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(54) Title: A METHOD AND APPARATUS FOR MIXING MICROLITER FLUID VOLUMES BY APPLICATION OF ACOUSTIC SIGNALS

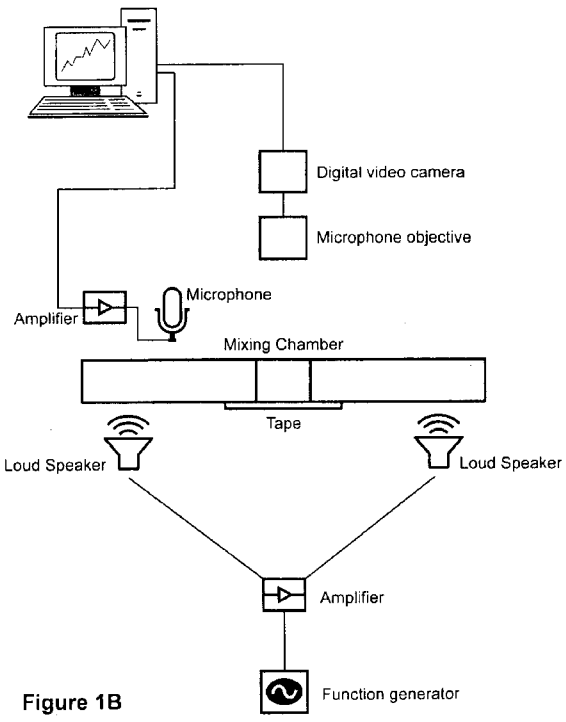

Figure 1B

(57) Abstract: The present disclosure teaches the facilitation of fluid mixing in microliter volume environments. Enabled herein is enhanced mixing of molecular and cellular participants in a biochemical, chemical or physiological transformation within microliter volume environments. The enhanced mixing of microliter volumes of reactant fluid enables greater efficacy of interaction between the transformation participants in the fluid. The present disclosure teaches the use of limiting amounts of transformation participants in an efficient manner in microliter environments.

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A METHOD AND APPARATUS FOR MIXING MICROLITER FLUID VOLUMES BY APPLICATION OF ACOUSTIC SIGNALS

FILING DATA

[0001] This application is associated with and claims priority from US Provisional Patent Application No. 61/434,315, filed on 19 January, 2011, entitled "A Method", the entire contents of which, are incorporated herein by reference.

FIELD

[0002] The present disclosure teaches the facilitation of fluid mixing in microliter volume environments. Enabled herein is enhanced mixing of molecular and cellular participants in a biochemical, chemical or physiological transformation within microliter volume environments. The enhanced mixing of microliter volumes of reactant fluid enables greater efficacy of interaction between the transformation participants in the fluid. The present disclosure teaches the use of limiting amounts of transformation participants in an efficient manner in microliter environments.

BACKGROUND

[0003] Bibliographic details of the publications referred to by author in this specification are collected alphabetically at the end of the description.

[0004] Reference to any prior art in this specification is not, and should not be taken as. an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

[0005] Biochemical, chemical and physiological transformations form the basis of numerous assays in diagnosis, disease control, industrial processes, and the development and monitoring of therapeutic product use and development. The use of volumes of

greater than microliter amounts can reduce efficiency, increase cost and is often not possible due to the low level of participating molecules in the transformation. This is particularly the case when large populations need to be screened for biosecurity, disease control or for treating the young or infirmed. It also restricts gene expression studies.

[0006] For example, understanding the molecular basis of cell biology requires correlating gene expression with cell behavior or function. Unfortunately, current technologies for quantifying gene expression, particularly where large numbers of genes are assessed simultaneously, require large amounts of RNA to be extracted from many cells (e.g. 0.1-1μg of RNA or RNA from ~50,000 cells is recommended for Affymetrix (Registered Trademark) Whole-Transcript Expression Analysis. The resulting gene expression profile thus represents an average across a very large number of different cell behaviors and correlating gene expression back to the behavior of any particular cell is problematic. A way around this is to "purify" the cell sample using, for example, laser-capture microdissection (LCM) or fluorescence-activated cell sorting (FACS). However, these techniques are expensive and time-consuming, there will always be a degree of contamination with other cells, and, in the case of FACS, may itself affect gene expression.

[0007] Ideally, gene expression would be measured in a single-cell. Although this can be achieved (Aumann *et al.* (2008) *Exp Neurol 213*:419-430; Cauli *et al.* (1997) *J Neurosci 17*:3894-3906; Neuhoff *et al.* (2002) *J Neurosci 22*:1290-1302; Stahlberg and Bengtsson. (2010) *Methods 50*:282-288), the data are largely qualitative and only a small number of genes can be assessed per cell. This is due to the limiting amount of RNA that can be harvested from a single-cell (<10pg). Essentially, the low amount of input RNA from a single-cell degrades before it can be converted into a stable cDNA copy by the reverse transcription (RT) reaction, the efficacy of which rapidly diminishes as RNA concentrations decrease (Curry *et al.* (2002) *BioTechniques 32*:768, 770, 772, 754-765; Stahlberg *et al.* (2004) *Clin Chem 50*:509-515).

[0008] Microliter volumes of solution are not easily mixed using standard methods such as shaking, triturating or vortexing due to the physical restrictions on the induction of turbulence (Batchelor (1967) *An Introduction to Fluid Dynamics*, Cambridge University Press). Hence, small volumes must be stirred artificially (Ottino and Wiggins (2004) *Philos transact A Math Phys Eng Sci 362*:923-935; Wiggins and Ottino (2004) *Philos Transact A Math Phys Eng Sci 362*:937-970).

[0009] Liu *et al.* (2003) *Anal. Chem. 75*:1911-1917 developed a process of cavitation microstreaming. This process is predicated on trapped air bubbles resting on a solid surface generating circular flows when set into vibration by an acoustic field. Acoustic microstreaming results from nonlinear rectification of the oscillatory fluid motion due to the soundwaves (Riley (2001) *Fluid Mech. 33*:43-65). However, this is only detected when there is a large gradient in the acoustic field. A small air bubble facilitates a small radius of curvature between a liquid and a gas thereby the soundwaves distort around the small radius. However, microstreaming based on the generation of small bubbles requires specialized microdevices or the use of high-frequency surface-acoustic waves (Petkovic-Duran (2009) *BioTechniques 47*:827-834, and references cited therein). Furthermore, the cavitation required can cause mechanical shearing of biological components.

[0010] Disclosed herein is an acoustic microstreaming method to improve the efficiency of biochemical, chemical and physiological transformations in microliter environments which does not rely on small air bubbles.

VALIDATION

[0011] Some aspects described herein have, since the priority date, been published in the article: "Acoustic microstreaming increases the efficiency of reverse transcription reactions comprising single-cell quantities of RNA", Boon *et al.* (2011), *BioTechniques 50*:116-119. This article subsequently achieved the status of the 4th most read *BioTechniques* publication of 2011 (*BioTechniques*, 28 December, 2011). Aspects were also published in

the article: "Increasing cDNA yields from single-cell quantities of mRNA in standard laboratory reverse transcriptase reactions using acoustic microstreaming", Boon *et al.* (2011), *J. Vis. Exp. 53*:e3144.

SUMMARY

[0012] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or method step or group of elements or integers or method steps but not the exclusion of any other element or integer or method step or group of elements or integers.

[0013] Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

[0014] Nucleotide sequences are referred to by a sequence identifier number (SEQ ID NO). The SEQ ID NOs correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is provided in Table 1. A sequence listing is provided after the claims.

Table 1

Sequence Listing

SEQ ID NO:	Description
1	Nucleotide sequence of Hprt forward primer (20mer)
2	Nucleotide sequence of Hprt reverse primer (20mer)
3	Nucleotide sequence of Nurr1 forward primer (23mer)
4	Nucleotide sequence of Nurr1 reverse primer (23mer)

[0015] As used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a fluid" includes one or more fluids, "an agent" includes a single agent, as well as two or more agents; reference to "the disclosure" includes single or multiple aspects taught by the disclosure; and so forth. Aspects disclosed herein are encompassed by the term "invention". All aspects of the invention are enabled within the width of the claims.

[0016] Acoustic microstreaming results from soundwaves propagating around a small obstacle which creates a mean flow near the obstacle. As taught herein, an acoustic microstreaming method is provided which ensures the system has a liquid-air interface within a droplet with a small radius of curvature, causing the droplet to oscillate. The oscillation does not rely on the need for air bubbles introduced either by cavitation or manually. Hence, it is a proviso herein that air bubbles are not required for the acoustic oscillation to occur. The present disclosure teaches mixing of fluid in volumes of from about 0.1μl to about 150μl thereby enhancing the interaction between entities within the fluid. The meniscus of fluid in a small container provides an appropriately small radius thereby facilitating a large gradient in the acoustic field. The ability to ensure adequate mixing between entities also results in enhanced yields of the products or effects of the interactions between the entities. The mixing of fluid is based on acoustic microstreaming of small reaction volumes, the small reaction volume being from about 0.1μl to about 150μl.

[0017] Hence, the present disclosure is instructional for a method of mixing a fluid in a vessel, the method comprising providing from about 0.1μl to 150μl of fluid in the vessel so as to establish a discontinuity in acoustic impedance and applying an acoustic signal to cause mixing within the fluid. A second acoustic signal may also be applied, the first and second signals having respective frequencies each selected from about 1Hz to about 20,000Hz in an alternating or changing manner to effect chaotic mixing within the fluid. Multiple alternating or changing frequencies may also be employed. This includes selecting from 2 to 10 different frequencies and alternating or changing between the 2 to 10 frequencies in sequence. In an embodiment, a gradient in the acoustic field is generated by the liquid-air interface formed by the meniscus of the fluid in a small container of a size sufficient for oscillation to occur when an acoustic signal is supplied. In this regard, the liquid-air interface of the meniscus can undergo transition between one or more of linear translation, volume and/or shape, oscillation modes. In one aspect, the first fluid is a reaction fluid comprising participant entities in a biochemical, chemical or physiological transformation. Generally, the yield or extent of interaction between the participant

entities is greater than the yield or interaction obtained if the first fluid was mixed by a process selected from trituration, shaking and vortexing. The participant molecules comprise nucleic acid molecules, proteins, co-factors, polysaccharides, lipopolysaccharides, phospholipids, glycoproteins, chemical molecules and cells or cell extracts. Examples of nucleic acid molecules are genomic DNA, cDNA, RNA, mRNA, short double-stranded RNA, siRNA, RNAi, DNA:RNA hybrids, microRNAs and synthetic nucleic acid molecules. Examples of proteins include enzymes, cytokines and interleukins. One or more of the participants in the reaction may be immobilized to a solid support such as a bead or a wall of the container. The acoustic microstreaming may also be employed in a range of diagnostic procedures including ELISA reactions and screening of biological fluids such as blood, serum, plasma, saliva, sputum and the like including cell extracts directly or indirectly for the presence of enzymes, cytokines, nucleic acids and metabolites.

[0018] Hence, the present disclosure enables limiting amounts of input participant entities in a biochemical, chemical or physiological transformation to be transformed more efficiently in standard reaction microliter volumes without need of specialized equipment such as microfluidics.

[0019] In another embodiment, an assay to screen for a product of an interaction between entities in fluid is taught. The method comprises providing from about 0.1µl to about 150µl of the fluid in a reaction vessel so as to establish discontinuity in acoustic impedance and applying an acoustic signal to cause mixing of the entities within the fluid for a time and under conditions sufficient for the entities to interact to form a product. Air bubbles are not required prior to the application of soundwaves. A second acoustic signal may be applied, the first and second signals having respective frequencies selected from about 1Hz to about 20,000Hz in an alternating manner to effect chaotic mixing within the fluid. In an embodiment, the discontinuity in acoustic impedance is formed between the first fluid and a second fluid in the form of air above the meniscus formed by the first fluid in a container. In one aspect, the first fluid is a reaction fluid comprising participant entities in a biochemical, chemical or physiological transformation. Generally, the yield of product from interaction of the entities is greater than the yield obtained if the fluid was mixed by a

process selected from trituration, shaking and vortexing. The entities in the fluid may include nucleic acid molecules, proteins, co-factors, polysaccharides, lipopolysaccharides, phospholipids, glycoproteins, chemical molecules and cells or cell extracts. As above, the nucleic acid molecules may be selected from genomic DNA, cDNA, RNA, mRNA, short double-stranded RNA, siRNA, RNAi, DNA:RNA hybrids, microRNAs and synthetic nucleic acid molecule. One or more of the participants in the reaction may also be immobilized to a solid phase such as a bead or a wall of the container. By "fluid" includes tissue and cell extracts. Examples of proteins include enzymes, cytokines and interleukins.

[0020] In an embodiment, the enzyme is a reverse transcriptase. In this case the fluid comprises RNA and reverse transcriptase participants and the fluid in the vessel is subjected to mixing for a time and under conditions sufficient for cDNA to be generated. In an embodiment, the amount of RNA in the fluid is from about 0.005pg/μl to about 100pg/μl of fluid or is a single-cell equivalent.

[0021] In another embodiment, the fluid is a diagnostic reaction fluid such as in an ELISA or other immune-based assay including a radio immunoassay.

[0022] In an embodiment, the present disclosure enables a method for facilitating interaction between entities in a fluid involved in a transformation event, the fluid having volume of from about 0.1μl to about 150μl, the method comprising providing the fluid in a vessel so as to establish discontinuity in acoustic impedance and applying an acoustic signal to cause mixing of the entities within the fluid.

[0023] In an embodiment, a single signal is applied resulting in uniform mixing. In another embodiment, at least two signals are applied in an alternating or changing manner and each with a different frequency within the range of from about 1Hz to about 20,000Hz so as to induce chaotic mixing.

[0024] In an embodiment, the entities are biological entities which participate in a biochemical or physiological transformation under appropriate conditions.

[0025] Hence, the present specification is instructional for a method for facilitating a biochemical or physiological transformation in a reaction fluid comprising participant biological entities wherein the reaction volume is from about 0.1µl to about 150µl, the method comprising providing the reaction fluid in a vessel so as to establish an acoustic field and then subjecting the reaction fluid to an acoustic signal to cause mixing of the participant entities in the fluid for a time and under conditions sufficient for the biochemical or physiological transformation to occur.

[0026] In another embodiment, the entities are chemical entities. Hence, another aspect taught herein is a method for facilitating interaction between chemical entities in a fluid, the fluid having a volume of from about 0.1µl to about 150µl, the method comprising providing the fluid in a vessel so as to establish discontinuity in acoustic impedance and applying an acoustic signal to cause mixing of the entities within the fluid.

[0027] The acoustic mixing is referred to herein as acoustic microstreaming and it is a proviso that air bubbles are not required prior to application of the acoustic signal in order for the signal to induce a mean flow.

[0028] In an embodiment, the present specification enables a method for facilitating a transformation in a reaction fluid comprising participant molecules wherein the reaction fluid is from about 0.1µl to about 150µl, the method comprising subjecting the reaction fluid to mixing by acoustic microstreaming for a time and under conditions sufficient for the transformation to occur.

[0029] Taught herein is a method of mixing fluid comprising participants of a biochemical transformation in a vessel, the method comprising providing from about 0.1µl to 150µl of fluid in the vessel and applying an acoustic signal to cause mixing within the fluid, wherein the fluid has a liquid-air interface at the meniscus formed by the fluid in the vessel with a radius of curvature of a size which results in oscillation and mean flow following application of the acoustic signal, and wherein the fluid does not contain air bubbles prior

to application of the acoustic signal.

[0030] Further enabled herein is to the use of acoustic microstreaming to effect mixing in a reaction fluid volume of from 0.1µl to 150µl in the generation of a transformation event between participating molecules, cells or cell extracts within the reaction fluid.

[0031] Taught herein is a method for generating cDNA from mRNA in a fluid having a volume of from about 0.1µl to about 150µl, the method comprising applying an acoustic signal of from 1Hz to 20,000Hz to the fluid in the absence of air bubbles for a time and under conditions sufficient for components in the fluid to oscillate.

[0032] An apparatus is also provided comprising a vessel for receiving a fluid in a volume of from 0.1µl to 150µl to establish a discontinuity in acoustic impedance and an acoustic transmitter adapted to apply an acoustic signal to cause mixing within the fluid. In an embodiment, the vessel comprises ambient air to enable discontinuity in acoustic impedance to form between the fluid in the vessel and the air *via* the meniscus.

[0033] In another embodiment, an apparatus is provided for facilitating mixing of participants in a reaction fluid having a volume of from about 0.1µl to about 150µl, the apparatus comprising one or a multiplicity of reaction chambers proximal to an audio speaker operably linked to a function generator to generate an audio signal sufficient to include a vortex and dipole vibration of any reaction fluid in a chamber. When chaotic mixing is required, the function generator enables alternating signals at different frequencies.

[0034] In an embodiment, an acoustic microstreaming method is provided whereby an acoustic signal is applied to a fluid having a volume of from 0.1µl to 150µl in a container, wherein the fluid has a liquid-air interface at the meniscus formed by the fluid in the container with a radius of curvature of a size which results in oscillation and a mean flow by application of the acoustic frequency. In an embodiment, alternating frequencies are applied. The presence of air bubbles is not required for acoustic microstreaming to take

effect.

[0035] Abbreviations used in the subject specification are defined in Table 2.

Table 2

Abbreviations

Abbreviation	Description
CCD	Charged couple device
DI water	Deionized water
FACS	Fluorescence-activated cell sorting
Hprt	Hypoxanthine phosphoribosyl transferase
ICCD	Intensified charged couple device
LCM	Laser-capture microdissection
Nurr1	Nuclear receptor related 1
PCR	Polymerase chain reaction
PMT	Photon multiplying tube
qPCR	Quantitative PCR
RT	Reverse transcriptase

BRIEF DESCRIPTION OF THE FIGURES

[0036] Some figures contain color representations or entities. Color photographs are available from the Patentee upon request or from an appropriate Patent Office. A fee may be imposed if obtained from a Patent Office.

[0037] Figures 1A through C are diagrammatic representations of an acoustic microstreaming mixer. (A) The four mixing holes were tapered to tightly fit four standard 0.2ml RT reaction vials. A pair of audio speakers (75mm diameter, 4Ω, 20kHz) and a function generator (Wavetek model 145) were used to apply a sinusoidal signal onto an acrylic plate containing four RT reaction vials. (B) Acoustic microstreaming mixer design. At an applied frequency of 150±0.5Hz it was observed experimentally, using a low concentration of polymer fluorescent beads (Duke Scientific Corporation, Palo Alto, California, USA), a vortex-like flow pattern in all four RT reaction vials. In the present work, 150Hz was used to generate microstreaming in RT reaction vials and enhance mixing of RNA samples with RT reagents. (C) Schematic of how a simple vortex flow elongates the interface between two fluids (black and white), allowing diffusion to take place over a short timescale.

[0038] Figures 2A through E are graphical representations showing effects of micromixing during 25µl RT reactions on subsequent real-time quantitative PCR (qPCR). (A) Example of qPCR traces detecting Nurr1 cDNA following three RT reactions comprising a "single-cell equivalent" concentration of RNA (0.1pg/µl). "No mix" = previous RT reactions mixed *via* trituration of the sample by repeated aspiration into and out of a pipette, without micromixing, "mix5"=micromixing for the initial 5 minutes of RT; "mix60"=micromixing for the entire 60 minutes of RT. (B and C) Mean ± SE number of qPCR cycles to reach 50% maximum fluorescence for detection of Hprt (B, n=2 experiments) and Nurr1 (C, n=3 experiments) as a function of RNA concentrations in prior RT reactions that were triturated (no mix, light gray) or micromixed for the initial 5 minutes (mix5, dark gray) or the entire 60 minutes (mix60, black). Asterisks denote significant ($p<0.05$) differences compared with the no mix samples at the same

concentration (Tukey multiple comparisons test). (D) qPCR traces detecting Nurr1 cDNA following RT reactions comprising different RNA concentrations [1000 (black), 100 (yellow), 10 (blue) and 1pg/μl (red)]. (E) Melting curve analyses of qPCR products from the experiment shown in (D).

[0039] Figure 3 is a graphical representation showing the effects of acoustic microstreaming on mixing of 20pg/μl RNA in DI water. Without micromixing RNA relies on diffusion only; the mixing process is slow and PMT output (gray line) is stable. As soon as the micromixing is switched on, RNA mixes quickly with DI water. The PMT average signal output (black line, 10 point average) increased indicating rapid transport of RNA from the top to the bottom of the mixing chamber.

DETAILED DESCRIPTION

[0040] The present disclosure teaches a method for mixing a fluid in a small reaction volume. In an embodiment, the mixing is referred to as acoustic microstreaming. By "small reaction volume" is meant a volume of from about 0.1µl to about 150µl. Examples of small reaction volumes include approximately 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149 and 150µl. It is taught herein that more efficient mixing of entities in the fluid increases the efficacy of any interaction between the entities such as during a transformation event. This in turn leads to greater yields of transformation products and/or outcomes. Hence, the present disclosure instructs the use of limiting amounts of input participant entities in a biochemical, chemical or physiological transformation to be transformed more efficiently in standard reaction microliter volumes without need of specialized equipment such as microfluidics.

[0041] Acoustic microstreaming results from soundwaves propagating around a small obstacle which creates a mean flow near the obstacle. Whilst such an obstacle was previously an air bubble, the instant disclosure ensures the system has a liquid-air interface at the meniscus of a droplet in a small container with a small radius of curvature causing the droplet to oscillate following application of soundwaves. Hence, it is a proviso herein that air bubbles are not required and are not present prior to the application of soundwaves.

[0042] Accordingly, taught herein is a method of mixing a fluid in a vessel, the method comprising providing from about 0.1µl to 150µl of fluid in the vessel so as to establish a discontinuity in acoustic impedance and applying an acoustic signal to cause mixing within the fluid.

[0043] Enabled herein is a method for facilitating mixing of entities within a fluid, the fluid having a volume of from about 0.1μl to about 150μl, the method comprising providing the fluid in a vessel so as to establish a discontinuity in acoustic impedance and applying an acoustic signal to cause mixing of the entities within the fluid.

[0044] The present disclosure instructs a method for facilitating interaction between entities in a fluid involved in a transformation event, the fluid having volume of from about 0.1μl to about 150μl, the method comprising providing the fluid in a vessel so as to establish a discontinuity in acoustic impedance and applying an acoustic signal to cause mixing of the entities within the fluid.

[0045] In an embodiment, the present disclosure enables a method for facilitating a biochemical, chemical or physiological transformation in a reaction fluid comprising participant biological, chemical or physiological entities wherein the reaction fluid is from about 0.1μl to about 150μl, the method comprising subjecting the reaction fluid to mixing by acoustic microstreaming for a time and under conditions sufficient for the transformation to occur.

[0046] In an embodiment, the present disclosure enables a method for facilitating a biochemical, chemical or physiological transformation in a reaction fluid comprising participant biological, chemical or physiological entities wherein the reaction fluid is from about 0.1μl to about 150μl, the method comprising subjecting the reaction fluid to mixing by acoustic microstreaming in the absence of air bubbles for a time and under conditions sufficient for the transformation to occur.

[0047] Taught herein is a method of mixing fluid comprising participants of a biochemical transformation in a vessel, the method comprising providing from about 0.1μl to 150μl of fluid in the vessel and applying an acoustic signal to cause mixing within the fluid, wherein the fluid has a liquid-air interface at the meniscus formed by the fluid in the vessel with a radius of curvature of a size which results in oscillation and mean flow following

application of the acoustic signal, and wherein the fluid does not contain air bubbles prior to application of the acoustic signal.

[0048] The present disclosure teaches efficient mixing in small reaction volumes by providing a reaction fluid in a vessel so as to establish a discontinuity in acoustic impedance. The application of an acoustic signal causes mixing within the fluid, in the absence of cavitation. This is regarded as uniform or single signal mixing. In another embodiment, a second acoustic signal is applied to effect chaotic or multi-signal mixing. When chaotic or multi-signal mixing is required, generally the first and second signals are at first and second frequencies, respectively and are alternatively applied. This establishes flow patterns with streamlines that cross when the frequencies are alternatively applied. In a another embodiment, a further from 2 to about 10 frequencies are employed to effect multiple alternating or changing frequency mixing. The term "from 2 to about 10" includes 2, 3, 4, 5, 6, 7, 8, 9 and 10 frequencies.

[0049] The discontinuity in acoustic impedance is formed between the reaction fluid and a second fluid which is generally a gas such as ambient air. A meniscus of the fluid in a small container such as a well or vial provides an appropriately small radius thereby facilitating a large gradient in the acoustic field. In an embodiment, a gradient in acoustic field is generated of sufficient size by the small radius of curvature at the liquid-air interface formed by the meniscus of the fluid in the container. The discontinuity in acoustic impedance may also be regarded as an acoustic field gradient. The acoustic field gradient is not reliant on the presence of air bubbles and it is a proviso herein that air bubbles (generated either by cavitation or introduced manually) not be present prior to the application of soundwaves.

[0050] In an embodiment, an acoustic microstreaming method is provided whereby an acoustic signal is applied to a fluid having a volume of from 0.1μl to 150μl in a container, wherein the fluid has a liquid-air interface at the meniscus formed by the fluid in the container with a radius of curvature of a size which results in oscillation and a mean flow by application of the acoustic frequency. In an embodiment, alternating frequencies are

applied. It is a proviso that air bubbles not be present prior to the application of the soundwaves.

[0051] An aspect enabled herein includes a method for facilitating a biochemical, chemical or physiological transformation in a reaction fluid comprising participant biological or chemical entities wherein the reaction volume is from about 0.1µl to about 150µl, the method comprising providing the reaction fluid in a reaction vessel so as to establish a discontinuity in acoustic impedance and then subjecting the reaction fluid to an acoustic signal to cause mixing of the participant entities in the fluid for a time and under conditions sufficient for the transformation to occur, wherein the fluid does not contain air bubbles prior to the application of the acoustic signal.

[0052] In an embodiment, both a first and second signal is applied at first and second frequencies, respectively, in an alternating or changing manner so as to induce chaotic mixing. Hence, both uniform and chaotic mixing is contemplated herein.

[0053] Reference to "biological, chemical and physiological entities" in a fluid include any molecule, cell, cell extract or chemical agent which is or are involved directly or indirectly in a transformation event. The term "transformation event" includes, for example, an enzymatic reaction, a chemical or biochemical reaction, an antibody binding event, a ligand-receptor interaction, as well as cellular transformation such as inducing a cell or cell extract to produce an effector molecule such as a cytokine. The latter is encompassed by a physiological transformation event.

[0054] Examples of entities include nucleic acid molecules, proteins, co-factors, antibodies, carbohydrates, glycoproteins, lipids, polysaccharides, lipopolysaccharides, chemical molecules and the like. Examples of nucleic acid molecules include RNA and its various forms such as mRNA, single- or double-stranded RNA, short interfering, RNAs, RNAi molecules, DNA, DNA:RNA hybrids and synthetic nucleic acids. Examples of proteins include enzymes, cytokines, interleukins, etc. Examples of chemical molecules are catalysts and chemical compounds. Other examples include samples such as blood,

serum, plasma and cell extracts. In an aspect, the fluid is used in a diagnostic reaction such as an ELISA or other immunoassay or nucleic acid based assay. In an aspect, one or more of the entities are immobilized to a solid support such as a bead or a wall of the container.

[0055] In an embodiment, the reaction fluid may comprise one or more cells which, after exposure to a stimulant such as an antigen, produce immune effector molecules. The transformation is then the production of the effector molecules following stimulation of an immune cell. As indicated above, this is an example of a physiological transformation. Other transformation events are referred to as biochemical or chemical transformation.

[0056] Hence, by a "transformation event" is meant any cause and effect resulting from adequate mixing of entities which participate in the transformation for a time and under conditions sufficient for the transformation to occur.

[0057] Accordingly, the present disclosure teaches a method for facilitating an event which occurs following interaction between two or more entities in a fluid, the fluid comprising from about 0.1μl to 150μl, the method comprising subjecting the fluid to mixing by acoustic microstreaming for a time and under conditions sufficient to facilitate the event.

[0058] Enabled herein is a method for facilitating an event which occurs following interaction between two or more entities in a fluid, the fluid comprising from about 0.1μl to 150μl, wherein the fluid has a liquid-air interface at the meniscus formed by the fluid in a container with a radius of curvature of a size which results in oscillation and a mean flow following application of an acoustic signal. The fluid does not require air bubbles to the application of the acoustic signal.

[0059] By "acoustic microstreaming" is meant either single signal mixing (uniform mixing) or multi-signal mixing using two or more different frequencies (chaotic mixing). In particular, acoustic microstreaming is the process of providing a fluid in a vessel so as to establish a discontinuity in acoustic impedance so that when an acoustic signal is applied,

there is mixing of the fluid as well as entities within the fluid. In essence, soundwaves propagate around a small obstacle such as the interface between a fluid layer and a solid phase to create a mean flow near the obstacle. The obstacle is not an air bubble. Acoustic microstreaming is achieved at audio frequencies by ensuring the system has a liquid-air interface with a small radius of curvature, causing the fluid to oscillate. In essence, the liquid in a small container forms meniscus with an appropriately small radius of curvature. This causes the droplet to oscillate upon application of the soundwaves. Oscillation does not require the presence of air bubbles.

[0060] Reference to a "vessel" includes a well, vial, container, tube, microtube, or other walled receptacle adapted to receive a fluid in a volume of 0.1µl to 150µl.

[0061] When a participating molecule is nucleic acid molecule, then the nucleic acid molecule is generally present in the 0.1µl to 150µl reaction fluid in an amount of from 0.005pg/µl to about 100pg/µl of reaction fluid. This range includes 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and 100pg/µl reaction fluid. In an embodiment, the nucleic acid molecule is RNA and is subject to, for example, reverse transcriptase.

[0062] The subject teachings enable a method for generating cDNA from mRNA in a reaction volume comprising the mRNA and other participating molecules required for a reverse transcription reaction wherein the volume of the reaction fluid is from about 0.1µl to 150µl, the method comprising subjecting the reaction fluid to mixing by acoustic microstreaming for a time and under conditions sufficient for cDNA to be generated from the mRNA. It is a proviso herein that acoustic microstreaming is applied to fluid without air bubbles.

[0063] Generally, the method enables generation of greater amounts of cDNA from single-

cell equivalent amounts of mRNA than would otherwise be achievable by conventional procedures.

[0064] Consequently, another aspect taught herein is a method for generating cDNA from single-cell equivalent amounts of mRNA in a reaction fluid comprising the mRNA and other participating molecules required for a reverse transcription reaction wherein the volume of the reaction fluid is from about 0.1μl to about 150μl, the method comprising providing the reaction fluid in a reaction vessel so as to establish a discontinuity in acoustic impedance and then subjecting the reaction fluid to an acoustic signal to cause mixing in the fluid for a time and under conditions sufficient for cDNA to be generated from mRNA and wherein the fluid does not contain air bubbles prior to the application of the acoustic signal.

[0065] Taught herein is a method for generating cDNA from mRNA in a fluid having a volume of from about 0.1μl to about 150μl, the method comprising applying an acoustic signal of from 1Hz to 20,000Hz to the fluid in the absence of air bubbles for a time and under conditions sufficient for components in the fluid to oscillate.

[0066] In an embodiment, a single signal is provided to facilitate uniform mixing. In another embodiment, both a first and second signal is applied at first and second frequencies, in an alternate manner so as to induce chaotic mixing.

[0067] The use of acoustic microstreaming facilitates enhanced yield and/or enhance interaction between participating molecules. Conveniently, the yield of product obtained in a reaction vessel or extent of interaction between participating molecules is greater than the yield or interaction obtained if the reaction fluid was mixed by a process selected from trituration, shaking or vortexing.

[0068] Another aspect enabled herein is a method for conducting an immunoassay comprising mixing immunoassay components in a reaction fluid to from about 0.1μl to 150μl, the method comprising providing the reaction fluid in a reaction vessel so as to

establish a discontinuity in acoustic impedance and then subjecting the reaction fluid to an acoustic signal to cause mixing in the fluid for a time and under conditions sufficient for the product of the immunoassay to form, and deleting the product.

[0069] When a signal is applied, it is at a frequency within the audio frequency range of about 1Hz to about 20,000Hz, including from about 10 to about 20,000Hz, including about 20 to about 20,000Hz, including from about 50 to 500 Hz. When chaotic mixing is required, at least two signals are used at first and second frequencies, respectively. Hence, a first frequency is selected from about 1 to about 20,000Hz and a second frequency is selected from about 1 to about 20,000Hz wherein the first and second frequencies may each be the same or one or other or both may change. Specific frequencies useful in the practice of the present invention include 50Hz, 100Hz, 150Hz, 200Hz and 250Hz. Each may be +/- 10Hz. Multiple alternating frequencies of from 2 to about 10 frequencies may also be employed.

[0070] The present disclosure teaches the use of acoustic microstreaming to effect mixing in a reaction fluid volume of from 0.1µl to 150µl in the generation of a transformation event between participating molecules with the reaction fluid.

[0071] The acoustic microstreaming may result in uniform or chaotic mixing.

[0072] Hence, the present disclosure teaches a method which enables mixing of fluid in volumes of from about 0.1µl to about 150µl, the fluid forming a meniscus between in a container having a liquid-air interface with a small radius of curvature whereby application of sound frequencies to the fluid causes the fluid to oscillate.

[0073] The present disclosure teaches an assay to screen for a product of an interaction between entities in fluid, the method comprising providing from about 0.1µl to about 150µl of the fluid in a reaction vessel so as to establish a discontinuity in acoustic impedance and applying an acoustic signal to cause mixing of the entities within the fluid for a time and under conditions sufficient for the entities to interact to form a product. As

indicated above, both uniform and chaotic mixing may be used.

[0074] In an embodiment, large scale screening of blood on blood derivatives (e.g. serum, plasma, etc.) is performed for biomarkers of disease, state of health and vaccination history.

[0075] An apparatus is also provided comprising a vessel for receiving a fluid in a volume of from 0.1μl to 150μl to establish a discontinuity in acoustic impedance and an acoustic transmitter adapted to apply an acoustic signal to cause mixing within the fluid. In an embodiment, the vessel comprises ambient air to enable a discontinuity in acoustic impedance to form between the reaction fluid in the vessel and the air. In an embodiment, the meniscus of the fluid in a container provides an appropriately small radius of curvature such that upon application of the soundwaves, the fluid oscillates. The fluid does not require or contain air bubbles prior to the application of the acoustic signal.

[0076] In another embodiment, an apparatus is provided for facilitating mixing of participants in a reaction fluid having a volume of from about 0.1μl to about 150μl, the apparatus comprising one or a multiplicity of reaction chambers proximal to an audio speaker operably linked to a function generator to generate an audio signal sufficient to include a vortex and dipole vibration of any reaction fluid in a chamber. When chaotic mixing is required, the function generator enables alternating signals at different frequencies. The reaction chambers are designed so as to facilitate the formation of a meniscus by the reaction fluid with a small radius of curvature. The audio signal then induces oscillation of the fluid. The fluid does not require or contain air bubbles prior to the application of an acoustic signal.

EXAMPLES

[0077] Aspects disclosed herein are is further described by the following non-limiting Examples. In these Examples, the materials and methods set out below are employed.

Acoustic Microstreaming

[0078] As detailed elsewhere (Petkovic-Duran *et al.*, 2009 *supra*; Ottino and Wiggins, 2004 *supra*; Wiggins and Ottino, 2004 *supra*; Tho *et al.*, 2007 *supra*; Tho *et al.* (2007) *Journal of Fluid Mechanics* 576:191-233) acoustic microstreaming is a phenomenon where soundwaves propagating around a small obstacle create a mean flow near the obstacle. An acoustic microstreaming based device (Figure 1) is provided in which acoustic microstreaming is achieved at audio frequencies by ensuring the system has a liquid-air interface with a small radius of curvature, causing the entire drop to oscillate. The meniscus of a drop in a small well or vial provided an appropriately small radius. Most significantly, existing PCR vials or other standard laboratory consumables are utilized. A frequency of 150Hz was selected which resulted in a vortex pattern. Air bubbles are not required and are not present prior to application of soundwaves.

Reverse transcription (RT)

[0079] Total RNA was isolated from snap frozen acutely prepared adult mouse midbrain slices using the PicoPure (Trademark) RNA Isolation Kit (Arcturus, CA, USA) and DNase treated according to the manufacturer's instructions. The concentration of this "stock" RNA was determined using a NanoDro (Trademark) 1000 Spectrophotometer (Thermo Scientific, DE, USA). In a sterile, nuclease-free 200µl thin-walled PCR tube, 0.5µg of random hexamer primers (Promega) was added to serial dilutions of the RNA stock in a total volume of 11µl and heated to 70°C for 5 minutes. The tube was chilled on ice for 5 minutes and centrifuged briefly. After adding RNA AMV Reverse Transcriptase (30 U; promega), 5X Reaction Buffer, dNTP mix (1mM final), RNasin (Registered Trademark) Ribonuclease Inhibitor (40U; Promega), the RT mix was incubated for 60 minutes at 37°C, with or without micromixing.

qPCR

[0080] After reverse transcription, specific cDNA products were quantified using a Corbett Rotor-gene RG-3000 qPCR machine (Corbett Research, Sydney, Australia). The final qPCR reaction mix consisted of 1µl of RT mix, 0.25U AmpliTaq Gold (Registered Trademark) DNA polymerase (Applied Biosystems, CA, USA), 1 x gold PCR buffer, 200µM dNTPs, 1mM $Mg(OAc)_2$, 5 x SYBR (Registered Trademark) [Invitrogen, Victoria, Australia], 5µM specific primers (see Supplementary information), in a total volume of 20µl. After the 95°C for 5 minute hot start, cycling parameters were 95°C for 15 seconds denaturation, 60°C for 15 seconds annealing, 72°C for 20 seconds extension and signal acquisition.

Optical detection and quantification of micromixing of single-cell quantities of RNA

[0081] After DNase1 treatment, 1µg of isolated total RNA was fluorescently labeled by coupling Alexa (Registered Trademark) Fluor 488 to the purine bases using a ULYSIS Nucleic Acid Labeling Kit (Molecular Probes, Eugene, OR, USA). Briefly, the ethanol precipitated (1/10 volume of 3M sodium acetate and 2X volumes of ethanol) total RNA were re-suspended in 24µl of ULS (Trademark) labeling buffer and denatured at 95°C for 5 minutes. After chilling on ice, 1µl of labeling reagent was added, and then the reaction was incubated at 90°C for 10 minutes. The reaction was stopped by plunging the reaction tube into an ice bath. Uncoupled fluorophore was removed by passing the reaction mix through a Sephadex (Trademark) gel G-100 column by centrifugation at 1300g. The recovered RNA was diluted to 1ng/µl by RNase and DNase free water.

[0082] For the higher concentration of RNA (1ng/µl) tested, an Intensified CCD (PI-MAX, Princeton Instruments) camera was used to visualize the mixing. The ICCD camera software provided the greyscale mean and standard deviation of each frame taken. However, the ICCD camera was not sensitive enough to enable visualization of the lower RNA concentration (20pg/µl) tested. For the lower RNA concentration a 488nm Sapphire Laser (Coherent Inc, Santa Clara, USA) was used for excitation of fluorescent-labeled RNA. The emission light was collected with a Photon Multiplying tube (PMT)

[Hamamatsu Photonics, Hamamatsu City, Japan] which was focused at the bottom of the mixing chamber. Data from the PMT were logged with a M8784 counting board (Hamamatsu Photonics, Hamamatsu City, Japan) and a PCI bus add-in board.

EXAMPLE 1

Enhancement of biochemical transformation by micromixing

[0083] RT reactions were performed in standard 0.2ml PCR tubes with RNA concentrations ranging from 1ng/µl to 0.1pg/µl in a total reaction volume of 25µl. At the lower end of this range, the RT reaction mix contains approximately the amount of RNA present in a single-cell (hereafter referred to as a "single-cell equivalent"). Two different micromixing protocols were tested: micromixing for only the initial 5 minutes of the 60 minute reaction ("mix5") and micromixing throughout the entire reaction ("mix60"). Also compared was the absence of micromixing ("no mix") in which standard laboratory mixing (trituration of the sample by repeated aspiration into and out of a pipette) was performed. The cDNA yield from these RT reactions was assessed by performing qPCR using primers designed to amplify two test genes expressed by dopaminergic (DA) neurons of the midbrain: hypoxanthine phosphoribosyl-transferase (Hprt), a commonly measured "housekeeping" gene present at relatively low-levels (low abundance) and constant amounts in all cell-types, and the orphan nuclear receptor Nurr1, a low abundance transcription factor present in adult midbrain DA neurons.

[0084] The number of qPCR cycles required to reach detection threshold (arbitrarily defined as 50% maximum fluorescence in this study) for both Hprt (Figure 2B) and Nurr1 (Figure 2C) was significantly reduced when micromixing was applied during the RT reaction ($p<0.001$ for Hprt and $p=0.03$ for Nurr1, two-way ANOVAs). Thus, micromixing offered improvement above and beyond current standard laboratory practice. This shows micromixing increased the amount of RT reaction product (cDNA) and, therefore, improved RT reaction efficiency. The degree of improvement was dependent on RNA concentration (see below), but was maximal (on average) at ~9 qPCR cycles for Hprt (at 10pg/µl, Figure 2B) and ~15 cycles for Nurr1 (at 0.1 pg/µ l, Figures 2A and 2C). The extent of this improvement in terms of yields is shown when the yields from different concentrations of RNA are compared (Figures 2B and 2C). It is apparent that, in the absence of micromixing, ~100 fold more RNA was required to produce qPCR signals equivalent to those obtained when reactions were micromixed. In other words, for Hprt, RT of 100pg/µl RNA without

micromixing resulted in a qPCR signal equivalent to RT of 1pg/µl RNA with micromixing (Figure 2B), and for Nurr1, RT of 10pg/µl RNA without micromixing resulted in a qPCR signal equivalent to RT of 0.1pg/µl RNA with micromixing (Figure 2C). Therefore, micromixing resulted in a ~1 00-fold increase in cDNA product.

[0085] The effects of micromixing depended on RNA concentration, with low, single-cell equivalent concentrations benefiting significantly more than higher concentrations (Figures 2B, 2C and 2D). In the case of Hprt (Figure 2B), micromixing for 60 min provided a better yield from the lowest RNA concentration (1pg/µl) than micromixing for 5 minutes, whereas micromixing for longer periods had no benefit for the two higher RNA concentrations ($p=0.008$, two-way ANOVA). There did appear to be a benefit in micromixing the lowest RNA concentration (0.1pg/µl) for a longer period. The same conclusion can be drawn from the data in Figure 2D, which illustrate six qPCR runs for Nurr1 cDNA detection at each of four different RNA concentrations (1ng/µl black traces, 100pg/µl yellow traces, 10pg/µl blue traces, and 1pg/µl red traces). Duplicate samples were run under each of the three different treatments (no mix, mix5, and mix60) at each concentration (i.e. six traces/concentration). At the two highest concentrations (black and yellow traces) micromixing made no or very little difference to the signal. At the next lowest concentration micromixing began to make a difference (10pg/µl, blue traces), and micromixing made its greatest contribution at the lowest concentration (1pg/µl, red traces). When 1pg/µl samples (red traces) were micromixed throughout the entire RT reaction (mix60), fewer cycles were required to reach the detection threshold (50% maximum fluorescence) than unmixed samples or samples micromixed for 5 minutes, some of which were undetectable after 40 cycles. Moreover, melting curve analysis of the qPCR products from the 1pg/µl samples revealed that only those micromixed for 60min (red "mix60" traces in Figure 2E) yielded the appropriate product, i.e. an amplicon identical to that obtained in all the higher concentration samples. By contrast, the remaining 1pg/µl samples (no mix and mix5), as well as the negative controls (gray traces), contained alternative amplicon products (i.e. false positives such as primer dimers). In other words, micromixing throughout the entire RT reaction permitted detection of an otherwise undetectable transcript from single-cell equivalent quantities of RNA.

EXAMPLE 2

Optical detection and quantification of micromixing of single-cell quantities of RNA

[0086] From the results in Example 1, it was concluded that micromixing has beneficial effects at low RNA concentrations (single-cell equivalent), but insignificant effects at higher RNA concentrations. The purpose of the next experiment was to visualize the RNA during the micromixing process to see whether it improves mixing of RNA and RT reagents, particularly at low RNA concentrations. If so, this is the likely source of the improvement seen at the qPCR level.

[0087] To do this, RNA was fluorescent-labeled and imaged before, during and after micromixing. Note that 25µl of deimmunized (DI) water was used to model the RT reaction mixture in these experiments. Although some components of the RT reaction mixture have higher viscosity, their concentration in the RT reaction mixture is small so this difference is unlikely to alter the conclusion drawn. At a higher RNA concentration (1ng/µl), the RNA moved very slowly with the vortex once the micromixer was switched on. The standard deviation of the image intensity did not change significantly before and after the micromixer was switched on. In contrast, at a much lower RNA concentration (20pg/µl) micromixing produced much faster and thorough mixing of the labeled RNA. The PMT was focused to collect the light from the bottom of the mixing chamber. It initially recorded some light emitted from the 1µl drop of 20pg/µl RNA placed on the top of the mixing chamber. With the micromixer switched off, the PMT output remained unchanged for 5 minutes (Figure 3). This indicates that diffusion alone is very slow, causing negligible transport of the introduced RNA from the top to the bottom of the mixing chamber over 5 minutes. However, as soon as the micromixer was switched on, the PMT output increased indicating rapid transport of RNA from the top to the bottom of the mixing chamber (Figure 3). The labeled RNA then appears to mix with the DI water, evidenced by an irregular and gradually reducing signal from the PMT. This is typical of any system in which a small quantity of introduced tracer abruptly begins mixing into a larger vessel (Tatterson (1991) *Fluid mixing and gas dispersion in agitated tanks*, McGraw-Hill,

New York). Decay of the irregularities and establishment of a new, stable output level, indicating mixed products, appeared to take about 3 seconds.

[0088] The results of Examples 1 and 2 demonstrate that micromixing during RT reactions comprising low or "single-cell equivalent" amounts of RNA in small but conventional laboratory reaction volumes (25µl) significantly enhances qPCR detection of two low-abundance mRNA transcripts (Hprt and Nurr1). This shows that micromixing increases the efficiency of such RT reactions over and above what can be achieved using standard laboratory mixing techniques (e.g. trituration). Although not intending on limiting the present invention to any one theory on mode of action, micromixing may increase the rates of interactions between reagents (mRNA, primers, reverse-transcriptase, and dNTPs). Micromixing alone provides significant and adequate benefit without the need for further specialized equipment and methods. Micromixing, therefore, provides a novel, inexpensive and readily implemented alternative to microfluidics-based approaches to enhance cDNA yield from single-cell amounts of RNA.

[0089] In the broader context, micromixing of fluids comprising entities which participate in a transformation event enhances the transformation event. This will be the case whether the transformation is a biochemical or chemical reaction or a cell or cells being stimulated to produce an effect such as an immune effector molecule.

EXAMPLE 3

Optimization of frequencies

[0090] Multiple RT reactions are performed in standard 0.2ml PCR tubes with RNA concentrations ranging from 1ng/μl to 0.1pg/μl in a total reaction volume of 25μl. Each reaction is subject to: (i) a different frequency; and/or (ii) different combinations of 2 or more alternate frequencies (to effect chaotic mixing). cDNA yield is then measured to determine which frequency or which combination of alternating frequencies resulted in maximum biotransformation to cDNA. Optimal frequencies and optimal alternating frequencies are then determined.

EXAMPLE 4

Optimization of reaction conditions

[0091] Once the optimal frequencies are determined from Example 3, mixing times are then varied, including conducting continuous mixing, static mixing and pre-mixing. Optimal mixing times are then selected based on optimal cDNA yield or percentage transformation of RNA. Mixing experiments are also varied as to concentration of reagents to optimize concentration of reactants.

EXAMPLE 5

Single-cell reactants

[0092] Micromixing is performed as described above on RNA harvested directly from a single-cell. No further purification of the RNA is performed. Micromixing is then optimized given the contaminating proteins, lipids, DNA, organelles and the like. Both single and alternating multiple frequencies are employed. Output measurements include cDNA generation.

EXAMPLE 6

Improving single-cell gene expression profiling

[0093] The demonstrated ~100-fold increase in RT reaction product afforded by micromixing enables more sensitive measurement of gene expression in a single-cell.

[0094] Two aspects are tested:

(1) **Measurement of expression of 100s of genes in a single cell.**

Without micromixing only 3-5 genes per cell can be measured. This is tested by performing single-cell RT reactions with and without micromixing then hybridizing the reaction products onto various commercially available microarrays. It is anticipated that the micromixed samples will produce stronger signals from individual probes (genes) on the microarrays as well as greater numbers of positive probes (detectable genes).

(2) **Quantification of how much of each gene is expressed in a single cell.**

Without micromixing, only qualitative assessments of whether genes are expressed or not can be made. This is tested by performing single-cell RT reactions with and without micromixing then real-time PCR (qPCR) to quantify the amounts of different genes present. It is anticipated that micromixing will increase the fidelity of conversion of inputted RNA to cDNA, such that the amount of cDNA present (measured by qPCR) more accurately reflects the amount of RNA present in the cells. It is further tested whether micromixing enables quantification of gene expression by comparing identical cells that have been treated (or not) with a drug (e.g. growth factor) that is known to increase the expression of particular target genes. It is anticipated that without micromixing, treated and untreated cells will show the same amount of target gene expression, whereas micromixing will reveal the increase in target gene expression due to treatment.

EXAMPLE 7

Chemical and biochemical reactions

[0095] Micromixing also has application in other chemical and biochemical reactions. For example, purification of RNA from formalin fixed paraffin embedded (FFPE) tissues requires a series of lengthy reactions to reverse formalin modification of RNA as well as release of the RNA from the tissue. These reactions are proposed to be aided by micromixing to decrease the time required, increase the yield of RNA, and enable detection of otherwise undetectable transcripts.

EXAMPLE 8

ELISA

[0096] ELISA reactions are set up in volumes of from 0.1µl to 150µl. The reactions are subject to unified or chaotic mixing and output of the ELISA measured. This facilitates, for example, large scale screening of samples such as blood, serum and plasma, for biomarkers of disease, state of health and vaccination history.

[0097] Those skilled in the art will appreciate that aspects of aspects described herein are susceptible to variations and modifications other than those specifically described. It is to be understood that these aspects include all such variations and modifications. All of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of the steps or features are encompassed by the aspects taught herein as the invention.

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CLAIMS:

1. A method of mixing a fluid comprising participants of a biochemical transformation in a vessel, said method comprising providing from about 0.1µl to 150µl of fluid in the vessel and applying an acoustic signal to cause mixing within the fluid, wherein the fluid has a liquid-air interface at the meniscus formed by the fluid in the vessel with a radius of curvature of a size which results in oscillation and mean flow following application of the acoustic signal, and wherein the fluid does not contain air bubbles prior to application of the acoustic signal.

2. The method of Claim 1 further comprising applying a second acoustic signal, the first and second signals having respective frequencies each selected from about 1Hz to about 20,000Hz in an alternating manner to effect chaotic mixing within the fluid.

3. The method of Claim 2 further comprising applying a subsequent frequency selected from about 1Hz to about 20,000Hz in a multiple alternating or changing manner.

4. The method of Claim 1 wherein the yield or extent of interaction between the participant entities is greater than the yield or interaction obtained if the first fluid was mixed by a process selected from trituration, shaking and vortexing.

5. The method of Claim 4 wherein the participant molecules are selected from nucleic acid molecules, proteins, co-factors, polysaccharides, lipopolysaccharides, phospholipids, glycoproteins, chemical molecules and cells or cell extracts.

6. The method of Claim 5 wherein the nucleic acid molecules are selected from genomic DNA, cDNA, RNA, mRNA, short double-stranded RNA, siRNA, RNAi, DNA:RNA hybrids, microRNAs and synthetic nucleic acid molecules.

7. The method of Claim 5 wherein the protein is an enzyme, cytokine or interleukin.

8. The method of Claim 7 wherein the enzyme is a reverse transcriptase.

9. The method of Claim 5 wherein the cell is an immune cell.

10. The method of any one of Claims 1 to 9 wherein the first fluid volume is from about 5µl to about 50µl.

11. The method of any one of Claims 1 to 10 wherein the frequency of the signal is selected from about 50Hz to about 500Hz.

12. The method of Claim 11 wherein the frequency of the signal is selected from about 100Hz to about 250Hz.

13. The method of any one of Claims 1 to 12 wherein the fluid comprises RNA and a reverse transcriptase and the fluid in the vessel is subjected to mixing for a time and under conditions sufficient for cDNA to be generated.

14. The method of Claim 13 wherein the amount of mRNA in the fluid is from about 0.005pg/µl to about 100pg/µl of fluid.

15. The method of Claim 13 wherein the amount of mRNA in the fluid is a single-cell equivalent.

16. A method of generating cDNA from mRNA in 0.005pg/µl to about 100pg/µl of fluid said method comprising providing from 0.1µl to 150µl of fluid in a vessel and applying an acoustic signal to cause mixing of a reverse transcriptase and the mRNA and measuring cDNA output wherein the fluid has a liquid-air interface at the meniscus formed by the fluid in the vessel with a radius of curvature of a size which results in oscillation and mean flow following application of the acoustic signal, and wherein the fluid does not contain air bubbles prior to application of the acoustic signal.

17. The method of Claim 16 further comprising applying a second acoustic signal, the first and second signals having respective frequencies selected from about 1Hz to about 20,000Hz in an alternating manner to effect chaotic mixing within the fluid.

18. An assay to screen for a product of an interaction between biochemical entities in fluid, said method comprising providing from about 0.1µl to about 150µl of said fluid in a reaction vessel and applying an acoustic signal to cause mixing of the entities within the fluid for a time and under conditions sufficient for the entities to interact to form a product, wherein the fluid has a liquid-air interface at the meniscus formed by the fluid in the vessel with a radius of curvature of a size which results in oscillation and mean flow following application of the acoustic signal, and wherein the fluid does not contain air bubbles prior to application of the acoustic signal.

19. The assay of Claim 18 further comprising applying a second acoustic signal, the first and second signals having respective frequencies selected from about 1Hz to about 20,000Hz in an alternating manner to effect chaotic mixing within the fluid.

20. The assay of Claim 18 further comprising applying a subsequent frequency selected from about 1Hz to about 20,000Hz in a multiple alternating manner.

21. The assay of any one of Claims 18 to 20 wherein the yield of product from interaction of the entities is greater than the yield obtained if the fluid was mixed by a process selected from trituration, shaking and vortexing.

22. The assay of Claim 21 wherein the entities are selected from nucleic acid molecules, proteins, co-factors, polysaccharides, lipopolysaccharides, phospholipids, glycoproteins, chemical molecules and cells or cell extracts.

23. The assay of Claim 21 wherein the nucleic acid molecules are selected from genomic DNA, cDNA, RNA, mRNA, short double-stranded RNA, siRNA, RNAi, DNA:RNA hybrids, microRNAs and synthetic nucleic acid molecules.

24. The assay of Claim 21 wherein the protein is an enzyme, cytokine or interleukin.

25. The assay of Claim 24 wherein the enzyme is a reverse transcriptase.

26. The assay of Claim 22 wherein the cell is an immune cell.

27. The assay of any one of Claims 18 to 26 wherein the first fluid volume is from about 5μl to about 50μl.

28. The assay of any one of Claims 18 to 27 wherein the frequency of the signal is selected from about 50Hz to about 500Hz.

29. The assay of Claim 28 wherein the frequency of the signal is selected from about 100Hz to about 250Hz.

30. The assay of Claim 18 to 29 wherein the fluid comprises RNA and a reverse transcriptase and the fluid in the vessel is subjected to mixing for a time and under conditions sufficient for cDNA to be generated.

31. The assay of Claim 30 wherein the amount of mRNA in the fluid is from about 0.005pg/μl to about 100pg/μl of fluid.

32. The assay of Claim 30 wherein the amount of mRNA in the fluid is a single-cell equivalent.

33. An apparatus comprising a vessel for receiving a fluid comprising biochemical components in a volume of from about 0.1μl to about 150μl to establish a discontinuity in acoustic impedance and an acoustic transmitter adapted to apply an acoustic signal to cause mixing within the fluid in the absence of air bubbles wherein the fluid has a liquid-air interface at the meniscus formed by the fluid in the vessel with a radius of curvature of a

size which results in oscillation and mean flow following application of the acoustic signal, and wherein the fluid does not contain air bubbles prior to application of the acoustic signal.

34. The apparatus of Claim 33 wherein the vessel comprises ambient air to enable an acoustic field gradient to form between the fluid in the vessel and the air.

35. The apparatus of Claim 33 or 34 wherein the acoustic transmitter is adapted to provide alternating signals with different frequencies, each selected from about 1Hz to about 20,000Hz.

36. An apparatus is provided for facilitating mixing of biochemical participants in a reaction fluid having a volume of from about 0.1μl to about 150μl, the apparatus comprising one or a multiplicity of reaction chambers proximal to an audio speaker operably linked to a function generator to generate an audio signal sufficient to include a vortex and dipole vibration of any reaction fluid in a chamber wherein the fluid has a liquid-air interface at the meniscus formed by the fluid in the vessel with a radius of curvature of a size which results in oscillation and mean flow following application of the acoustic signal, and wherein the fluid does not contain air bubbles prior to application of the acoustic signal.

37. The apparatus of any one of Claims 33 to 36 when used in the method or assay of any one of Claims 1 to 32.

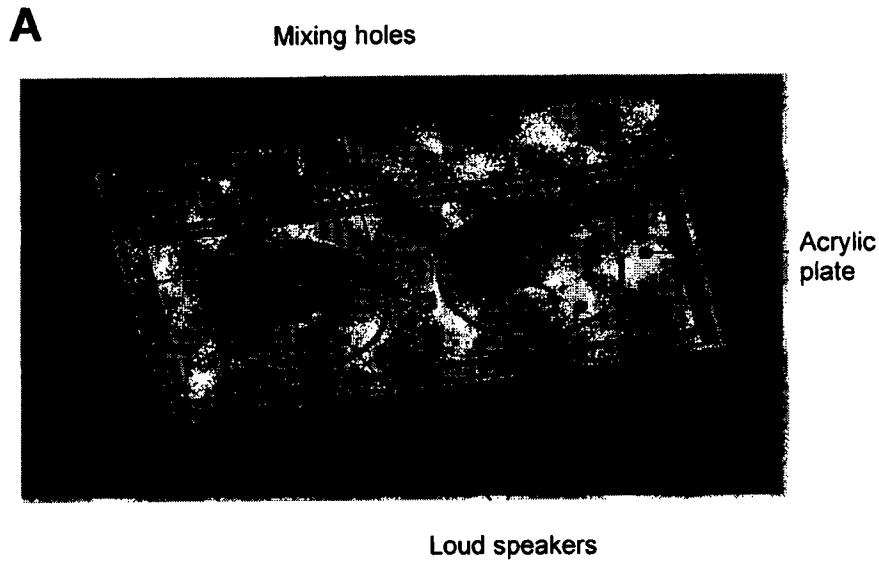

Figure 1A

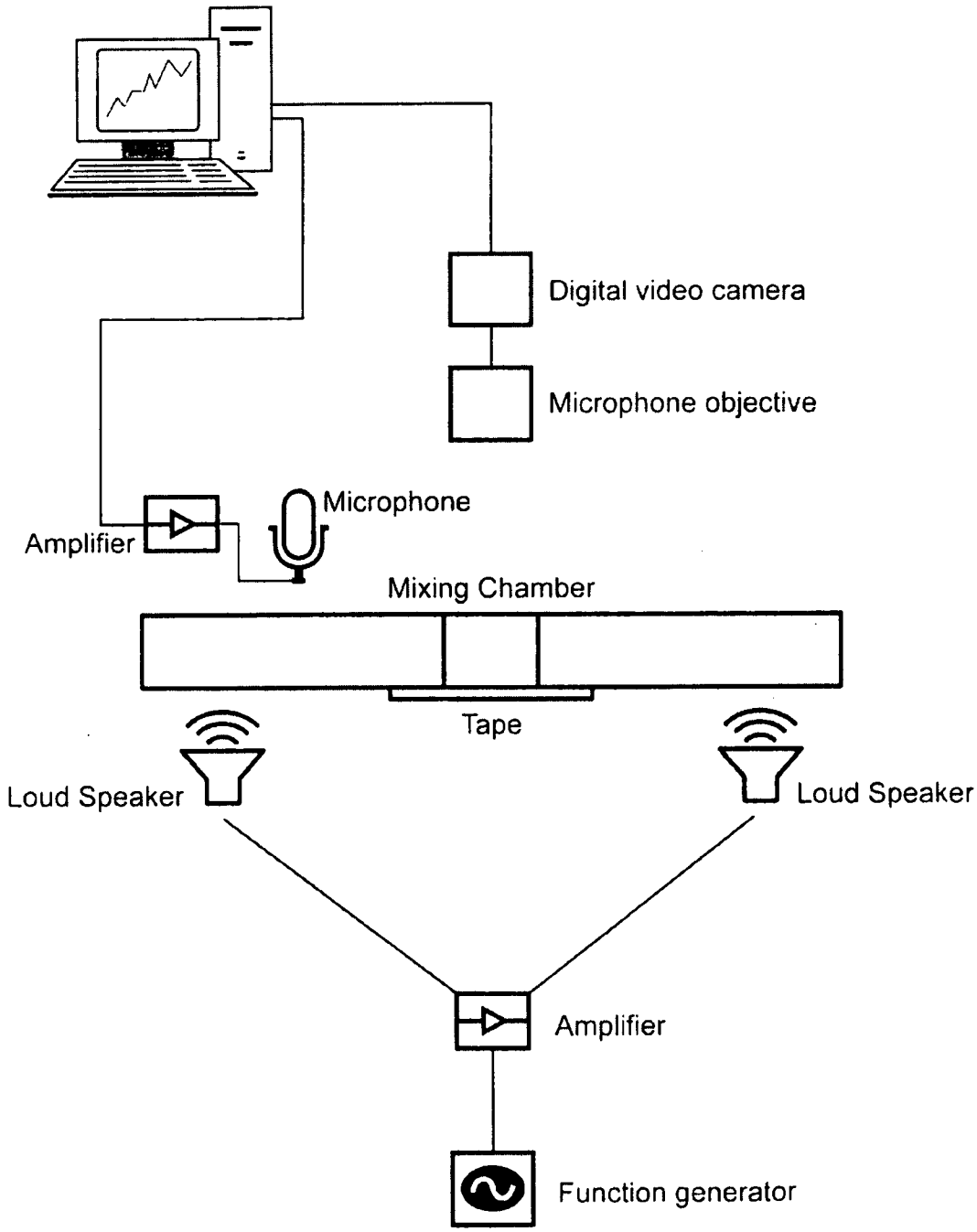
Digital video camera
Microphone objective
Microphone
Amplifier
Mixing Chamber
Tape
Loud Speaker
Loud Speaker
Amplifier
Function generator

Figure 1B

C

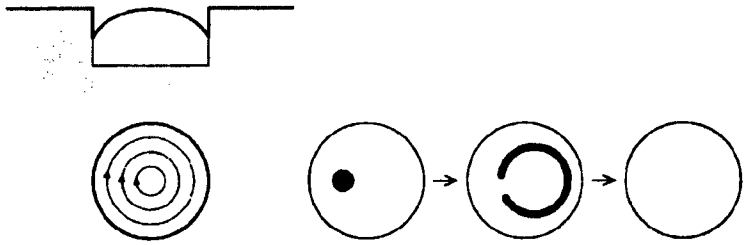

Figure 1C

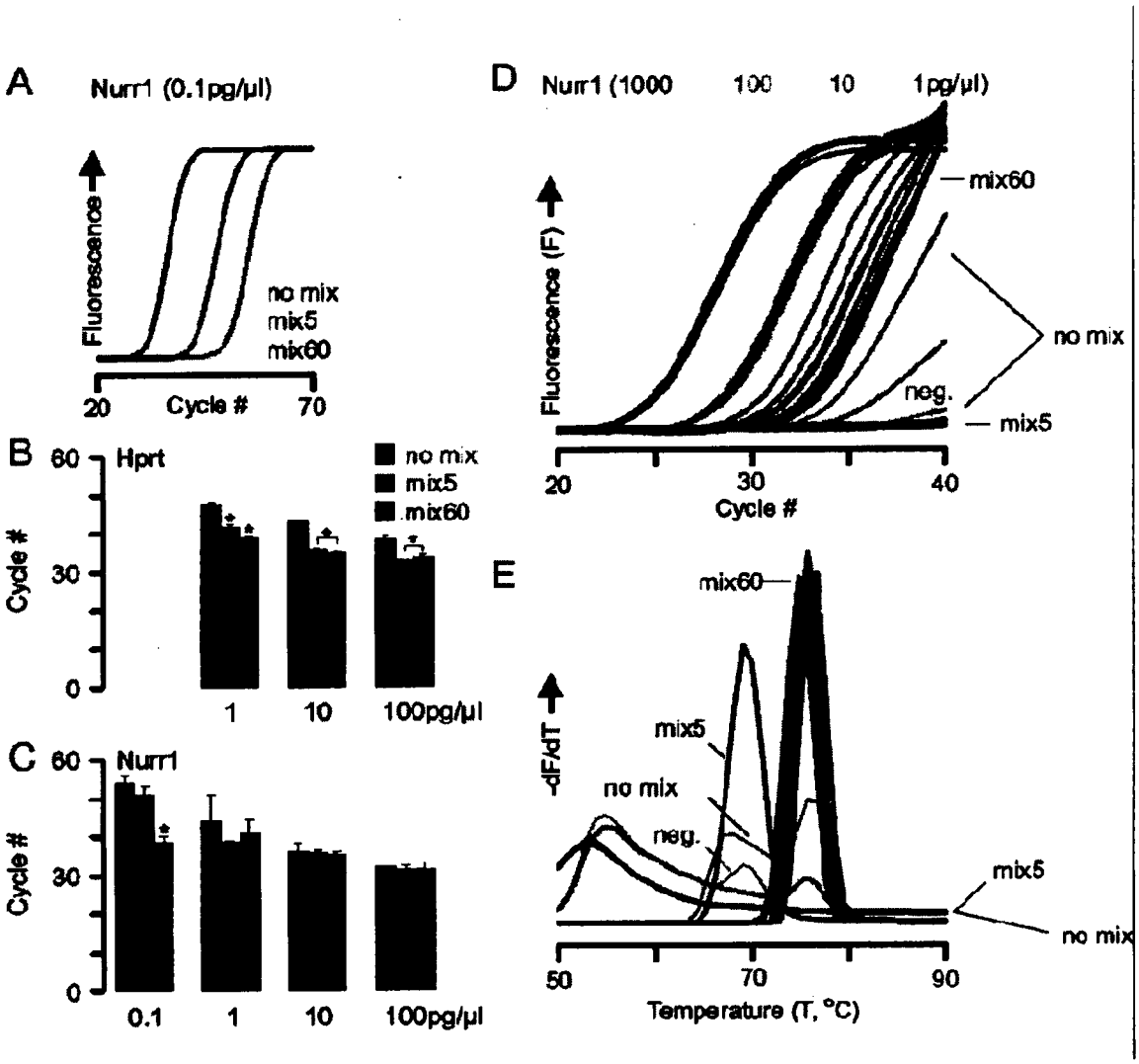

Figure 2A to E

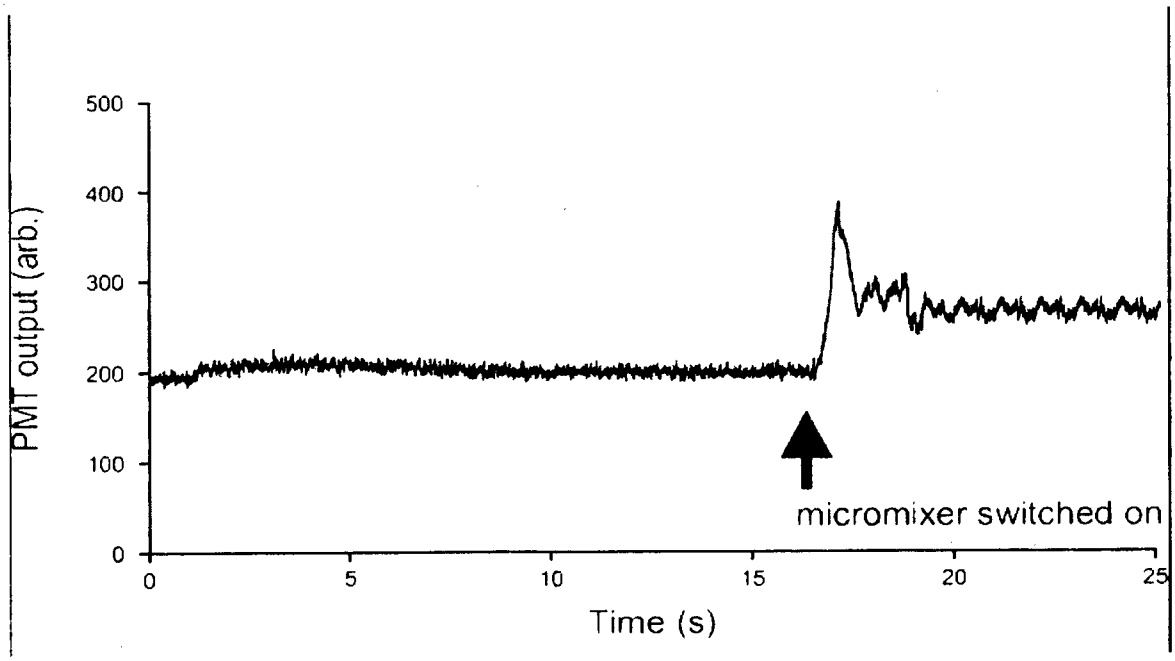
PMT output (arb.)
500
400
300
200
100
0
0
5
10
15
20
25
Time (s)
micromixer switched on

Figure 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2012/000041

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

B01F 11/02 (2006.01) ***B81B 7/04*** (2006.01) ***C12N 13/00*** (2006.01)
B01J 19/10 (2006.01) ***C12M 1/02*** (2006.01)
B01L 3/00 (2006.01) ***C12M 1/42*** (2006.01)

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)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPODOC and WPI: /IC/EC B01F11 or B81B or B01L3 and keywords ((+mixing or microstreaming) and (well or vial or microfluidic or slide or tray) and (acoustic or sound or speaker or amplifier or +sonic+)); EPODOC and WPI: /IC/EC and keyword(s) ((well or vial or slide or ampoule or microtiter or microarray or microcavit+ or microdepression) and meniscus and (acoustic or sound or +sonic)), ((well or vial or slide or ampoule or microtiter or microarray or microcavit+ or microdepression) and (acoustic or sound or +sonic) and +drop+ and (mixing or agitating)), ((well or vial or slide or ampoule or microtiter or microarray or microcavit+ or microdepression) and meniscus and (RNA or DNA or protein or cell+ or enzyme or nucleic)); Google Patents and keyword(s) (microstreaming meniscus), (mixing acoustic meniscus OR interface droplet well OR vial), (microliter "fluid mixing" frequency); esp@acenet and keyword(s) (wells AND C12N13/00), (microstreaming).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/105616 A1 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 12 October 2006 See the abstract; page 4 lines 14-15, page 9 lines 4-20, page 13 lines 21-23, page 15 lines 7-11 and page 16 lines 7-28; **claims 1-5, 7, 10-11**; and figure 4.	1-4, 10-12, 18-21, 27-29, 33-37
Y		5-9, 22-26
X	US 2006/0275883 A1 (Rathgeber et al.) 07 December 2006 See the abstract; para. [0001]-[0016], [0081], [0086]; and figures 1-5.	1, 4, 10, 18, 21, 27, 33-34, 36-37
Y		5-9, 22-26

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"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	Date of mailing of the international search report
09 February 2012	20 February 2012
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **JOHN DEUIS** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6283 2146

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2012/000041

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☐ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

   Please note, although the specification contains a [nucleic acid and/or an amino acid] sequence, it was not used for the purposes of this search.

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2004/0257906 A1 (Scriba et al.) 23 December 2004 See the abstract; and para. [0001]-[0003]. Note, for the Y indications; WO 2006/105616 and US 2004/0257906 are combined for claims 5-9, 22-26, US 2006/0275883 and US 2004/0257906 are combined for claims 5-9, 22-26.	5-9, 22-26

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2012/000041

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	2006105616	AU	2006230821	US	2009034360		
US	2006275883	EP	1596972	EP	1596974	JP	2006519091
		JP	2006519685	US	8038337	US	2007264161
		US	2011188337	WO	2004076046	WO	2004076047
US	2004257906	EP	1420875	JP	2005504623	WO	03018181

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX