volume (V) of 1fL < V < 50nL; and allowing the molecular species to self-assemble into substantially uniformly sized droplets by removing the first liquid. (A payload, carried in the first and/or the second liquids, or added after the droplets form, may also be present.) In one embodiment, the droplets thus formed have a mean outer diameter, D, of from 20nm to 1000nm, with a coefficient of variation of less that 10%, more preferably less than 7%, most preferably less than 5%. In some embodiments, even tighter size distributions are provided, e.g., 4%CV, 3% CV, or at most 2%CV.

[0098] The mean outer diameter of the collection of droplets is brought as close as desired to a particular value (e.g., 100nm, 200nm, etc.), which can be selected so that the droplets are correctly sized to deliver a payload to a desired cellular site of action. For example, in one embodiment, D = 100nm. More generally, the collection of droplets has a mean outer diameter, D, of from 20 nm to 1000 nm, from 25 to 500 nm, from 50 to 200 nm, from 75 to 125nm, from 90 to 110nm, or (e.g.) from 95 to 105nm, with a narrow size distribution (optionally Gaussian) about the mean diameter, D; i.e., with a coefficient of variation of less that 10%, more preferably less than 7%, most preferably less than 5%. In some embodiments, even tighter size distributions are provided, e.g., 4%CV, 3% CV, or at most 2%CV.1%.

[0099] The outer diameters of individual droplets, as well as the mean outer diameter of a collection of droplets, can be determined using a suitable measurement technique, for example, photon correlation spectroscopy, freeze fracture and electron microscopy, or by using the "Coulter principle," whereby voltage potential fluctuation in a small orifice determines droplet size. Alternatively, other techniques for determining droplet size and size distributions, presently known or discovered in the future, are used. In general, photon correlation spectroscopy is preferred over electron microscopy because it is significantly faster.

[00100] Advantageously, however, actual measurement is not required, as the method of manufacture is designed to ensure that the droplets have the desired mean diameter and narrow size distribution, as described below.

[00101] It is contemplated that all manner of droplets can be prepared with a desirably narrow size distribution as described herein, regardless of the lipid(s) and/or other chemical species that comprise the droplets. The droplets can be monolayer vesicles, or bilayer vesicles (formed, e.g., of amphipathic, aka amphiphilic, lipids), and can be multilamellar or,

more preferably, unilamellar. Phospholipids, such as phosphatidylethanol amines, are a type of amphipathic lipid capable of self-assembling into droplets in water. A non-limiting list of lipids capable of self-assembling into droplets is found in U.S. Patent No. 7,083,572, col. 20, lines 23-59, which is hereby incorporated by reference herein.

[00102] Non-limiting examples of payloads include amino acids, proteins, enzymes, natural and synthetic nucleic acids (e.g., DNA, RNA, siRNA, plasmids, etc.), dyes, contrast agents, radiolabeled compounds, fluorescent compounds, medicaments, organic compounds, inorganic compounds (e.g., gold and/or other metallic droplets; semiconducting droplets, e.g., nanodots), and mixtures of these and/or other substances. The payload can have any desired chemical form, including atomic, molecular, and ionic. In one embodiment, the collection of droplets is carried in a liquid medium, which typically is water or some other aqueous medium. For example, the aqueous medium can further comprise a pH buffer. Non-limiting examples of buffers include saline, ammonium sulfate, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), TRIS (tris(hydroxymethyl)aminomethane), and mixtures thereof.

[00103] Where a payload is included, it can be carried by or coupled to the droplets in a number of ways familiar to persons skilled in the art. For example, the payload can be carried within the hollow interior of the droplets (which, in most cases, also hold water). The payload can be coupled to or carried on the inner and/or outer walls of the droplets. The payload can be enmeshed within the mono- or bi-layers that form the droplets. In some embodiments, the payload extends from the interior to the exterior of the droplets.

[00104] As indicated above, in view of the tight size distribution, the collection of droplets can be characterized by a mean outer diameter and a standard deviation there from. Thus, in one embodiment of the invention, a composition for delivering a payload to a patient comprises a collection of droplets sufficient in quantity to be administered as a pharmaceutical (for example, 10^7 or more droplets), wherein the droplets have a mean outer diameter, D, of from 25nm to 1000 nm, with a CV of less than or equal to 10%. In some cases, the collection of droplets will have a spread of outer diameters that can be characterized by a so-called normal distribution, with a probability function, P(x), where x is distance (outer diameter) in nanometers. Systemic errors in the droplets production process (and/or the manufacturing process(es) used to make various components of the apparatus used to make the collection of droplets), as well as other factors, may, in some cases, result in the droplets having a size distribution other than that characterized by the classic Gaussian probability function.

[00105] For emulsion PCR droplet production, generally the first liquid is hydrophillic and the second liquid is non-aqueous. Thus, in one embodiment, the first liquid comprises an aqueous solvent. The first liquid may also include a pH buffer. Non-limiting examples include saline, ammonium sulfate, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), TRIS (tris(hydroxymethyl)aminomethane), and mixtures thereof. Nonlimiting examples of the second fluid include hydrocarbons (e.g., hexane), chlorinated hydrocarbons (e.g., dichloromethane, chloroform, trichloroethylene, etc), halogenated oils (fluorinated oils, etc), silicone oils, aromatic compounds (e.g., toluene), ethers (if sufficiently hydrophobic), mixtures thereof, as well as other organic solvents or compounds or mixtures of such materials). Mixtures of organic compounds can be used. Of particular interest is an embodiment in which the first liquid comprises one or more PCR reagents, i.e., linkers, beads, primers, DNA templates, nucleic acids, etc, and the second liquid is a hydrophobic oil containing a surfactant.

[00106] As characterized, the invention contemplates that droplets having a mean volume of from anywhere from 1fL to 1nL are formed. In some embodiments, however, the mean droplet volume is confined to a narrower range, namely, from 27 fL to 125 pL, with an outer diameter ranging from 3 to 50 micrometers. In one embodiment of the invention, droplets of the first liquid are formed by ejecting the first liquid through at least one nozzle directly into a laminar flow of the second liquid. This is schematically illustrated in Fig. 1. A nozzle 1 is in fluid flow communication with a channel 2 through which moves a laminar flow of a second liquid 3. At time t_1, the nozzle ejects a first droplet 4 of a first liquid into the channel and is carried downstream by the second liquid. At time t_2, a second droplet 5 of the first liquid is ejected through the nozzle into the channel and also is carried downstream. At time t_3, a third droplet is ejected from the nozzle into the channel and carried downstream. The first, second, and third droplets of the first liquid are substantially immiscible with (or no more than sparingly soluble in) the second liquid, and each droplet is separated in space by a distance determined by the nozzle ejection rate and the rate of laminar flow of the moving liquid in the channel. The flow of liquids in the channel is substantially laminar (non-turbulent), and a sufficient delay is provided between each droplet such that the droplets substantially retain their integrity as they flow through the channel. In the special case where droplets are produced either synchronously or otherwise ejected by a nozzle in response to a signaling event, droplet production may be timed in such a way that two or more droplets merge or are otherwise ordered.

[00107] Figure 1 illustrates one embodiment of an apparatus for making substantially uniformly sized droplets and other small droplets according to the invention. Referring to Figure 2, there is shown an apparatus 10 having a bottom microfluidics channel plate 12, a top microfluidics channel plate 14, an inlet/outlet manifold 16 (having a liquid inlet port 18 at

one end and a liquid outlet port 20 at the opposite end), thermoelectric heater/coolers 22, and a radiator 24 (which are thermally coupled to the inlet/outlet manifold and provide a way of supplying and/or removing heat to and/or from the apparatus). A plurality of nozzles 26 are positioned below the bottom microfluidics channel plate and provide a means for ejecting precisely controlled droplets of a first liquid into the apparatus. In the embodiment shown, 64 nozzles are depicted schematically. One or more nozzles can be provided as an inkjet printer head (e.g.) or as stand alone nozzles capable of ejecting discrete droplets. The apparatus also includes one or more nozzle actuators 27, which provide a carefully controlled impulse to eject a precisely sized bubble (droplet) of the first liquid through each nozzle. Non-limiting examples of nozzle actuators include piezoelectric actuators and thermal bubble actuators. In one embodiment, each nozzle is driven by a separate actuator. In another embodiment, a single actuator drives two or more nozzles. Inkjet printer heads, nozzles, and actuators, and the associated electronics and software to drive them, are well known in the art.

[00108] The top and bottom microfluidics channel plates 14, 12 mate in fluid-tight fashion and together form a microfluidics channel at the plates' interface. Referring now to Figure 3, the top microfluidics channel plate 14 is shown in greater detail. The plate includes an inlet channel 28, an outlet channel 30, and a plurality of transverse liquid channels 32 that extend from the inlet channel to the outlet (exit) channel. In the embodiment shown, 64 such transverse channels are provided. One end of the inlet channel includes an opening that allows liquid to flow into the channel from the inlet port 18 in the inlet/outlet manifold. Similarly, one end of the exit channel has an opening that allows liquid to exit to the outlet port 20 (also referred to as the product outlet port) in the inlet/outlet manifold. The outlet port, in turn, can be coupled to a pervaporation unit either directly or via a fluid conduit.

[00109] Additional detail of the transverse liquid channels and their relationship with the plurality of nozzles is shown in Figures 4 and 5. As indicated, the transverse liquid channels 32 are substantially smaller in cross-section than the inlet and outlet channels. In Figure 5, a cross-sectional view taken along lines B-B, one of the nozzles 26 is shown extending into one of the transverse liquid channels 32. The transverse liquid channel has opposite sidewalls 33, 34, a bottom 35 formed by the bottom microfluidics channel plate, and a top 36 formed by the top microfluidics channel plate. The nozzle extends from the bottom of the transverse liquid channel up into the interior of the channel through an opening (not shown) in the bottom of the bottom microfluidics channel plate. Figure 6 is an exaggerated schematic view of Figure 4 along lines B-B. Figure 7 is an exaggerated schematic view of Figure 6 along lines C-C showing a portion of a top microfluidics channel plate 14 rotated out and away from the bottom microfluidics channel plate 12 and the pressure chamber 13 and pressure

chamber plate 15, revealing one of the plurality of transverse liquid channels 32, with a nozzle 26 protruding up from the bottom of the channel into the interior.

[00110] It will be appreciated that the dimensions of the inlet and outlet channels, the transverse liquid channel, and the nozzles are exceedingly small, with even smaller nozzle diameters (i.e., the inner diameter of the ejection orifice at the tip of each nozzle). Nonlimiting examples include: transverse channels: about 1 to about 100 μm (with small dimensions being preferred); inlet and outlet channels: the same size as, or slightly larger than, the transverse channels; nozzle orifices: about 0.01 to about 50 μm, preferably about 0.01 to about 10μm. Small transverse channels permit small droplets to be obtained and require less carrier fluid in the system, yielding a higher titre of the final composition, i.e., more droplets (droplets) per milliliter. Smaller nozzles permit smaller droplets to be generated, which should yield a greater number of droplets in the final composition per unit volume, e.g., more droplets per mL.

[00111] In the embodiment shown in Figures 2 through 7, each of the plurality of nozzles has substantially the same nozzle diameter, and each nozzle has a proximal end coupled, directly or indirectly, to one or more liquid reservoirs (not shown), and a distal end that extends into the interior of a corresponding one of the plurality of transverse liquid channels. Alternate embodiments, however, are also within the scope of the invention. For example, the nozzles need not necessarily have the same nozzle diameter. In addition, each nozzle can be flush with the bottom of a corresponding transverse liquid channel, or some nozzles can protrude into, while others are flush with, a corresponding transverse liquid channel, etc. Two or more nozzles can extend into a single transverse channel.

[00112] In general, the materials used to construct the microfluidics channel paths are selected to be non-corrosive in the presence of water and organic solvents, and cleaning regimens of soap, steam, and/or chlorides. Nonlimiting examples include silicon, glass, gold, chrome, NiCo (nickel cobalt alloy), and stainless steel. In one embodiment, the top and bottom plates are held together by Van der Waal forces to form a fluid-tight assembly which is housed in a stainless steel assembly held together by threaded fasteners (not shown) that span the radiator to the I/O manifold, with the thermoelectric heater/coolers held in compression between them. In another embodiment the channels are formed by deposited layers of materials typical of electrodeposition or photodeposition. Optionally, a thermally conductive lubricant can be applied to the upper and/or lower surfaces of the heater/coolers to facilitate heat transfer between the radiator and the I/O manifold.

[00113] The thermoelectric heater/coolers allow the temperature of the microfluidics channels and the nozzles to be controlled, which can be desirable for a number of reasons.

First, controlling the temperature allows the surface tension at the first liquid/second liquid interface (e.g., the solvent/water interface at the nozzle orifices) to be modulated. If the fluids are cold, the surface tension will be greater. Second, in the microfluidics channels, viscosity is controlled via temperature. Third, in the general mixing of the fluids, it is important to maintain a good separation between the different fluid types. The droplets, if too "hot" might tend to "blur" into the other liquid, due to an increase in solubility.

[00114] In some embodiments, where heat-sensitive compounds are present, it is contemplated that the apparatus will be operated above or below room temperature (~25 °C), in the range of about 30 to about 200 °F (-1 to 92 °C), with about 30 to about 80 °F (1 to 100 °C) being most desirable for most PCR droplet chemistry. In other embodiments, where more thermally stable materials are employed, e.g., where the droplets being formed are solid organic polymer beads, or where the apparatus may be operated at even higher temperatures, e.g., about 300 °F (147 °C). Accordingly, it is contemplated that the apparatus will be operated at a temperature of from about 20 to about 300 °F (-7 to 147 °C, or, alternatively, about 20 to about 200 °F (-7 to 92 °C), or alternatively, about 20 to about 100 °F (-7 to 37 °C).

[00115] It is also contemplated that the pressures of the first and second liquids in the apparatus is carefully controlled. In one embodiment, each of the liquids has, independently, a pressure of 2000 psi or less, e.g., from 10-100 psi; more typically 20-100 psi (excluding the pervaporation unit, which, in one embodiment, is expected to operate at a higher pressure). In another embodiment, either or both liquids have a pressure that exceeds 100 psi. The two liquids can be supplied by a pressure supply system, which is coupled to the nozzles and the inlet port of the inlet/outlet manifold.

[00116] One embodiment of a pervaporation unit is depicted in Figure 8. The pervaporation unit 70 includes a lower housing 72 and a membrane support plate 74, which together define a vacuum chamber 76. An O-ring (not shown) seated in a channel 78 along the top periphery of the lower housing ensures that a gas-tight seal is maintained between the housing and the membrane support plate. A vacuum port 80 in the lower housing can be connected to a vacuum pump (not shown) and permits gasses to be evacuated from the vacuum chamber to create and maintain a reduced pressure inside the vacuum chamber. In some embodiments, a cold trap (not shown) is located in line between the vacuum port and the vacuum pump, allowing the permeate (e.g., organic solvent) to be captured for disposal or, more preferably, reuse. A selectively permeable membrane 82 is sandwiched between a fluid flow plate 84 and the membrane support plate 74. An inlet/outlet manifold 86 sits atop the fluid flow plate. Thermoelectric heaters/coolers 88 and a radiator 90 allow heat to be supplied to or removed from the pervaporation unit as needed. A fluid inlet port 92 in the

inlet/outlet manifold can be coupled to the outlet port of the droplet generator (Fig. 2), while pervaporation products (e.g., droplets in water, emulsions, solid polymer beads, nanodots, other small droplet systems) can be removed from the unit through a fluid outlet port 94. Inlet and outlet ports 96 and 98 are also provided in the fluid flow plate 84, and provide access to the selectively permeable membrane 82. Peripheral components such as a power supply and a microprocessor or other logic controller (not shown) can be coupled to the thermoelectric heaters/coolers and, as with the droplet generator, allow the temperature of fluids in the unit to be closely monitored and controlled. In one embodiment, the flow of fluids through the droplet generator and the pervaporation unit, and the temperature of the fluids, are carefully regulated by a shared microprocessor or other logic controller.

[00117] Figure 9 illustrates the underside of the fluid flow plate 84, which faces the selectively permeable membrane 82. The plate includes openings 96 and 98 and a serpentine channel 100 cut into the lower face, the channel extending from one opening to the other. The serpentine channel provides an extended pathway for a laminar flow of droplets of the first liquid carried by the second liquid, and serves to minimize turbidity while maintaining maximum surface contact between the moving liquids and the selectively permeable membrane. Reduced pressure on the underside of the membrane (facing the enclosed chamber) facilitates the steady, yet controlled, removal of the first liquid along the length of the serpentine channel.

[00118] In operation, the pervaporation unit is brought to and maintained at temperature, which may be higher or lower than ambient and within the range of about 20 to about 170 °F (-7 to 76 °C), as dictated by the physical chemistry of the combination of fluids being separated. For the production of droplets, in which the lipids must self-assemble, the preferred temperature range is about 32 to about 80 °F (0 to 26 °C). For the production of other droplet types, the range may be much higher because of the inherent stability of the chemistry and more efficient operation of the pervaporation process at higher pressure and temperature differentials. In the case of handling temperature-sensitive molecules or materials, the pervaporation unit is capable of maintaining any temperature with a lower limit defined by the freezing point of the aqueous media.

[00119] Figure 10 illustrates one embodiment of a system for delivering pressurized liquids to a droplet generator. The system 140 includes first and second liquid storage tanks 142, 144, which hold, respectively, the first liquid (e.g., an aqueous solvent from which droplets are formed) and the second liquid (e.g., fluorinated oil or another non-aqueous medium). For emulsion PCR applications, nucleic acid samples and PCR reagents are also present in the first liquid storage tank. The contents of these tanks are coupled to the droplet generating apparatus 162 by lines 146 and 148. A gas tank 150, preferably filled with an

inert gas, such as argon, neon, helium, etc., is coupled to gas regulators 151 and 152, which supply the first 142 and second 144 fluid tanks, which in turn are coupled to a pair of solenoid valves 154 and 156, respectively. The valves allow pressurized fluid to be metered into the first fluid reservoir 158 and second fluid reservoir 160, respectively. Liquid reservoirs drive the feed of liquids into the droplet generating unit 162.

[00120] In some embodiments, the solenoid valves 154 and 156 are coupled to logic controllers 164 and 166 coupled to a computer control unit (e.g., a microprocessor, CPU, computer, etc.) 168, which is coupled to the droplet generating unit 162. Alternatively, the logic controllers 164 or 166 for the solenoid valves are part of the computer control unit 168. System commands (i.e., commands for activating/deactivating the nozzle actuator(s) associated with the droplet generator unit 162; for controlling the first liquid pressure/flow through the inlet port in the droplet generator; etc.) can be input into the control unit to operate the overall system. In some embodiments, a power supply is provided to provide power to the various electrical components of the system.

[00121] The droplet generator unit 162 dispenses a stream of droplets directly where?, or, optionally, the droplet generating unit can direct droplets directly to a thermocycler 170, and an assay 172, which may contain optics, imaging, electronic or other means of droplet interrogation to generate data and results.

[00122] The droplet generator 162 also comprises a microfluidics 174 area containing the nozzles 26, nozzles actuators 27, and transverse channels 32 as shown, for example, in Figs. 2-9, including droplet merging area(s) and which also may optionally provide for a transparent viewing area to allow the microscope 176 optical access to droplets as they are being formed. The microfluidics 174 area is illuminated by a light emitting diode (LED) 178 that is driven by the computer control unit 168. A video capture unit 180 (e.g., a CCD chip) captures the image and transmits it to the computer control unit. In some embodiments the LED 178 is part of the droplet generating unit 162. In other embodiments, the LED is incorporated into the microscope 176.

[00123] As shown in Fig. 11, a method and process using the system of the present invention is diagrammed in a step-wise flow chart.

[00124] By controlling the concentration of lipids in the first liquid, the pressures of the first and second liquids, and the size of the droplets generated in the droplet generator, a collection of substantially uniformly sized droplets for emulsion PCR , having a mean diameter, D, is formed. Collections of other substantially uniformly sized small droplets are prepared in an analogous manner. The system is controlled externally by a computer with display and user interface 182 (i.e., a personal computer with a monitor and keyboard, a

tablet computer, or a handheld device). The image from the video capture unit 80 appears on the computer with display and user interface, and may optionally be stored there for future reference or other process. Displaying an image allows the device user to change device settings in real time.

[00125] Advantageously, the method and apparatus described herein allows for site-specific biological vectors, i.e., compositions for delivering a therapeutic payload to a patient, in particular, a collection of substantially uniformly sized droplets sufficient in quantity to be administered as a therapeutic delivery system.

[00126] Droplet size is a function of the number of molecules comprising the droplet. Likewise, any small droplet is sized according to the chemistry and quantity of material comprising the droplet, provided, of course, that the material is compacted or otherwise shaped or oriented in such a way that size is a direct function of material volume. Beginning with a specific concentration of solute in a suitable solvent (e.g. lipids in chloroform), each droplet of a specified size will have the same number of lipid molecules distributed in it. Upon removal of the solvent, each droplet or other droplet will have substantially the same number of molecules in it, with a variance that is linearly related to the variance in the volume of the droplets. Thus, if the droplets vary in volume by about 2 to about 3%, and they are made of the same solution, then droplets derived from these droplets will also vary in solute material content and thus, size, by about 2 to about 3%.

[00127] Controlling droplet/droplet size is therefore a function of controlling droplet size and solute concentration. Droplet size is controlled by using nozzles having substantially uniformly sized and shaped ejection orifices, and by adjusting the electrical pulse creating the droplets. The latter is a fine-tuning technique. In one embodiment, droplet volume is corrected (brought toward normality) by up to about 10% by adjusting the electrical pulse(s) that drive the nozzle actuator(s). This is similar to the way inkjet printers are adjusted to ensure that each droplet of ink is the same size. In the embodiment shown in FIG. 2, there are 64 nozzles, and each one has a "fingerprint" of sorts. To ensure that each nozzle produces, e.g., a 10 pL droplet, rather than 9pL droplet, the voltage or current in the pulse wave to the nozzle's actuator is adjusted as necessary. This can be done iteratively. In one embodiment, the apparatus further includes a feedback mechanism in which a computer and droplet size detector are used to measure droplet size, compare it to a desired value, and then adjust the electric pulse(s) driving the actuator(s) to correct droplet size as necessary.

[00128] Figure 12 shows the details of the emulsion and droplet formation within the microfluidics unit 174 that includes the components as disclosed and shown in Figs. 2-9. As shown, the actuators 27 are under the control of the computer control unit 168.

[00129] One can calculate the size of a droplet containing a specified number of molecules if the density or the area in space occupied by a given molecule is known or can be determined. This information can then be used to calculate a specific droplet size, and the corresponding volume of solvent in each droplet will determine the concentration of the starting solvent solution. In theory, droplets nearly as large as the droplets themselves can be formed by using very concentrated solutions, at least in the case where the droplets are solid, i.e., not droplets. At the other extreme, very dilute solutions can yield very small droplets, e.g., a 100 nm diameter droplet containing just 300,000 lipid molecules is prepared from a very dilute solution of lipids in solvent. Solute concentration can be adjusted directly, by adding additional solute to solvent or by diluting the solution with additional solvent.

[00130] In some embodiments of the present invention, a relatively concentrated solution of solute in solvent (e.g., lipids in chloroform) is prepared and stored in a first tank. A second tank contains neat solvent (e.g., chloroform). The two tanks are coupled to the droplet generator by one or more conduits and valves which, in turn, are coupled to the system's logic control, so that precise amounts of the solution and solvent can be metered out as needed. The ratio of the solution and neat solvent can be automatically adjusted to produce any desired concentration of solute in solution, from very rich to very dilute. Each of the nozzles can then be selected to eject a particular sized droplet (plus or minus some variance). Alternatively, a variable solvent system is combined with a series of different sized droplet generators, making it possible to achieve any range of droplets from big to small, i.e., about 5 nanometers to about 100 micrometers for solid droplets, and from about 20 nanometers to about 1 micrometer for droplets.

[00131] In another embodiment of the invention, nucleic acids are formed into microdroplets and merged with PCR reagents prior to undergoing elongation and other downstream assays. Figure 11 describes an example emulsion PCR workflow wherein nucleic acid samples (e.g., DNA, RNA) are purified, and optionally mixed with additional reagents (e.g., buffer and other PCR reagents), prior to being formed into an emulsion of droplets. Separately, other PCR related reagents are prepared and formed into an emulsion. The droplets from the two emulsions are prepared either separately or coincidentally, and merged, prior to undergoing any one or all of the additional steps comprising a complete PCR assay, inclusive of thermocycling, droplet selection, and interrogation and interpretation.

[00132] Advantageously, the process is digital. A computer or other microprocessor issues an electric pulse and a droplet is ultimately ejected from the machine. This is a tremendous improvement over the analog processes of open loop hydrodynamic focusing or the condensation reactions that other droplet manufacturers use because, in a given run, one will know exactly how many droplets were made. Counting very small droplets is a serious

technical challenge. Using systems currently available, to count the droplets in a sample, one processes the entire sample through a droplet detector and, each time a droplet is detected, an electric pulse is sent to a counter. The present invention essentially proceeds in the reverse fashion, and thus provides both a droplet maker and counter all in one.

[00133] The precise control of droplet size by the method and apparatus described herein, and the use of substantially inert materials such as silicon, glass, chrome, nickel, and selected polymers allow the invention to be used to form substantially uniformly sized droplets. The following non-limiting examples demonstrate the breadth of possible material combinations, as well as their applicability to a number of industries.

[00134] The invention has been described with reference to various embodiments, figures, and examples, but is not limited thereto. For example, in another aspect of the invention, droplets having externally modified surfaces are prepared by incorporating external molecular components in the second fluid. The fluids chemically interact with, and become bound to, the small droplets as they form. For example, the surfaces of uniformly sized small droplets can be modified by any number of chemical processes, including coating and ligand attachment, by mixing the compounds for those processes into the second fluid. The droplets can be further modified in one or more subsequent steps by adding fluids in-line, downstream of the described droplet forming processes, and by adding any number of additional linear or batch processing steps. Persons having ordinary skill in the art will appreciate that the invention can be modified in a still other ways without departing from the invention, which is limited only by the appended claims and equivalents thereof.

WHAT IS CLAIMED IS:

1. An apparatus for preparing an emulsion of uniform microdroplets, comprising:
at least one first fluid inlet port in liquid communication with a pressure chamber;
at least one nozzle;
a pressure chamber in liquid communication with the at least one nozzle;
at least one transverse liquid channel;
at least one second fluid inlet port in liquid communication with the at least one transverse liquid channel;
an outlet port in liquid communication with the at least one transverse liquid channel;
at least one nozzle actuator coupled to the at least one nozzle; and
an electronic nozzle actuator computer control unit.

2. An apparatus as recited in claim 1, wherein each of the at least one nozzle is coupled to a corresponding transverse liquid channel.

3. An apparatus as recited in claim 1, wherein the at least one actuator comprises piezoelectric actuators.

4. An apparatus as recited in claim 1, further comprising a thermoelectric temperature control system to control fluid viscosity.

5. An apparatus as recited in claim 1, further comprising a light emitting diode linked to the electronic nozzle actuator computer control unit.

6. An apparatus as recited in claim 1, further comprising an integrated video capture device capable of providing a real time image of a droplet stream in the at least one transverse channel.

7. An apparatus as recited in claim 1, wherein the electronic nozzle actuator computer control unit is capable of varying one or more aspects of electronic functions, including polarity, amplitude, waveform, and frequency.

8. An apparatus as recited in claim 1, further comprising a first liquid reservoir coupled to each of the at least one nozzle, and a second liquid reservoir coupled to the second liquid inlet port.

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9. An apparatus as recited in claim 1, further comprising a pressurization system for moving liquids from the first and second reservoirs into the at least one nozzle and the first and second fluid inlet ports, respectively.

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10. An apparatus as recited in claim 1, wherein each of the at least one nozzle has an ejection orifice diameter of from about 0.05 μm to about 50 μm.

11. An apparatus as recited in claim 1, wherein each of the at least one nozzle has an
10 ejection orifice diameter of from about 0.1 μm to about 25 μm.

12. An apparatus as recited in claim 1, further comprising at least one droplet detector.

13. An apparatus as recited in claim 1, wherein the fluid channel at the point of nozzle
15 junction is capable of supporting laminar fluid flow.

14. An apparatus as recited in claim 1, wherein the nozzles are sized such that they are capable of producing uniform droplets with a coefficient of variation of less than about 10%.

15. An apparatus as recited in claim 1, wherein the at least one nozzle is sized such that it
20 is capable of producing uniform droplets with a coefficient of variation of less than about 7%.

16. An apparatus as recited in claim 1, wherein the at least one nozzle is sized such that it is capable of producing uniform droplets with a coefficient of variation of less than about 5%.

25

17. An apparatus as recited in claim 1, wherein at least one droplet detector is linked to the at least one nozzle actuator.

18. An apparatus as in claim 1, wherein detection of one or more droplets in one part of
30 the system enables formation of one or more droplets in another part of the system.

19. An apparatus as recited in claim 1, wherein the at least one nozzle is two or more nozzles which are positioned and capable of being timed such that droplets are ejected from the at least one nozzle with temporal synchronicity such that the droplets merge together.

35

20. An apparatus as recited in claim 1, wherein the at least one first fluid inlet port and the at least one second fluid inlet port are supplied at least in part by an automated fluid handling station.

21. An apparatus as recited in claim 20, wherein the mean droplet volume is between about 1 pL and about 1 fL.

22. A method of making substantially uniformly sized droplets using the apparatus of claim 1, comprising:

forming droplets of a first liquid in a laminar flow of a second liquid, each droplet having a mean droplet volume of between about 1nL and about 1fL, wherein the first and second liquids are no more than sparingly soluble in one another.

23. A method as recited in claim 22, wherein the first liquid contains one or more PCR reagents from among the collection of nucleic acids, bases, DNA templates, capture beads or buffers dissolved, dispersed, or suspended therein.

24. A method as recited in claim 22, wherein one nozzle generates greater than 20,000 droplets per second.

25. A method as recited in claim 22, wherein one nozzle generates greater than 70,000 droplets per second.

26. A method as recited in claim 22, wherein the first liquid is aqueous and the second liquid is hydrophobic.

27. A method as recited in claim 22, wherein the droplets are formed sequentially to comprise a sample of greater than 2,000,000 droplets.

28. A method for merging two droplets using the apparatus of claim 1, wherein the at least one nozzle is actuated with timing such that the resultant droplets merge.

29. A method as recited in claim 28, wherein a piezoelectric material provides a motive force for ejecting a first liquid through the at least one nozzle.

30. A method as recited in claim 28, wherein thermoexpansion provides a motive force for ejecting a first liquid through the at least one nozzle.

31. A method as recited in claim 28, wherein the two droplets are produced within the same channel.

32. A method as in 28, wherein the two droplets are produced in separate channels.

33. A method as in 28, wherein the two droplets are produced by sharing the same impulse.

34. A method as in 28, wherein the two droplets are produced by alternating an impulse such that one nozzle produces droplets as a fraction or multiple of another nozzle.

35. A method as in 28, wherein the two droplets are the same size or different sizes.

36. A method as recited in claim 28, wherein the two droplets are formed sequentially to comprise a sample of greater than 2,000,000 droplets.

37. A method as recited in claim 28, further comprising an oil-in-water emulsion.

38. A method of making a collection of substantially uniformly sized droplets using the apparatus of claim 1, comprising:

forming droplets of a first liquid containing one or more aqueous reagents dissolved, suspended, or dispersed therein by ejecting droplets of the first liquid from at least one nozzle directly into the non-aqueous laminar flow of the second liquid, wherein the second liquid is substantially immiscible in water, and wherein each droplet has a volume of from about 0 to about 100 pL.

39. A method as recited in claim 38, wherein the droplets have a volume of from about 0.5 to about 5000 fL.

40. A method as recited in claim 38, wherein at least one droplet contains at least one reagent selected from the group consisting of PCR reagents; primers, nucleic acids, salts, nucleotides, peptides, antibodies, aptamers, polymerase, and mixtures thereof.

41. A method as recited in claim 38 wherein the at least one droplet contains a nucleic acid to be amplified.

42. A method as recited in claim 41, wherein the outlet port is in fluid communication with a PCR workflow process.

43. A method as recited in claim 42, wherein the workflow process is selected from among thermal cycling, nucleic acid elongation, emulsion breaking, filtration, sequencing, or combinations thereof.

44. A method as recited in claim 40, wherein the outlet port is directed into one or more capture containers or wells on a multi-well plate.

45. A method as recited in claim 40, wherein a droplet contains at least one bead-based reagent.

46. A method as recited in claim 40, wherein combining mixing reagents wherein two or more droplets are merged by virtue of timing their formation.

47. A method as recited in claim 40 wherein the droplets merge in the presence of an electromagnetic field

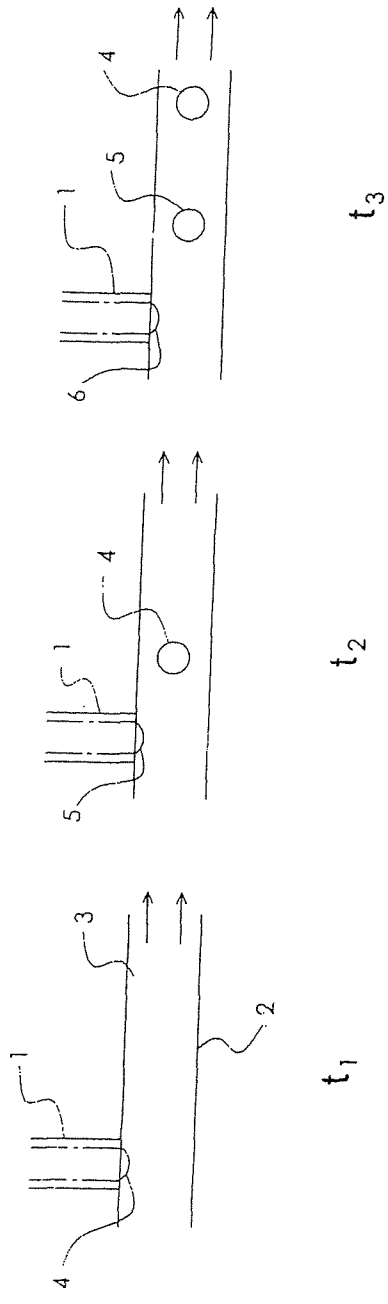


FIG. 1

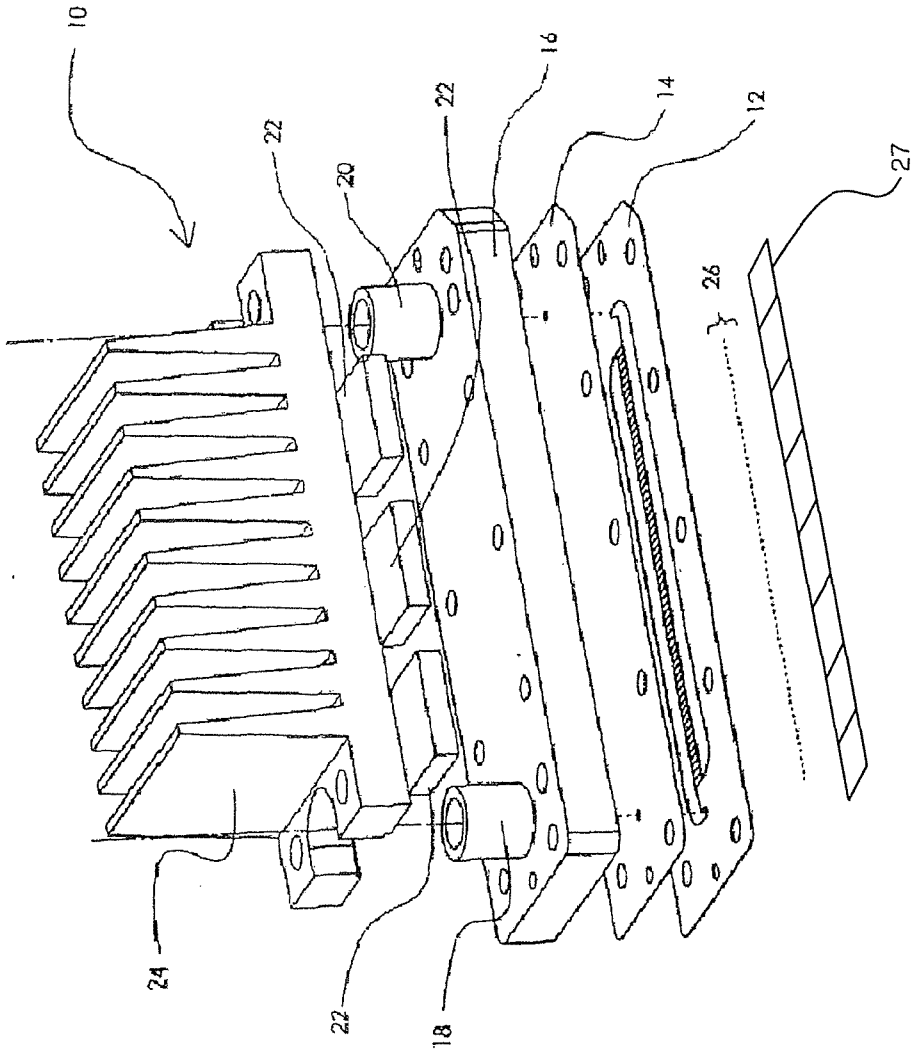


FIG. 2

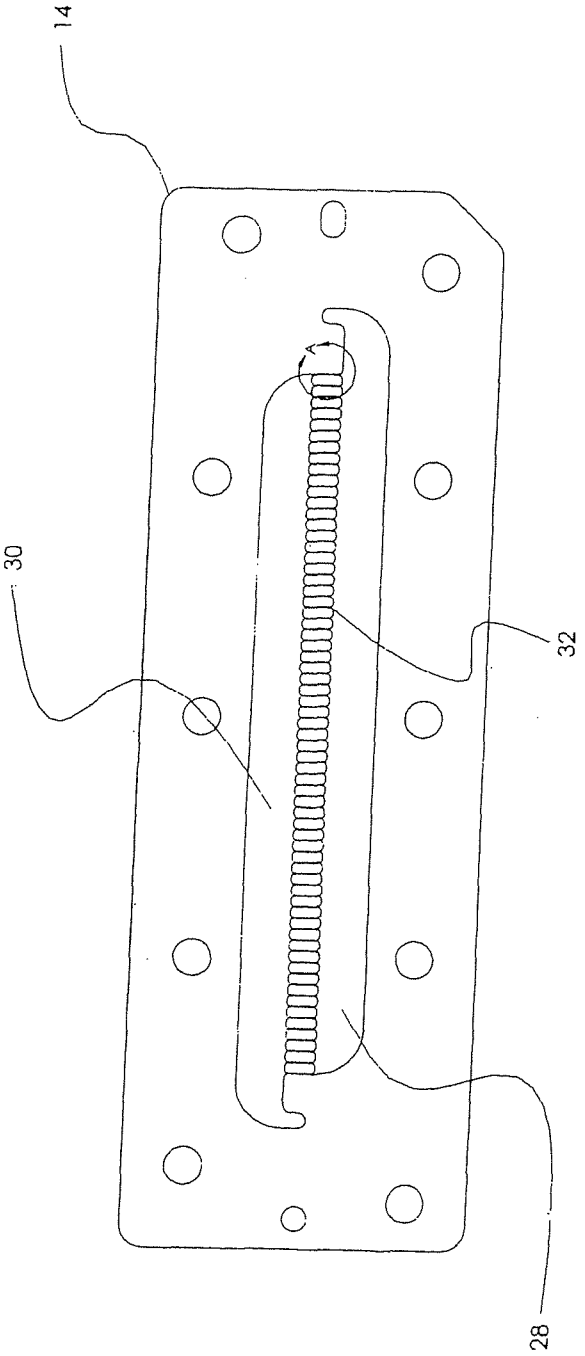


FIG. 3

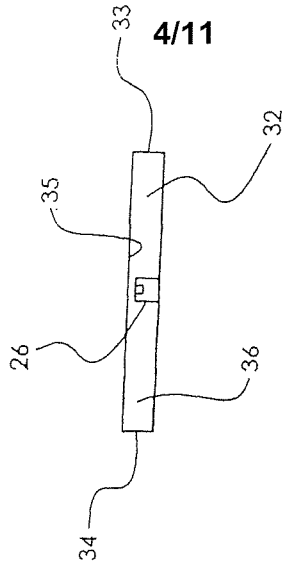


FIG. 5

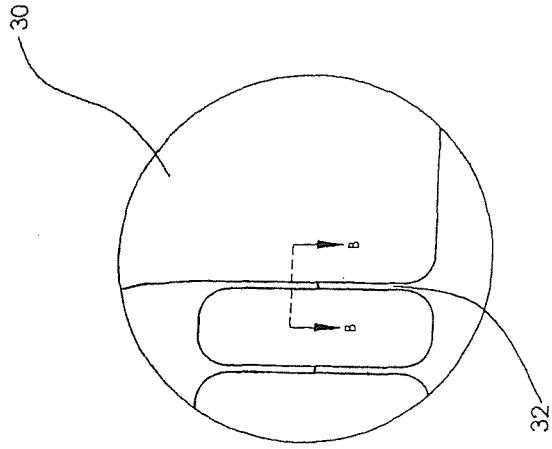


FIG. 4

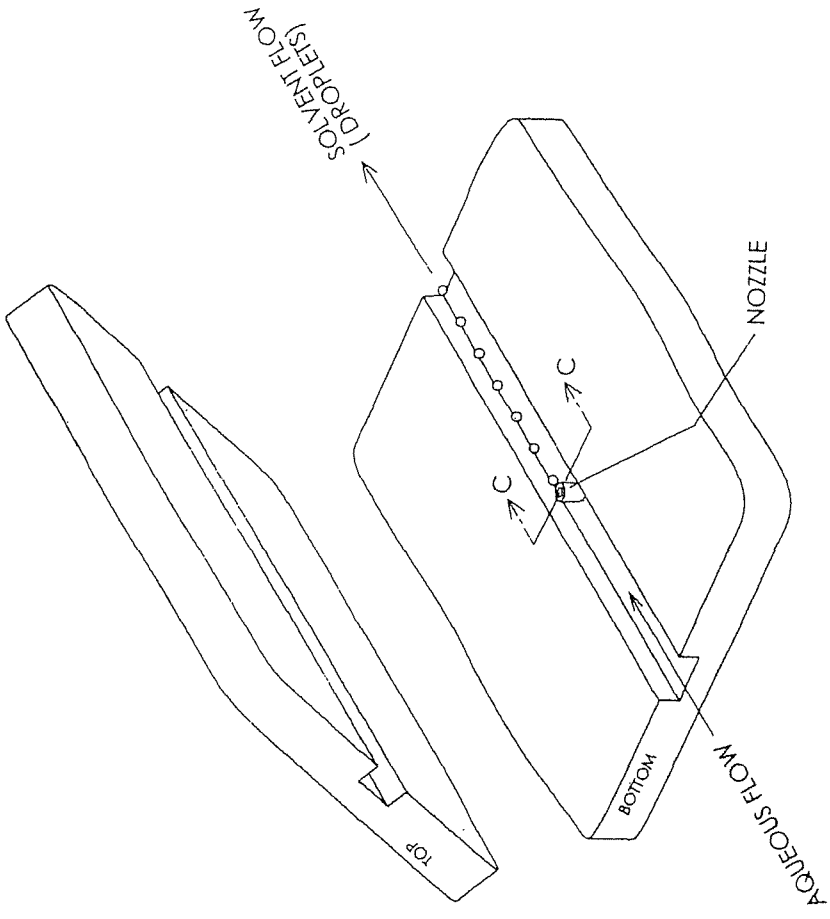


FIG. 6

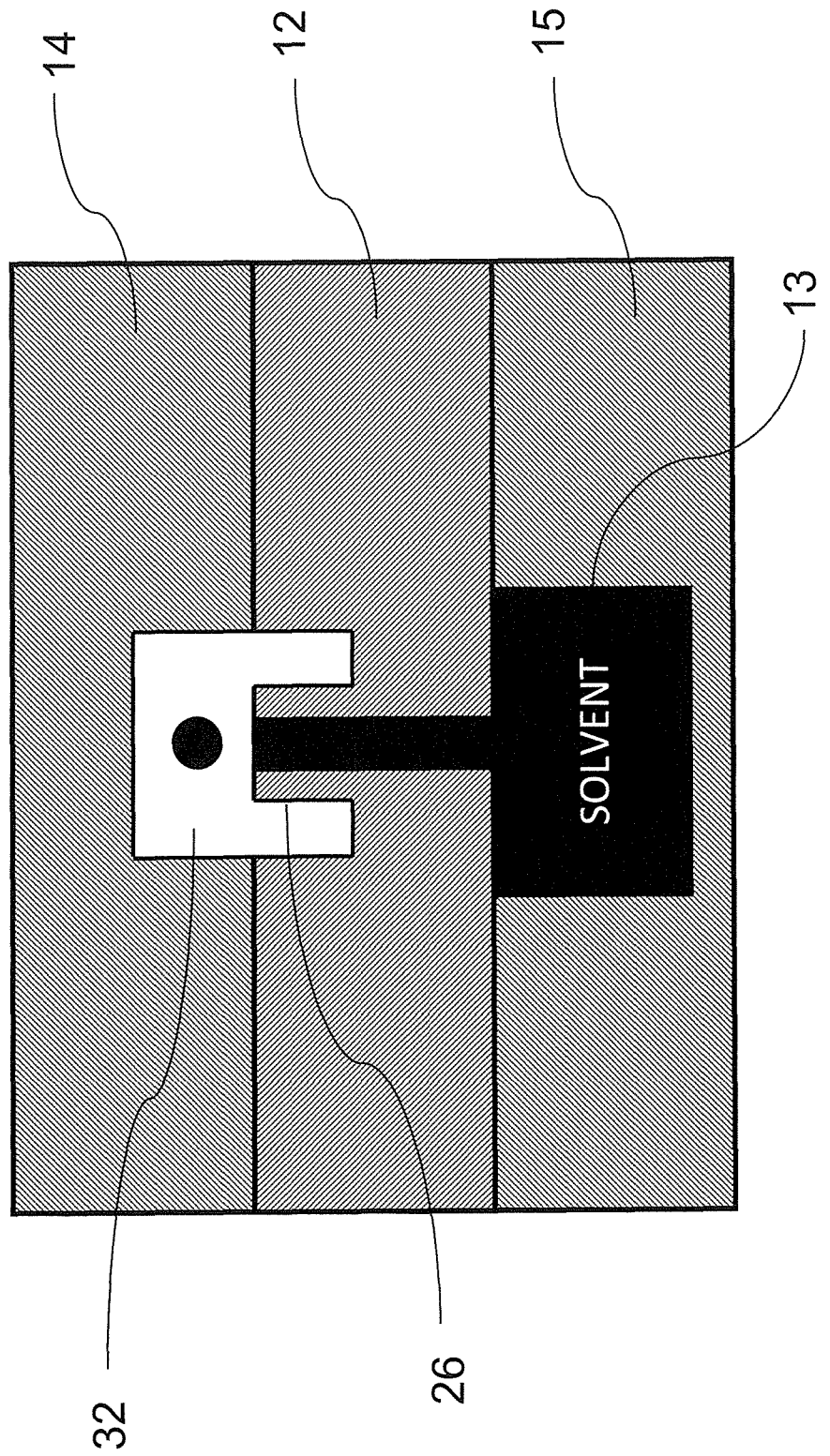


FIG. 7

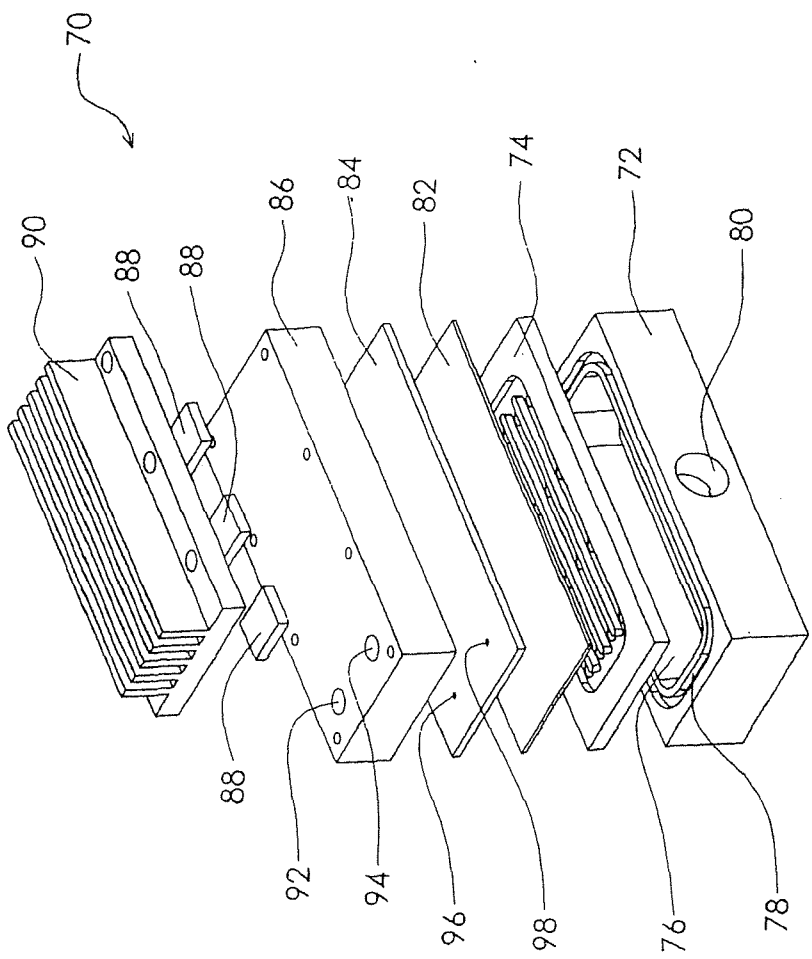


FIG. 8

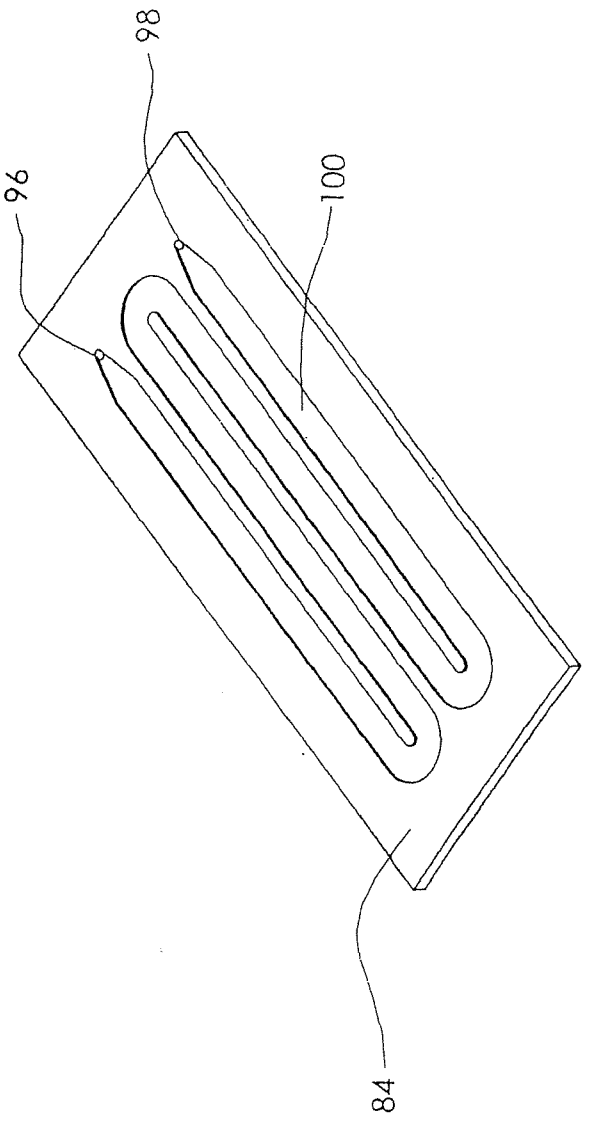


FIG. 9

FIG. 10

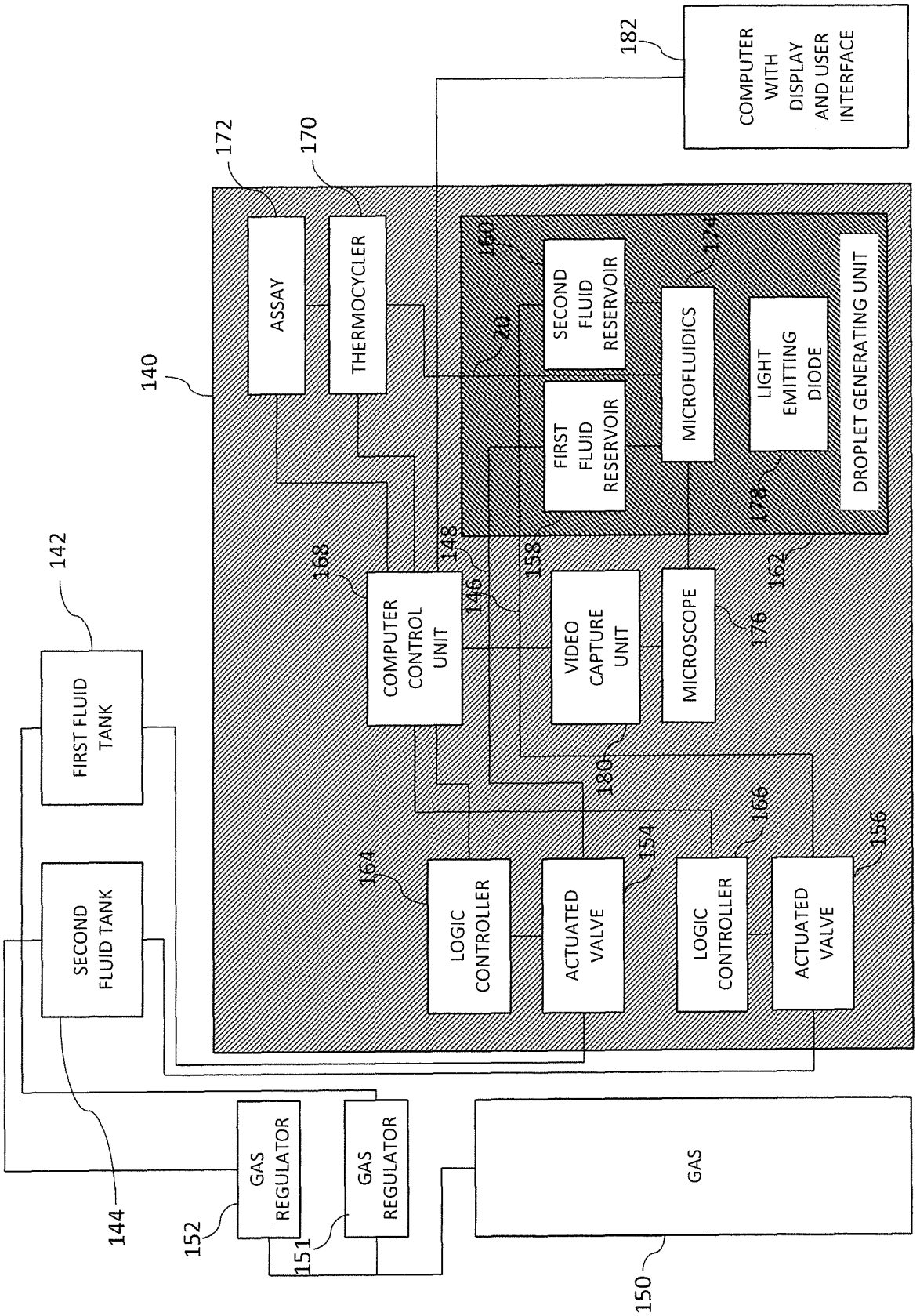

COMPUTER WITH DISPLAY AND USER INTERFACE
182
ASSAY
THERMOCYCLER
172
170
140
SECOND FLUID RESERVOIR
MICROFLUIDICS
LIGHT EMITTING DIODE
DROPLET GENERATING UNIT
FIRST FLUID RESERVOIR
160
174
178
COMPUTER CONTROL UNIT
VIDEO CAPTURE UNIT
MICROSCOPE
168
148
146
158
162
176
FIRST FLUID TANK
142
SECOND FLUID TANK
144
LOGIC CONTROLLER
ACTUATED VALVE
LOGIC CONTROLLER
ACTUATED VALVE
164
154
166
156
180
GAS REGULATOR
GAS REGULATOR
152
151
GAS
150

FIG. 11

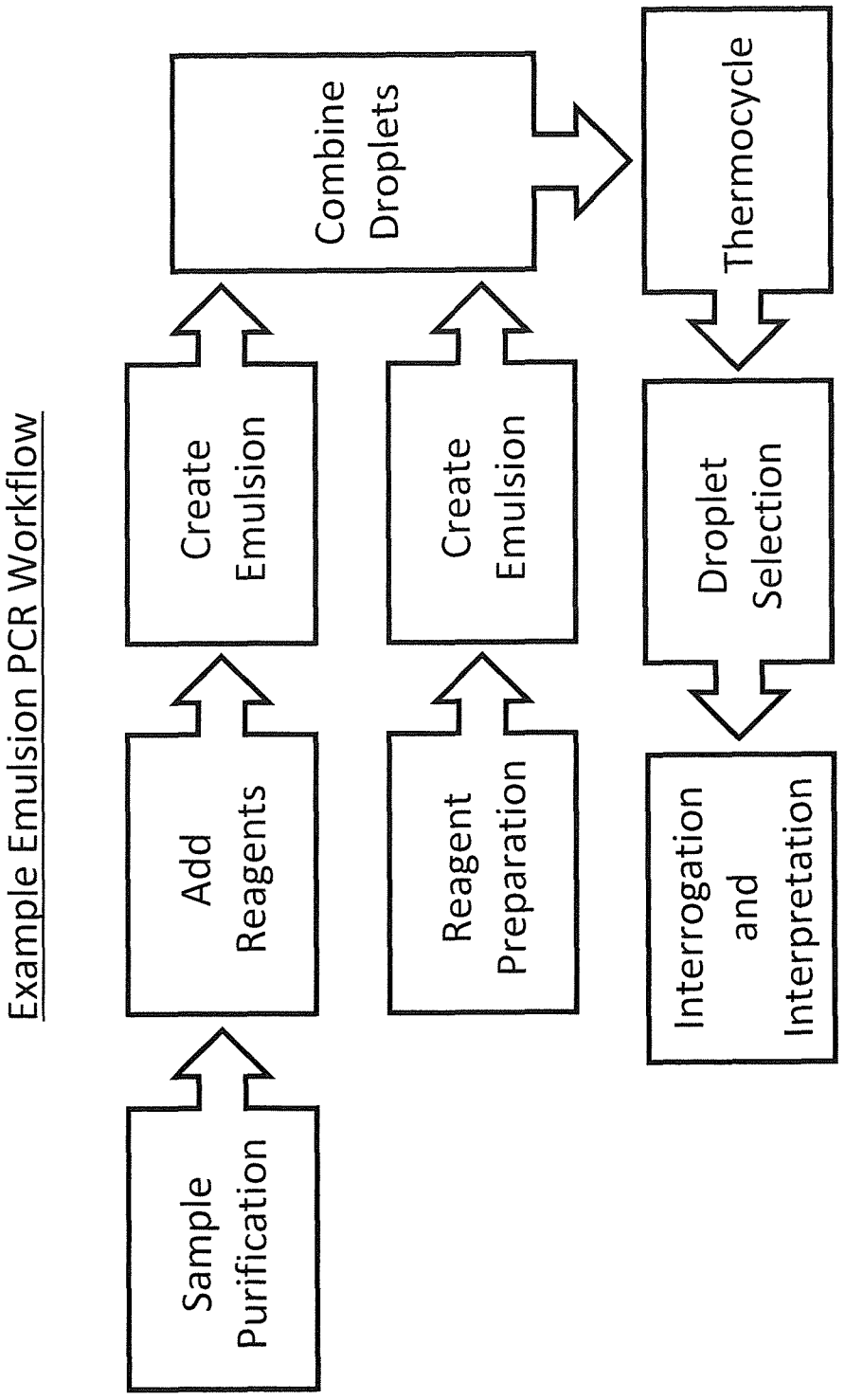
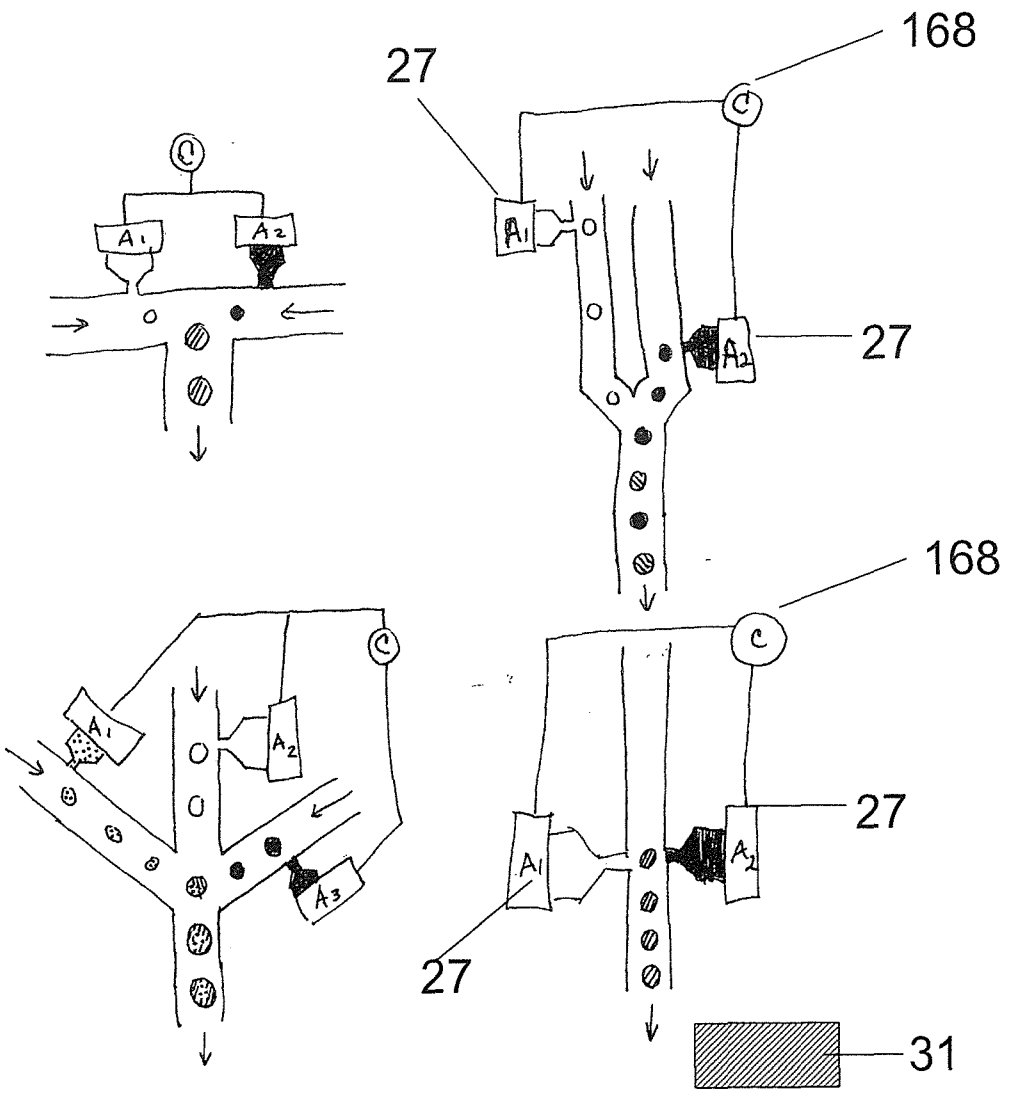

Example Emulsion PCR Workflow
Sample Purification
Add Reagents
Create Emulsion
Reagent Preparation
Create Emulsion
Combine Droplets
Thermocycle
Droplet Selection
Interrogation and Interpretation

FIG. 12



REPRESENTITIVE TIMING, LAYOUTS, OF DROPLET MERGING CONFIGURATIONS

A = actuator (27)
C = controller, electronic, for actuator(s). (168)