



US008709762B2

(12) **United States Patent**
Hindson(10) **Patent No.:** **US 8,709,762 B2**
(45) **Date of Patent:** **Apr. 29, 2014**(54) **SYSTEM FOR HOT-START AMPLIFICATION VIA A MULTIPLE EMULSION**(75) Inventor: **Benjamin J. Hindson**, Livermore, CA (US)(73) Assignee: **Bio-Rad Laboratories, Inc.**, Hercules, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 147 days.

(21) Appl. No.: **13/039,233**(22) Filed: **Mar. 2, 2011**(65) **Prior Publication Data**

US 2011/0217736 A1 Sep. 8, 2011

Related U.S. Application Data

(60) Provisional application No. 61/309,837, filed on Mar. 2, 2010.

(51) **Int. Cl.****C12N 9/12** (2006.01)**C12P 19/34** (2006.01)(52) **U.S. Cl.**USPC **435/91.2**; 134/14; 134/561; 435/194(58) **Field of Classification Search**

USPC 435/91.2, 194

See application file for complete search history.

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ABSTRACT

System, including methods, apparatus, compositions, and kits, for making and using compound droplets of a multiple emulsion to supply an amplification reagent, such as a heat-stable DNA polymerase or DNA ligase, to an aqueous phase in which the compound droplets are disposed. The compound droplets may be induced to supply the amplification reagent by heating the multiple emulsion, to achieve hot-start amplification.

14 Claims, 2 Drawing Sheets

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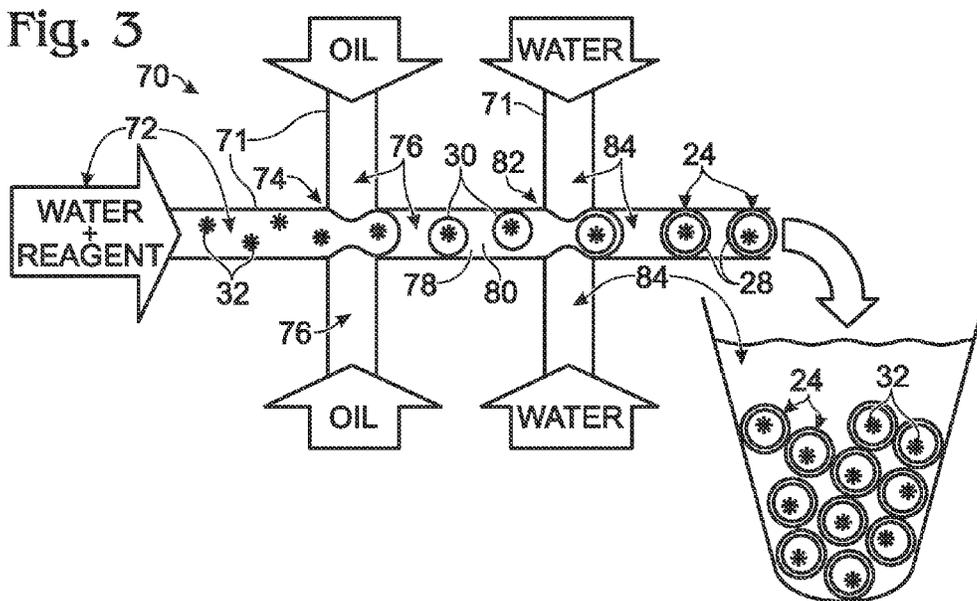
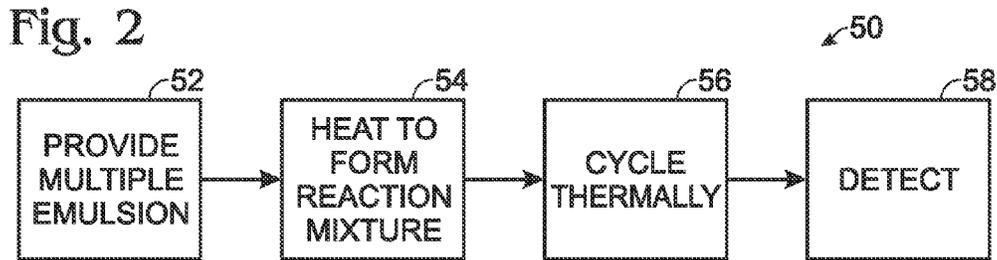
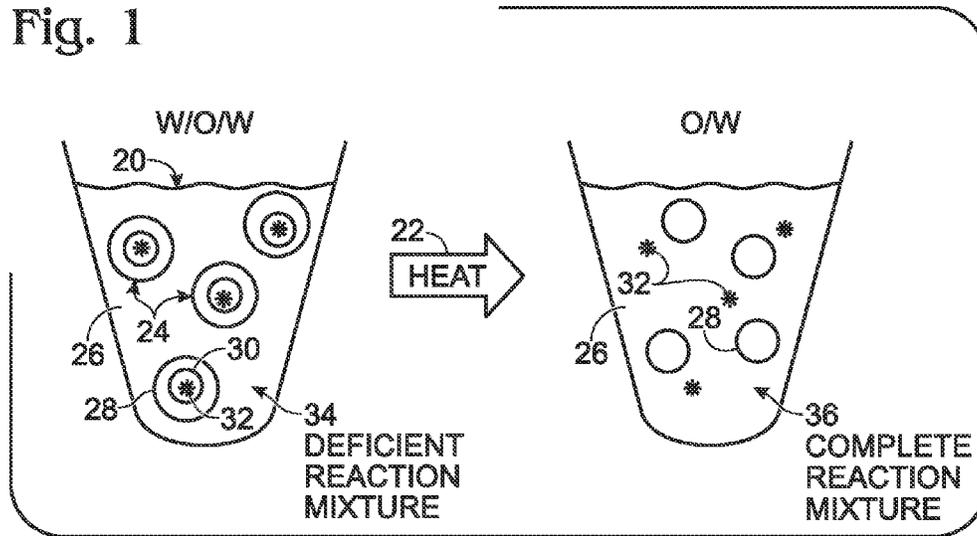
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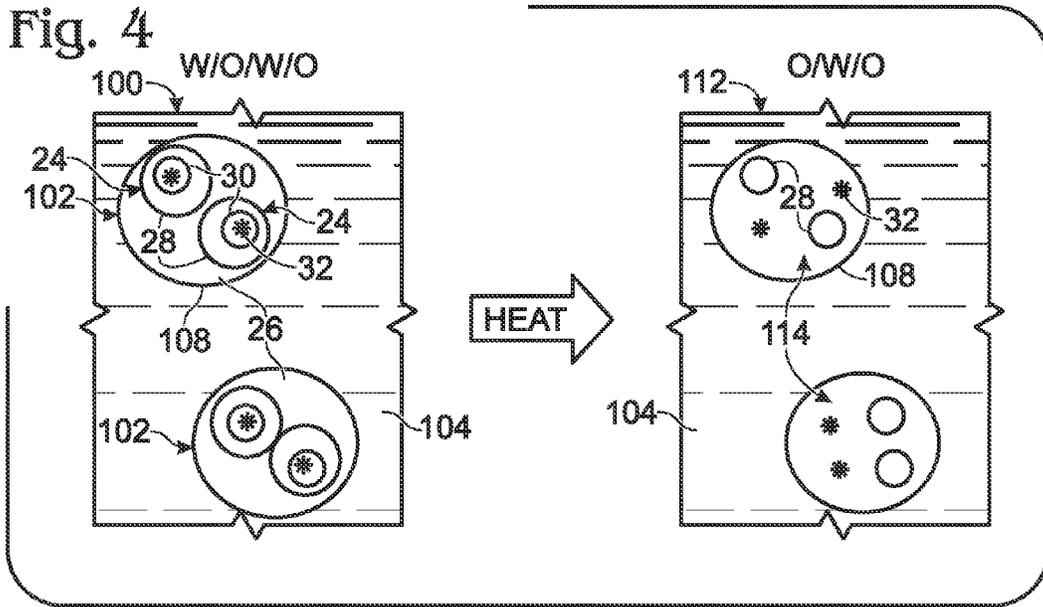
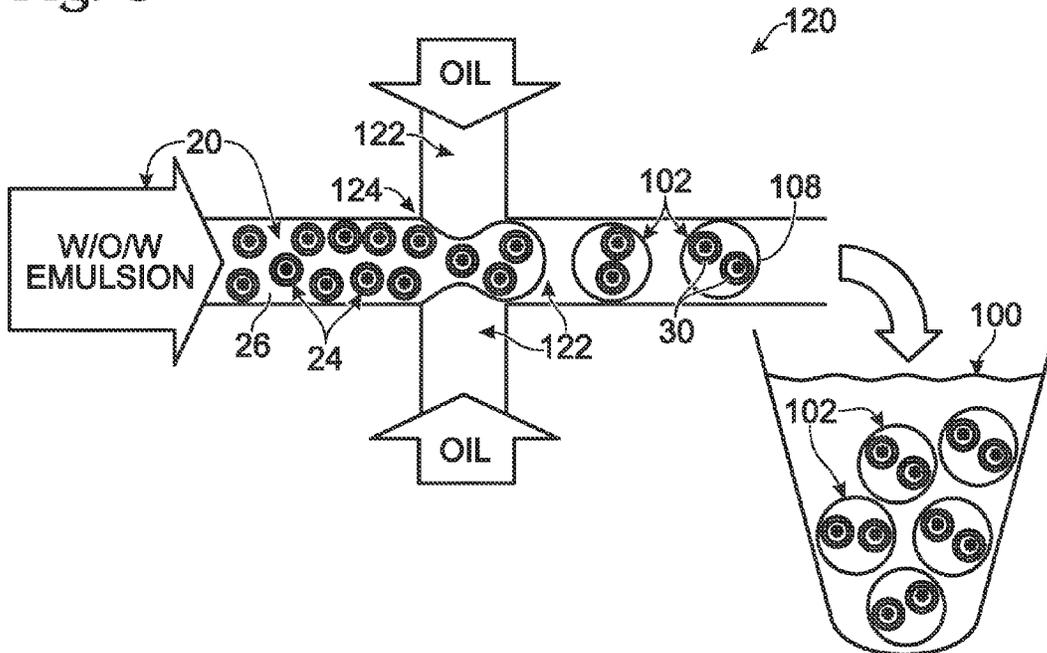


Fig. 5



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SYSTEM FOR HOT-START AMPLIFICATION VIA A MULTIPLE EMULSION

CROSS-REFERENCE TO PRIORITY APPLICATION

This application is based upon and claims the benefit under 35 U.S.C. §119(e) of U.S. Provisional Patent Application Ser. No. 61/309,837, filed Mar. 2, 2010, which is incorporated herein by reference in its entirety for all purposes.

CROSS-REFERENCES TO OTHER MATERIALS

This application incorporates by reference in their entireties for all purposes the following materials: U.S. Pat. No. 7,041,481, issued May 9, 2006; U.S. patent application Ser. No. 12/586,626, filed Sep. 23, 2009; U.S. patent application Ser. No. 12/862,542, filed Aug. 24, 2010; and U.S. patent application Ser. No. 12/976,827, filed Dec. 22, 2010.

INTRODUCTION

The polymerase chain reaction (PCR) utilizes repeated rounds of replication of a nucleic acid template, which may produce an exponential amplification of the template's copy number. PCR amplification relies on changes in the temperature of a reaction mixture to produce repeated cycles of template replication. Each replication cycle may be driven by a thermal cycle that involves (1) template denaturation at a higher temperature, (2) annealing of one or more primers to the denatured template at a lower temperature, and (3) extension of the annealed primers with a heat-stable DNA polymerase, such as Taq DNA polymerase, at a lower or intermediate temperature, to form complementary strands of the template.

Interfering side reactions can occur in a PCR mixture before the first thermal cycle is initiated, if an active reaction mixture is formed before heating. In particular, primer annealing may be inaccurate when the reaction mixture is formed at a lower temperature than the annealing temperature utilized for PCR amplification. In this case, the reaction mixture generally is heated to a denaturation temperature through a temperature range starting at or below room temperature. As a result, DNA polymerase is active and can extend primers that are annealed incorrectly, such as to form primer dimers and/or non-specific products. These undesired products can consume primers and deoxynucleotide triphosphates and can function as templates that compete with the desired amplification reaction. Accordingly, premature primer extension can reduce the efficiency and yield of the amplification reaction, complicate interpretation of results, and, in some cases, may cause failure to amplify the specified target.

Strategies have been developed to delay formation of a competent amplification mixture until the mixture has been heated above the annealing temperature, to effect a "hot-start." Generally, in these strategies, an essential PCR reagent is inactive and/or sequestered until the reaction has been heated sufficiently.

The PCR reagent, such as a heat-stable polymerase (e.g., Taq DNA polymerase), may be held in an inactive complex by an antibody or an aptamer until the polymerase is released from the complex by heating. However, locking the polymerase in an inactive complex can be expensive and inefficient.

In another approach, the polymerase may be kept separate from other components of the reaction mixture. For example, the polymerase may be sequestered from the bulk aqueous

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solution by a wax layer that melts above the annealing temperature to unite the polymerase with the other reaction components and form a complete reaction mixture. In another example, the polymerase may be disposed in a wax bead that melts to release the polymerase. However, reaction mixtures using a distinct phase to sequester the polymerase can be inefficient to assemble because the distinct phase may not be amenable to standard fluid transfer techniques.

There remains a need for other approaches to perform hot-start amplification of nucleic acids.

SUMMARY

The present disclosure provides a system, including methods, apparatus, compositions, and kits, for making and using compound droplets of a multiple emulsion to supply an amplification reagent, such as a heat-stable DNA polymerase or DNA ligase, to an aqueous phase in which the compound droplets are disposed. The compound droplets may be induced to supply the amplification reagent by heating the multiple emulsion, to achieve hot-start amplification.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic flow diagram illustrating an exemplary multiple emulsion being heated to promote fusion of aqueous droplets with a continuous aqueous phase, to form a mixture competent for nucleic acid amplification, in accordance with aspects of present disclosure.

FIG. 2 is a flowchart presenting an exemplary method of sample analysis using a multiple emulsion to controllably combine a sequestered reagent with other amplification reagents, in accordance with aspects of the present disclosure.

FIG. 3 is a schematic view of an exemplary system for forming compound droplets for use in the multiple emulsions of FIGS. 1 and 2, in accordance with aspects of the present disclosure.

FIG. 4 is a schematic flow diagram illustrating an exemplary multiple emulsion being heated to promote fusion of inner aqueous droplets with outer aqueous droplets that enclose the inner aqueous droplets, to form a complete reaction mixture for nucleic acid amplification in each outer droplet, in accordance with aspects of the present disclosure.

FIG. 5 is a schematic view of an exemplary system for forming the multiple emulsion of FIG. 4, in accordance with aspects of the present disclosure.

DETAILED DESCRIPTION

The present disclosure provides a system, including methods, apparatus, compositions, and kits, for making and using compound droplets of a multiple emulsion to supply an amplification reagent, such as a heat-stable polymerase or ligase, to an aqueous phase in which the compound droplets are disposed. The compound droplets may be induced to supply the amplification reagent by heating the multiple emulsion, to achieve hot-start amplification. The aqueous phase may be an incomplete reaction mixture for amplification due to a lack of the amplification reagent carried by the compound droplets. The amplification reagent may be sequestered in the compound droplets until an elevated temperature is reached, such as at least an annealing temperature, which may promote droplet fusion with the aqueous phase. Accordingly, the reaction mixture may not become complete (and competent for primer extension and/or ligation) until a suitable annealing stringency is achieved, thereby reducing formation of primer dimers and non-specific products.

A method of nucleic acid amplification is provided. In the method, a multiple emulsion may be provided that includes compound droplets disposed in an aqueous phase, which may be a continuous phase or a dispersed phase. The compound droplets may include at least one amplification reagent, such as a DNA polymerase (e.g., a heat-stable DNA polymerase, such as Taq polymerase), a heat-stable ligase, one or more dNTPs, magnesium (e.g., Mg^{2+}), at least one primer for a nucleic acid target, or a combination thereof. Each compound droplet may include at least one aqueous droplet enclosed by an oil droplet, and the aqueous droplet may contain the at least one amplification reagent. In any event, the multiple emulsion may be heated such that the at least one amplification reagent is released to the aqueous phase to form a reaction mixture. The reaction mixture may be cycled thermally to promote amplification of a nucleic acid target.

Another method of nucleic acid amplification is provided. In the method, a multiple emulsion may be provided that includes compound droplets disposed in an aqueous phase. The aqueous phase may lack an effective amount of at least one PCR reagent that is contained by the compound droplets. The multiple emulsion may be heated such that the at least one PCR reagent is added to the aqueous phase from the compound droplets to form a reaction mixture capable of PCR amplification of a nucleic acid target, if present, in the reaction mixture. The reaction mixture may be cycled thermally to encourage PCR amplification of the nucleic acid target.

Yet another method of nucleic acid amplification is provided. In the method, a multiple emulsion may be provided that includes compound droplets disposed in an aqueous phase, with each compound droplet including an oil droplet enclosing at least one aqueous droplet. The aqueous droplet may contain a heat-stable polymerase. The multiple emulsion may be heated such that aqueous droplets of the compound droplets fuse with the aqueous phase to supply an effective amount of the heat-stable polymerase to the aqueous phase, to form a reaction mixture capable of PCR amplification of a nucleic acid target, if present, in the reaction mixture. The reaction mixture may be cycled thermally to encourage PCR amplification.

A composition for nucleic acid amplification is provided. The composition may a multiple emulsion including compound droplets disposed in an aqueous phase, with each compound droplet including an oil droplet enclosing at least one aqueous droplet. The aqueous droplet may contain at least one amplification reagent. Heating the multiple emulsion may cause aqueous droplets of the compound droplets to fuse with the aqueous phase to supply the at least one reagent to the aqueous phase. Heating may form a reaction mixture capable of amplification of a nucleic acid target, if present, in the reaction mixture.

A kit is provided for use in nucleic acid amplification. The kit may include a first composition including compound droplets containing an amplification reagent. The kit also may include at least one second composition that is aqueous and that includes another amplification reagent.

Further aspects of the present disclosure are presented in the following sections: (I) definitions, (II) exemplary fusion of a multiple emulsion, (III) exemplary method of sample analysis using a multiple emulsion, (IV) exemplary system for forming compound droplets, (V) exemplary fusion within compound droplets, (VI) exemplary system for forming compound droplets that fuse internally, and (VII) selected embodiments.

I. Definitions

Technical terms used in this disclosure have the meanings that are commonly recognized by those skilled in the art. However, the following terms may have additional meanings, as described below.

Emulsion—a composition comprising liquid droplets disposed in an immiscible liquid. The droplets are formed by at least one dispersed phase, and the immiscible liquid forms a continuous phase. The continuous phase can also or alternatively be termed a carrier and/or a carrier phase. The dispersed phase (or at least one of the dispersed phases of a multiple emulsion) is immiscible with the continuous phase, which means that the dispersed phase (i.e., the droplets) and the continuous phase (i.e., the immiscible liquid) do not mix to attain homogeneity. The droplets are isolated from one another by the continuous phase and encapsulated (i.e., enclosed/surrounded) by the continuous phase.

The droplets of an emulsion may have any uniform or non-uniform distribution in the continuous phase. If non-uniform, the concentration of the droplets may vary to provide one or more regions of higher droplet density and one or more regions of lower droplet density in the continuous phase. For example, droplets may sink or float in the continuous phase.

An emulsion may be monodisperse, that is, composed of droplets of uniform size, or may be polydisperse, that is, composed of droplets of various sizes. If monodisperse, the droplets of the emulsion may vary in size by a standard deviation of the volume (or diameter) that is less than about 50%, 20%, 10%, 5%, 2%, or 1% of the average droplet volume (or diameter). Droplets generated from an orifice may be monodisperse or polydisperse.

A multiple emulsion disclosed herein may be a heat-sensitive emulsion. A heat-sensitive emulsion is any emulsion that can be induced to fuse when heated, such as to a temperature of less than about 90° C. or 95° C. For example, a heat-sensitive emulsion may be configured to fuse substantially when heated to at least about 50° C., 60° C., 70° C., 80° C., or 90° C., among others.

An emulsion may have any suitable composition. The emulsion may be characterized by the predominant liquid compound or type of liquid compound in each phase. The predominant liquid compounds in the emulsion may be one or more aqueous phases and one or more nonaqueous phases. The nonaqueous phase may be referred to as an oil phase comprising at least one oil, which generally includes any liquid (or liquefiable) compound or mixture of liquid compounds that is immiscible with water. The oil may be synthetic or naturally occurring. The oil may or may not include carbon and/or silicon, and may or may not include hydrogen and/or fluorine. The oil may be lipophilic or lipophobic. In other words, the oil may be generally miscible or immiscible with organic solvents. Exemplary oils may include at least one silicone oil, mineral oil, fluorocarbon oil, vegetable oil, or a combination thereof, among others.

In exemplary embodiments, the oil is a fluorinated oil, such as a fluorocarbon oil, which may be a perfluorinated organic solvent. A fluorinated oil may be a base (primary) oil or an additive to a base oil, among others. Exemplary fluorinated oils that may be suitable are sold under the trade name FLUORINERT (3M), including, in particular, FLUORINERT Electronic Liquid FC-3283, FC-40, FC-43, and FC-70. Another example of an appropriate fluorinated oil is sold under the trade name NOVEC (3M), including NOVEC HFE 7500 Engineered Fluid.

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Droplet—a small volume of a first liquid that is encapsulated by an immiscible second liquid, such as a continuous phase of an emulsion (and/or by a larger droplet). The volume of a droplet, and/or the average volume of droplets in an emulsion, may, for example, be less than about one microliter (or between about one microliter and one nanoliter or between about one microliter and one picoliter), less than about one nanoliter (or between about one nanoliter and one picoliter), or less than about one picoliter (or between about one picoliter and one femtoliter), among others. A droplet (or droplets of an emulsion) may have a diameter (or an average diameter) of less than about 1000, 100, or 10 micrometers, or about 1000 to 10 micrometers, among others. A droplet may be spherical or nonspherical. A droplet may be a simple droplet or a compound droplet.

Compound droplet—a droplet in which at least one droplet encloses at least one other droplet. A compound droplet includes at least two immiscible liquids, with one of the liquids surrounding the other liquid in the compound droplet to form at least one droplet within a droplet. A droplet that is surrounded by another droplet may be described as an inner droplet, which may or may not be the innermost droplet of a compound droplet. A droplet enclosing another droplet may be described as an outer droplet, which may or may not be the outermost droplet of a compound droplet. In contrast to a compound droplet, a simple droplet is not surrounded by another droplet.

Multiple emulsion—an emulsion including compound droplets. A multiple emulsion can be characterized according to the level of encapsulation of its constituent compound droplets, with a higher-order emulsion having more levels of encapsulation than a lower-order emulsion. For example, a double emulsion contains compound droplets structured as one more droplets within an enclosing droplet, a triple emulsion contains compound droplets structured as a droplet within a droplet within a droplet, and so on. In contrast, a single emulsion contains simple droplets in a continuous phase.

Surfactant—a surface-active substance capable of reducing the surface tension of a liquid in which it is present. A surfactant, which also or alternatively may be described as a detergent and/or a wetting agent, may incorporate both a hydrophilic portion and a hydrophobic portion, which may collectively confer a dual hydrophilic-hydrophobic character on the surfactant. A surfactant may, in some cases, be characterized according to its hydrophilicity relative to its hydrophobicity. Each dispersed and/or continuous phase may incorporate at least one surfactant. Each aqueous phase may include at least one nonionic surfactant and/or ionic surfactant. In some embodiments, the aqueous phase may include a surfactant that is a block copolymer of polypropylene oxide and polyethylene oxide. More particularly, the surfactant may be a block copolymer of polypropylene oxide and polyethylene oxide sold under the trade names PLURONIC and TETRONIC (BASF). In some embodiments, the surfactant may be a nonionic block copolymer of polypropylene oxide and polyethylene oxide sold under the trade name PLURONIC F-68. In some embodiments, the surfactant of the aqueous phase may be a water-soluble and/or hydrophilic fluorosurfactant. Exemplary fluorosurfactants for the aqueous phase are sold under the trade name ZONYL (DuPont), such as ZONYL FSN fluorosurfactants. In some cases, the surfactant may include polysorbate 20 (sold under the trade name TWEEN-20 by ICI Americas, Inc.). The concentration of a particular surfactant or total surfactant present in the aqueous phase and oil phase may be selected to stabilize compound droplets until they are heated to a desired fusion

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temperature. An exemplary concentration of surfactant for the aqueous phase is about 0.01 to 10%, 0.05 to 5%, 0.1 to 1%, or 0.5% by weight, among others.

A nonaqueous or oil phase may incorporate a hydrophobic surfactant. The nonaqueous phase may include one or more surfactants. The surfactants may include a nonionic surfactant, an ionic surfactant (a cationic (positively-charged) or anionic (negatively-charged) surfactant), or both types of surfactant. Exemplary anionic surfactants that may be suitable include carboxylates, sulphonates, phosphonates, and so on. The one or more surfactants may be present, individually or collectively, at any suitable concentration, such as greater than about 0.001% or 0.01%, or about 0.001% to 10%, 0.05% to 2%, or 0.05% to 0.5%, among others.

The one or more surfactants present in the nonaqueous phase (or oil phase) may be fluorinated surfactants (e.g., surfactant compounds that are polyfluorinated and/or perfluorinated). Exemplary fluorinated surfactants are fluorinated polyethers, such as carboxylic acid-terminated perfluoropolyethers, carboxylate salts of perfluoropolyethers, and/or amide or ester derivatives of carboxylic acid-terminated perfluoropolyethers. Exemplary but not exclusive perfluoropolyethers are commercially available under the trade name KRYTOX (DuPont), such as KRYTOX-FSH, the ammonium salt of KRYTOX-FSH (“KRYTOX-AS”), or a morpholino derivative of KRYTOX-FSH (“KRYTOX-M”), among others. Other fluorinated polyethers that may be suitable include at least one polyethylene glycol (PEG) moiety.

Fluorinated—including fluorine, typically substituted for hydrogen. Any of the fluorinated compounds disclosed herein may be polyfluorinated, meaning that such compounds each include many fluorines, such as more than five or ten fluorines, among others. Any of the fluorinated compounds disclosed herein also or alternatively may be perfluorinated, meaning that most or all hydrogens have been replaced with fluorine.

Sample—a compound, composition, and/or mixture of interest, from any suitable source(s). A sample is the general subject of interest for a test that analyzes an aspect of the sample, such as an aspect related to at least one analyte that may be present in the sample. Samples may be analyzed in their natural state, as collected, and/or in an altered state, for example, following storage, preservation, extraction, lysis, dilution, concentration, purification, filtration, mixing with one or more reagents, pre-amplification (e.g., to achieve target enrichment by performing limited cycles (e.g., <15) of PCR on sample prior to PCR), removal of amplicon (e.g., treatment with uracil-d-glycosylase (UDG) prior to PCR to eliminate any carry-over contamination by a previously generated amplicon (i.e., the amplicon is digestible with UDG because it is generated with dUTP instead of dTTP)), partitioning, or any combination thereof, among others. Clinical samples may include nasopharyngeal wash, blood, plasma, cell-free plasma, buffy coat, saliva, urine, stool, sputum, mucous, wound swab, tissue biopsy, milk, a fluid aspirate, a swab (e.g., a nasopharyngeal swab), and/or tissue, among others. Environmental samples may include water, soil, aerosol, and/or air, among others. Research samples may include cultured cells, primary cells, bacteria, spores, viruses, small organisms, any of the clinical samples listed above, or the like. Additional samples may include foodstuffs, weapons components, biodefense samples to be tested for bio-threat agents, suspected contaminants, and so on.

Samples may be collected for diagnostic purposes (e.g., the quantitative measurement of a clinical analyte such as an infectious agent) or for monitoring purposes (e.g., to deter-

mine that an environmental analyte of interest such as a biothreat agent has exceeded a predetermined threshold).

Reagent—a compound, set of compounds, and/or composition that is combined with a sample in order to perform a particular test(s) on the sample. A reagent may be a target-specific reagent, which is any reagent composition that confers specificity for detection of a particular target(s) or analyte(s) in a test. A reagent optionally may include a chemical reactant and/or a binding partner for the test. A reagent may, for example, include at least one nucleic acid, protein (e.g., an enzyme), cell, virus, organelle, macromolecular assembly, potential drug, lipid, carbohydrate, inorganic substance, or any combination thereof, and may be an aqueous composition, among others. In exemplary embodiments, the reagent may be an amplification reagent, which may include at least one primer or at least one pair of primers for amplification of a nucleic acid target, at least one probe and/or dye to enable detection of amplification, a polymerase, a ligase, nucleotides (dNTPs and/or NTPs), divalent magnesium ions, or any combination thereof, among others. In some embodiments, the reagent may be a PCR reagent, namely, a reagent involved in PCR amplification, such as a primer, a heat-stable polymerase, at least one nucleotide (dNTP or NTP), or magnesium, among others. An amplification reagent and/or a nucleic acid target each may be described as a reaction component.

The amplification reagent may be present at an effective amount, namely, an amount sufficient to enable amplification of a nucleic acid target in the presence of other necessary reagents. Exemplary effective amounts of PCR reagents are as follows: heat-stable DNA polymerase, 0.005 to 0.5 Units/ μ L; dNTPs, 50 μ M to 5 mM each; primers, 0.02 to 5.0 μ M each; and Mg^{2+} , 0.5 to 10 mM.

Nucleic acid—a compound comprising a chain of nucleotide monomers. A nucleic acid may be single-stranded or double-stranded (i.e., base-paired with another nucleic acid), among others. The chain of a nucleic acid may be composed of any suitable number of monomers, such as at least about ten or one-hundred, among others. Generally, the length of a nucleic acid chain corresponds to its source, with synthetic nucleic acids (e.g., primers and probes) typically being shorter, and biologically/enzymatically generated nucleic acids (e.g., nucleic acid analytes) typically being longer.

A nucleic acid may have a natural or artificial structure, or a combination thereof. Nucleic acids with a natural structure, namely, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), generally have a backbone of alternating pentose sugar groups and phosphate groups. Each pentose group is linked to a nucleobase (e.g., a purine (such as adenine (A) or guanine (G)) or a pyrimidine (such as cytosine (C), thymine (T), or uracil (U))). Nucleic acids with an artificial structure are analogs of natural nucleic acids and may, for example, be created by changes to the pentose and/or phosphate groups of the natural backbone. Exemplary artificial nucleic acids include glycol nucleic acids (GNA), peptide nucleic acids (PNA), locked nucleic acid (LNA), threose nucleic acids (TNA), and the like.

The sequence of a nucleic acid is defined by the order in which nucleobases are arranged along the backbone. This sequence generally determines the ability of the nucleic acid to bind specifically to a partner chain (or to form an intramolecular duplex) by hydrogen bonding. In particular, adenine pairs with thymine (or uracil) and guanine pairs with cytosine. A nucleic acid that can bind to another nucleic acid in an antiparallel fashion by forming a consecutive string of such base pairs with the other nucleic acid is termed “complementary.”

Replication—a process forming a copy (i.e., a direct copy and/or a complementary copy) of a nucleic acid or a segment thereof. Replication generally involves an enzyme, such as a polymerase and/or a ligase, among others. The nucleic acid and/or segment replicated is a template (and/or a target) for replication.

Amplification—a reaction in which replication occurs repeatedly over time to form multiple copies of at least one segment of a template molecule. Amplification may generate an exponential or linear increase in the number of copies as amplification proceeds. Typical amplifications produce a greater than 1,000-fold increase in copy number and/or signal. Exemplary amplification reactions for the assays disclosed herein may include the polymerase chain reaction (PCR) or ligase chain reaction (LCR), each of which is driven by thermal cycling. Thermal cycling generally involves cycles of heating and cooling a reaction mixture to perform successive rounds of denaturation (melting), annealing, and extension. The assays also or alternatively may use other amplification reactions, which may be performed isothermally, such as branched-probe DNA assays, cascade-RCA, helicase-dependent amplification, loop-mediated isothermal amplification (LAMP), nucleic acid based amplification (NASBA), nicking enzyme amplification reaction (NEAR), PAN-AC, Q-beta replicase amplification, rolling circle replication (RCA), self-sustaining sequence replication, strand-displacement amplification, and the like. Amplification may utilize a linear or circular template.

Amplification may be performed with any suitable reagents. Amplification may be performed, or tested for its occurrence, in an amplification mixture, which is any composition capable of generating multiple copies of a nucleic acid target molecule, if present, in the composition. An amplification mixture may include any combination of at least one primer or primer pair, at least one probe, at least one replication enzyme (e.g., at least one polymerase, such as at least one DNA and/or RNA polymerase, and/or at least one ligase), and/or deoxynucleotide (and/or nucleotide) triphosphates (dNTPs and/or NTPs), among others.

Hot-start amplification—amplification in which reaction (e.g., polymerization and/or ligation) does not occur significantly until an elevated temperature is reached. The elevated temperature may be at least about the annealing temperature for amplification, to reduce non-target amplification.

PCR—nucleic acid amplification that relies on alternating cycles of heating and cooling (i.e., thermal cycling) to achieve successive rounds of replication. PCR may be performed by thermal cycling between two or more temperature set points, such as a higher melting (denaturation) temperature and a lower annealing/extension temperature, or among three or more temperature set points, such as a higher melting temperature, a lower annealing temperature, and an intermediate extension temperature, among others. PCR may be performed with a heat-stable polymerase, such as Taq DNA polymerase (e.g., wild-type enzyme, a Stoffel fragment, Fast-Start polymerase, etc.), Pfu DNA polymerase, S-Tbr polymerase, Tth polymerase, Vent polymerase, or a combination thereof, among others. PCR generally produces an exponential increase in the amount of a product amplicon over successive cycles.

Any suitable PCR methodology or combination of methodologies may be utilized in the assays disclosed herein, such as allele-specific PCR, assembly PCR, asymmetric PCR, digital PCR, endpoint PCR, hot-start PCR, in situ PCR, intersequence-specific PCR, inverse PCR, linear after exponential PCR, ligation-mediated PCR, methylation-specific PCR, miniprimer PCR, multiplex ligation-dependent probe

amplification, multiplex PCR, nested PCR, overlap-extension PCR, polymerase cycling assembly, qualitative PCR, quantitative PCR, real-time PCR, RT-PCR, single-cell PCR, solid-phase PCR, thermal asymmetric interlaced PCR, touch-down PCR, universal fast walking PCR, or any combination thereof, among others.

Amplicon—a product of an amplification reaction. An amplicon may be single-stranded or double-stranded, or a combination thereof. An amplicon corresponds to any suitable segment or the entire length of a nucleic acid target.

Primer—a nucleic acid capable of, and/or used for, priming replication of a nucleic acid template. Thus, a primer is a shorter nucleic acid that is complementary to a longer template. During replication, the primer may be extended, based on the template sequence, to produce a longer nucleic acid that is a complementary copy of the template. Extension may occur by successive addition of individual nucleotides (e.g., by the action of a polymerase) or by attachment of a block of nucleotides (e.g., by the action of a ligase joining a pair of primers), among others. A primer may be DNA, RNA, an analog thereof (i.e., an artificial nucleic acid), or any combination thereof. A primer may have any suitable length, such as at least about 10, 15, 20, or 30 nucleotides. Exemplary primers are synthesized chemically. Primers may be supplied as at least one pair of primers for amplification of at least one nucleic acid target. A pair of primers may be a sense primer and an antisense primer that collectively define the opposing ends (and thus the length) of a resulting amplicon.

Probe—a nucleic acid connected to at least one label, such as at least one dye. A probe may be a sequence-specific binding partner for a nucleic acid target and/or amplicon. The probe may be designed to enable detection of target amplification based on fluorescence resonance energy transfer (FRET). An exemplary probe for the nucleic acid assays disclosed herein includes one or more nucleic acids connected to a pair of dyes that collectively exhibit fluorescence resonance energy transfer (FRET) when proximate one another. The pair of dyes may provide first and second emitters, or an emitter and a quencher, among others. Fluorescence emission from the pair of dyes changes when the dyes are separated from one another, such as by cleavage of the probe during primer extension (e.g., a 5' nuclease assay, such as with a TAQMAN probe), or when the probe hybridizes to an amplicon (e.g., a molecular beacon probe). The nucleic acid portion of the probe may have any suitable structure or origin, for example, the portion may be a locked nucleic acid, a member of a universal probe library, or the like. In other cases, a probe and one of the primers of a primer pair may be combined in the same molecule (e.g., AMPLIFLUOR primers or SCORPION primers). As an example, the primer-probe molecule may include a primer sequence at its 3' end and a molecular beacon-style probe at its 5' end. With this arrangement, related primer-probe molecules labeled with different dyes can be used in a multiplexed assay with the same reverse primer to quantify target sequences differing by a single nucleotide (single nucleotide polymorphisms (SNPs)). Another exemplary probe for droplet-based nucleic acid assays is a Plexor primer.

Label—an identifying and/or distinguishing marker or identifier connected to or incorporated into any entity, such as a compound, biological particle (e.g., a cell, bacteria, spore, virus, or organelle), or droplet. A label may, for example, be a dye that renders an entity optically detectable and/or optically distinguishable. Exemplary dyes used for labeling are fluorescent dyes (fluorophores) and fluorescence quenchers.

Reporter—a compound or set of compounds that reports a condition, such as the extent of a reaction. Exemplary report-

ers comprise at least one dye, such as a fluorescent dye or an energy transfer pair, and/or at least one oligonucleotide. Exemplary reporters for nucleic acid amplification assays may include a probe and/or an intercalating dye (e.g., SYBR Green, ethidium bromide, etc.).

Binding partner—a member of a pair of members that bind to one another. Each member may be a compound or biological particle (e.g., a cell, bacteria, spore, virus, organelle, or the like), among others. Binding partners may bind specifically to one another. Specific binding may be characterized by a dissociation constant of less than about 10^{-4} , 10^{-6} , 10^{-8} , or 10^{-10} M, among others. Exemplary specific binding partners include biotin and avidin/streptavidin, a sense nucleic acid and a complementary antisense nucleic acid (e.g., a probe and an amplicon), a primer and its target, an antibody and a corresponding antigen, a receptor and its ligand, and the like.

II. Exemplary Fusion of a Multiple Emulsion

FIG. 1 shows a schematic flow diagram illustrating an exemplary multiple emulsion **20** that is heated, indicated at **22**, to promote fusion of compound droplets **24** with an aqueous continuous phase **26**. Multiple emulsion **20** may, for example, be a water-in-oil-in-water (W/O/W) emulsion that is converted, at least partially, to an oil-in-water (O/W) emulsion by heat.

Each compound droplet **24** may include an outer droplet **28**, which may be an oil droplet. The outer droplet may enclose at least one inner droplet **30**, which may be an aqueous droplet. The outer droplet may enclose any suitable number of inner droplets, such as one to up to 10,000, or more. In any event, inner droplet **30** may be miscible with continuous phase **26** but may be prevented from merging with the continuous phase by the oil layer provided by outer droplet **28**. The terms “merge,” “coalesce,” and “fuse” are intended to be synonyms for the process of joining separate fluid phases, such as an aqueous droplet with a surrounding aqueous phase.

Aqueous droplet **30** may contain at least one amplification reagent **32**, which is indicated schematically in FIG. 1 by an asterisk (“*”). Continuous phase **26** may be deficient for the amplification reagent before heating. In other words, the reagent may be absent from continuous phase **26** or present substantially below the effective amount for amplification. Accordingly, the continuous phase, before heating, may provide a deficient or incomplete reaction mixture **34** for amplification, which is not capable of efficient amplification of a nucleic acid target, if present, in the deficient/incomplete reaction mixture. However, inner droplets **30** may be designed to complement the amplification reagents present in the continuous phase, such that fusion of inner droplets **30** with continuous phase **26** forms a complete reaction mixture **36** capable of amplification of the nucleic acid target, if present, with proper thermal cycling.

Further aspects of forming emulsions and fusing fluid phases are described in the references listed above under Cross-References, particularly U.S. patent application Ser. No. 12/586,626, filed Sep. 23, 2009; U.S. patent application Ser. No. 12/862,542, filed Aug. 24, 2010; and U.S. patent application Ser. No. 12/976,827, filed Dec. 22, 2010; which are incorporated herein by reference.

III. Exemplary Method of Sample Analysis Using a Multiple Emulsion

FIG. 2 shows a flowchart **50** presenting an exemplary method of sample analysis using a multiple emulsion to controllably combine amplification reagents. The steps shown in

FIG. 2 may be performed in any suitable order and combination and may be combined with any other steps or aspects disclosed elsewhere herein or in the references listed above under Cross-References, which are incorporated herein by reference.

A multiple emulsion may be provided, indicated at 52. The multiple emulsion may include compound droplets disposed in an aqueous phase that is continuous or dispersed. Each compound droplet may include an oil droplet enclosing at least one aqueous droplet, which may contain at least one amplification reagent, such as a heat-stable polymerase, a heat-stable ligase, one or more deoxynucleotide triphosphates, magnesium, one or more primers, or any combination thereof, among others. The amplification reagent is sequestered in an aqueous portion of the compound droplets and cannot react (e.g., cannot enable polymerization or ligation in the surrounding aqueous phase until released). The amplification reagent may be present in sufficient quantity to supply an effective amount of the reagent to the surrounding aqueous phase.

The multiple emulsion may be a segregated reaction mixture. The surrounding aqueous phase may provide an incomplete reaction mixture that contains an effective amount of each reaction component for an amplification reaction, except the reagent(s) contained in the compound droplet. The incomplete reaction mixture also may contain a sample to be tested for a target nucleic acid, or the sample may be added later, as described below.

The multiple emulsion may be formed from another emulsion composition that contains the compound droplets. The emulsion composition may be combined with other reaction components for amplification of a target to generate the multiple emulsion. For example, the other reaction components may be contacted with an aqueous continuous phase of the emulsion composition to create the incomplete reaction mixture surrounding the compound droplets.

In some embodiments, a master mix for amplification may be formed with the compound droplets and other reaction components. The master mix may include the compound droplets and a continuous phase that collectively contain all of the reagents necessary to perform amplification of a nucleic acid target, if combined with a suitable volume of the master mix. The master mix also may or may not contain reagents for reverse transcription, to permit RT-PCR analysis of samples. Portions of the master mix may be dispensed to a plurality of containers, such as to individual vials or to wells of a PCR plate, among others. The master mix may be dispensed with any suitable fluid transfer device, such as a pipette. At least one sample also may be dispensed to each container to form a segregated reaction mixture in the form of a multiple emulsion in each container.

The multiple emulsion may be heated, indicated at 54, to fuse aqueous droplets of the compound droplets with the surrounding aqueous phase, to create a reaction mixture. In some embodiments, heating may be provided as the temperature is increased for the initial melting step of an amplification reaction (e.g., PCR). In any event, heating destabilizes the emulsion and particularly an oil layer between the aqueous droplets and the continuous phase.

The reaction mixture may be cycled thermally, indicated at 56, to promote amplification of one or more nucleic acid targets.

A signal may be detected, indicated at 58, from the reaction mixture before, during, and/or after thermal cycling, to determine whether (and/or how much) amplification occurred in the mixture. Alternatively, or in addition, the amplified target may be isolated from the reaction mixture.

Further aspects of forming compound droplets, heating compound droplets to induce fusion, thermal cycling, signal detection, and data analysis are described in the references listed above under Cross-References, which are incorporated herein by reference, particularly U.S. patent application Ser. No. 12/586,626, filed Sep. 23, 2009; U.S. patent application Ser. No. 12/862,542, filed Aug. 24, 2010; and U.S. patent application Ser. No. 12/976,827, filed Dec. 22, 2010.

IV. Exemplary System for Forming Compound Droplets

FIG. 3 shows a schematic view of an exemplary system 70 for forming compound droplets 24 containing at least one reagent 32, for the multiple emulsion of FIG. 1. System 70 includes channels 71 that carry fluid to a serial arrangement of droplet generators formed where the channels intersect.

Reagent 32 may be part of an aqueous composition 72 ("water+reagent") that forms aqueous inner droplets 30 of compound droplets 24. In addition to reagent(s) 32, composition 72 may include at least one surfactant, a buffer, glycerol, salt, and/or the like. In some embodiments, the reagent may be or include a heat-stable polymerase or ligase. The polymerase may be recombinant or native Taq polymerase in an active configuration (i.e., the enzyme does not require heat activation if all required reaction components are available), or may be in an inactive configuration until heated.

During system operation, aqueous composition 72 may flow to a first droplet generator 74, where inner droplets 30 are formed as partitions of composition 72 enclosed by an oil phase 76. The oil phase may contain an oil 78 and at least one surfactant 80. For example, the oil phase may include a fluorinated oil and at least one fluorinated surfactant. In any event, the surfactant may be a biocompatible surfactant that facilitates generation of an emulsion that is stable at temperatures less than about 40° C., 50° C., 60° C., 70° C. or 80° C., among others, but unstable at temperatures greater than about 50° C., 60° C., 70° C., 80° C., or 90° C., among others. Also, the walls of the channel downstream of droplet generator 74, and upstream of the next droplet generator, may be hydrophobic to restrict wetting the walls with aqueous droplets 30.

The aqueous droplets in oil phase 76 may flow to a second droplet generator 82, where compound droplets 24 are formed in an aqueous continuous phase 84. The continuous phase may contain at least one surfactant, which may be the same as or a different surfactant from that present in aqueous droplets 30, and at the same or a different concentration. Also, the walls downstream of droplet generator 82 may be hydrophilic to restrict wetting of the walls with outer oil droplets 28 of compound droplets 24.

V. Exemplary Fusion within Compound Droplets

FIG. 4 shows a schematic flow diagram illustrating an exemplary multiple emulsion 100 that is heated to promote fusion within compound droplets 102. Emulsion 100 may be a water-in-oil-in-water-in-oil (W/O/W/O) emulsion that corresponds to emulsion 20 of FIG. 1, but in a partitioned configuration that disperses aqueous phase 26 (and enclosed compound droplets 24) in an immiscible continuous phase 104.

Compound droplets 102 each may be composed of at least three types of droplets: one or more inner aqueous droplets 30 enclosed by one or more oil droplets 28, which in turn are enclosed by an outer aqueous droplet 108 (composed of aqueous phase 26 of FIG. 1, but dispersed). Each inner droplet 30 may contain at least one amplification reagent 32. The com-

pound droplets may be disposed in continuous phase **104**, such as an oil phase including an oil and at least one surfactant.

Each compound droplet, and particularly inner droplets **30** and outer droplet **108** collectively, may provide an effective amount of each reaction component necessary to perform amplification of a nucleic acid target, if present in one of the aqueous phases of the compound droplet. However, at least one of the reaction components may be separated from the other reaction components by a layer of oil provided by oil droplets **28**, to prevent reaction before heat-induced fusion. Accordingly, fusion of inner droplets **30** with outer droplet **108**, in response to heat, may form an oil-in-water-in-oil (O/W/O) emulsion **112** that contains a complete reaction mixture **114** for amplification of a target in each outer droplet **108**.

VI. Exemplary System for Forming Compound Droplets that Fuse Internally

FIG. 5 shows a schematic view of an exemplary system **120** for forming multiple emulsion **100** of FIG. 4. A W/O/W emulsion (e.g., emulsion **20** of FIG. 1) and an oil phase **122** may feed a droplet generator **124** to form compound droplets **102** of multiple emulsion **100**. Droplet generator **124** may be structured generally as described for first droplet generator **74** of FIG. 3. Any number of inner droplets **30** may be disposed in each outer droplet **108**, such as 1 to 10,000, among others.

Heating emulsion **100** may produce O/W/O emulsion **112** (see Section V and FIG. 4), which releases the amplification reagent(s) (e.g., Taq DNA polymerase) and/or sample from the inner droplets.

VII. Selected Embodiments

This section presents selected embodiments of the present disclosure related to methods and compositions for coalescence of a continuous phase with inner droplets of compound droplets to form a reaction mixture.

1. A method of PCR amplification, comprising: (A) providing a multiple emulsion including compound droplets disposed in a continuous aqueous phase, each compound droplet including an oil droplet enclosing at least one aqueous droplet, the aqueous droplet containing at least one PCR reagent; (B) heating the multiple emulsion such that aqueous droplets of the compound droplets fuse with the continuous phase to supply an effective amount of the PCR reagent to the continuous phase, to form a reaction mixture capable of PCR amplification of a nucleic acid target, if present, in the reaction mixture; and (C) cycling the reaction mixture thermally to promote PCR amplification.

2. The method of paragraph 1, wherein the step of providing includes a step of providing a multiple emulsion having a continuous phase that lacks the PCR reagent.

3. The method of paragraph 1, further comprising a step of detecting one or more signals corresponding to PCR amplification of the nucleic acid target in the reaction mixture.

4. The method of paragraph 1, wherein the step of providing includes a step of providing a multiple emulsion containing a DNA polymerase in the at least one aqueous droplet.

5. The method of paragraph 4, wherein the step of providing includes a step of providing a multiple emulsion containing Taq DNA polymerase in the at least one aqueous droplet.

6. The method of paragraph 1, wherein the step of providing includes a step of providing a multiple emulsion in which the PCR reagent contained by the at least one aqueous droplet

is magnesium, at least one deoxyribonucleotide triphosphate, at least one nucleic acid primer, or a combination thereof.

7. The method of paragraph 1, wherein the step of providing a multiple emulsion includes a step of combining (a) a composition including the compound droplets and (b) an effective amount of each reaction component for the PCR amplification except the at least one PCR reagent.

8. The method of paragraph 1, wherein the step of providing includes a step of forming a composition comprising (a) a continuous phase including an effective amount of each reaction component for the PCR amplification except the at least one PCR reagent and the nucleic acid target and (b) a plurality of compound droplets containing the at least one PCR reagent, and a step of combining at least a volume portion of the composition and a sample that provides the nucleic acid target, if present, in the reaction mixture.

9. The method of paragraph 8, wherein the composition is a PCR master mix, and wherein the step of combining includes a step of dispensing volume portions of the PCR master mix and at least one sample to each of a plurality of containers.

10. The method of paragraph 1, wherein the step of providing a multiple emulsion includes a step of providing a multiple emulsion in which fusion of the at least one aqueous droplet with the continuous phase does not occur substantially until the multiple emulsion is heated to a temperature of at least 50° C.

11. A method of PCR amplification, comprising: (A) providing a first composition including compound droplets disposed in a continuous aqueous phase, each compound droplet including an oil droplet enclosing at least one aqueous droplet, the aqueous droplet containing a heat-stable DNA polymerase; (B) forming a second composition by combining the continuous aqueous phase with other reaction components for PCR amplification of a nucleic acid target, if present, in the second composition; (C) heating the second composition such that aqueous droplets of the compound droplets fuse with the continuous aqueous phase to supply an effective amount of the PCR reagent to the continuous phase, to form a reaction mixture capable of PCR amplification of a nucleic acid target, if present, in the reaction mixture; and (D) cycling the reaction mixture thermally to promote PCR amplification.

12. A composition for PCR amplification, comprising a multiple emulsion including compound droplets disposed in a continuous aqueous phase, each compound droplet including an oil droplet enclosing at least one aqueous droplet, the aqueous droplet containing at least one PCR reagent, wherein heating the composition causes aqueous droplets of the compound droplets to fuse with the continuous phase to supply an effective amount of the PCR reagent to the continuous phase, to form a reaction mixture capable of PCR amplification of a nucleic acid target, if present, in the reaction mixture.

13. The composition of paragraph 12, wherein the at least one aqueous droplet contains a heat-stable DNA polymerase.

14. A composition for use in PCR amplification, comprising a multiple emulsion including compound droplets disposed in a continuous aqueous phase, each compound droplet including an oil droplet enclosing at least one aqueous droplet, the aqueous droplet containing a heat-stable DNA polymerase.

The disclosure set forth above may encompass multiple distinct inventions with independent utility. Although each of these inventions has been disclosed in its preferred form(s), the specific embodiments thereof as disclosed and illustrated herein are not to be considered in a limiting sense, because numerous variations are possible. The subject matter of the inventions includes all novel and nonobvious combinations

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and subcombinations of the various elements, features, functions, and/or properties disclosed herein. The following claims particularly point out certain combinations and sub-combinations regarded as novel and nonobvious. Inventions embodied in other combinations and subcombinations of fea- 5 tures, functions, elements, and/or properties may be claimed in applications claiming priority from this or a related application. Such claims, whether directed to a different invention or to the same invention, and whether broader, narrower, equal, or different in scope to the original claims, also are regarded as included within the subject matter of the inven- 10 tions of the present disclosure.

The invention claimed is:

1. A method of nucleic acid amplification, comprising pro- 15 viding a composition comprising a plurality of compound droplets disposed in an aqueous phase, wherein each com- pound droplet comprise a plurality of aqueous droplets, each aqueous droplet enclosed by an oil droplet and each aqueous droplet containing at least one amplification reagent;

heating the composition to cause fusion of the aqueous droplets with the aqueous phase, such that the at least one amplification reagent is added to the aqueous phase to form at least one reaction mixture; and cycling the at least one reaction mixture thermally to encourage ampli- 20 fication of a nucleic acid target.

2. The method of claim 1, further comprising a step of detecting one or more signals corresponding to amplification of the nucleic acid target in the at least one reaction mixture.

3. The method of claim 1, wherein the aqueous droplets contain DNA polymerase.

4. The method of claim 3, wherein the DNA polymerase is a Taq DNA polymerase.

5. The method of claim 1, wherein the at least one ampli- 25 fication reagent includes magnesium, at least one deoxy- nucleotide triphosphate, at least one primer, or a combination thereof.

6. The method of claim 1, wherein the aqueous phase is continuous.

7. The method of claim 1, wherein the aqueous phase is dispersed.

8. The method of claim 1, 30 wherein the aqueous phase lacks an effective amount of at least one PCR reagent that is contained by the aqueous droplets, wherein;

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the at least one reaction mixture is capable of PCR ampli- fication of a nucleic acid target, if present, in the reaction mixture, and

wherein cycling the at least one reaction mixture thermally encourages PCR amplification of the nucleic acid target.

9. The method of claim 1, wherein fusion of the aqueous droplets with the aqueous phase occurs at least predominantly after the composition has been heated to a temperature of at least about 50° C.

10. The method of claim 8, wherein the aqueous phase in the composition provided includes an effective amount of each reaction component for the PCR amplification except the at least one PCR reagent.

11. A method of nucleic acid amplification, comprising providing a composition comprising a plurality of compound droplets disposed in an aqueous phase, wherein each com- 35 pound droplet comprise a plurality of aqueous droplets, each aqueous droplet enclosed by an oil droplet and each aqueous droplet containing a heat stable polymerase;

heating the composition such that the aqueous droplets with the aqueous phase, to form at least one reaction mixture capable of PCR amplification of a nucleic acid target, if present, in the at least one reaction mixture; and cycling the at least one reaction mixture thermally to encourage PCR amplification.

12. A composition for nucleic acid amplification, compris- 40 ing an aqueous phase that is dispersed or continuous; a plu- rality of compound droplet disposed in the aqueous phase; wherein each compound droplet comprise a plurality of aque- ous droplets, each aqueous droplet enclosed by an oil droplet and each aqueous droplet containing at least one amplifica- tion reagent, wherein heating the composition causes the aqueous droplets to fuse with the aqueous phase to supply the at least one amplification reagent to the aqueous phase.

13. The composition of claim 12, wherein the aqueous droplets contains a heat-stable DNA polymerase.

14. The composition of claim 12, where heating the com- 45 position forms at least one reaction mixture capable of ampli- fication of a nucleic acid target, if present, in the reaction mixture.

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