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(54) Title: METHODS, DEVICES AND SYSTEMS FOR EMULSION/DROPLET PCR

(57) Abstract: The present invention relates generally to the use of a class of surfactants for emulsion and droplet polymerase chain reaction ("PCR") mixtures. The class of surfactants consists of those having the chemical formula R-(OCH2CH2)₉-OH, wherein R is an alkyl group consisting of 12 to 18 carbons and n is 2 to 25. The present invention also relates to methods, devices, systems, and kits incorporating the above-described class of surfactants.

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**FIG. 1**

1. Forming an aqueous droplet comprising a polymerase chain reaction mixture
2. Surrounding the aqueous droplet with an oil phase
3. Adding a surfactant having Formula R-(OCH₂CH₂)₉-OH, wherein R is an alkyl group consisting of 12 to 18 carbons and n is 2 to 25
before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

with sequence listing part of description (Rule 5.2(a))

with international search report (Art. 21(3))
METHODS, DEVICES AND SYSTEMS FOR EMULSION/DROPLET PCE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of priority to U.S. Provisional Application No. 61/922,422, filed on December 31, 2013, which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

[0002] The present invention relates generally to surfactants for emulsion and droplet polymerase chain reaction ("PGR"). The present invention also relates to methods, devices, systems, and kits incorporating those surfactants.

SEQUENCE SUBMISSION

[0003] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is entitled 3400-265_ST25.txt, created on December 12, 2014 and is 1 kb in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

BACKGROUND

[0004] PGR is a biochemical technique used to amplify, quantify, and identify specific genes related to cancers, infectious diseases, forensics, and hereditary disorders. Exemplary references discussing PGR, which are incorporated by reference in their entirety, include: F. Moltzahn et al., Cancer Res. 2011, 71, 550-560; M.C. Strain et al., PLoS ONE 2013, 8, e55943; P. Liu et al., Lab. Chip 2011, 11, 1041-1048; J.A. Lounsbury et al., Lab. Chip 2013, 13, 1384-1393; and D. Pekin et al., Lab. Chip 2011, 11, 2156-2166. PGR is commonly performed in central laboratory environments and can involve thermocycling of 20 - 50 μL samples in well plates. The process to complete PGR, including sample preparation, can take several hours and require a number of
manual steps. The duration of PGR from sample preparation to DNA analysis can be reduced by integrating laboratory functions in micro total analysis systems.

[0005] An important and widely used element of micro total analysis systems is the droplet PGR component. Ultrafast droplet PGR can be performed with integrated heaters, wherein up to 40 thermal cycles can be conducted in less than six minutes. One such reference discussing this process, which is incorporated by reference in its entirety, is P. Neuzil, Nucleic Acids Res. 2006, 34, e77.

[0006] In droplet PGR, droplet microfluidics is used to monodispersly portion PGR reaction mixtures into microreactors surrounded by an oil phase. That is, PGR reaction mixtures can be formed as an aqueous droplet, which are surrounded by an oil phase. Typically, the droplets can differ in size, but are typically on the order of nanoliters or picoliters. At the larger end of this size range, DNA is quantified using a cycle threshold calibration curve. DNA has been absolutely quantified using a popular variation of PGR termed "digital PGR" by using picoliter droplets. The initial average DNA copy number per droplet in digital PGR is less than 1, implying a Poisson distribution with 0 or 1 copy in most droplets. One such reference discussing this process, which is incorporated by reference in its entirety, is N.R. Beer et al., Anal. Chem. 2007, 79, 8471-8475. In digital PGR, after thermocycling, only droplets that contain the target DNA fluoresce. The number of fluorescent and nonfluorescent droplets is counted to absolutely quantify the amount of target DNA according to Poisson statistics. Digital PGR is extremely useful and has been shown to quantify extremely rare targets, such as HIV DNA in infected patients undergoing effective treatment. See M.C. Strain et al., supra.

[0007] Though impactful, droplet PGR is in need of technical improvement. For example, as discussed in S.L. Angione et al., Anal. Chem., 2012, 84, 2654-2661, which is incorporated by
reference in its entirety, microreactors less than microliters in volume are impaired by surface
effects. While it is desirable to have an increase in surface area relative to the volume of the
droplet for quick transfer, this presents several potential problems. Irreversible adsorption of
amphiphilic proteins occurs as the droplet size becomes smaller and the ratio of the surface area
to the volume increases. This irreversible adsorption of proteins to hydrophobic interfaces
hinders microfluidic assay.

[0008] The adsorption of proteins is particularly problematic with droplet PGR, which makes
use of Taq polymerase (Taq Pol) to catalyze the reaction. Taq Pol, an enzyme derived from
thermophilic bacteria, is especially prone to absorption at hydrophobic interfaces because a large
volume fraction of it is hydrophobic. The aliphatic index characterizes the relative hydrophobic
volume of a protein and, in general, thermophilic bacterial proteins have large relative
hydrophobic volumes to aide in structural stability. The aliphatic index of Taq Pol is 98.6
(compared to the aliphatic index of BSA, which is 76.1). As discussed in F.C. Lawyer et al.,
Genome Res. 1993, 2, 275-287, which is incorporated by reference in its entirety, Taq Pol is
incredibly stable; even at DNA melting temperatures of 95 °C, the half-life of Taq Pol is 45 to 50
min. Thus, less stable proteins used in droplet microfluidics would sample a larger structural
space, resulting in the interaction with and irreversible denaturing of the less stable proteins on
the hydrophobic surface. Thus, adsorption is exacerbated when using unstable proteins in
microfluidic droplets.

[0009] Many techniques have been employed to overcome the adsorption of proteins, but these
methods have several different flaws. As discussed in S.L. Angione et al., Anal. Chem., 2012,
84, 2654-2661, which is incorporated by reference in its entirety, one technique employed to
overcome the adsorption of proteins is to increase the Taq Pol concentration in an attempt to
replace the adsorbed enzyme. This technique, however, is a wasteful solution that can require up to seven times the concentration of bulk PGR for optimal performance. The technique is further described in F. Wang et al., Biomed. Microdevices 2009, which is incorporated by reference in its entirety.

[0010] Another technique, as discussed in A.C. Hatch et al., Lab. Chip 2011, 11, 3838-3845, which is incorporated by reference in its entirety, is to increase the amount of surfactant used in the system. In addition to stabilizing droplets, large amounts of surfactant can competitively bind to the hydrophobic interface and reduce Taq Pol adsorption, though excessive surfactant can inhibit PGR. Similarly, bovine serum albumin may be included in the PGR mixture to competitively bind to the interface. Usually, both methods are employed simultaneously to create an emulsion, but as discussed in F. Diehl et al., Nat. Methods 2006, 3, 551-559 and R. Williams et al., Nat. Methods 2006, 3, 545-550, which are incorporated by reference in their entirety, for successful PGR, the emulsion must be generated on ice. Yet another technique employs the use of fluorinated oils and surfactants with Taq Pol at room temperature to create droplets for digital PGR. This technique, however, requires fluorocarbon specialty chemicals, which increases costs and concerns for the environment.

[0011] In view of the above, there is a need for PGR methods, systems, and kits that overcome the issues associated with protein adsorption in microfluidics that do not suffer from the above described flaws.

**SUMMARY**

[0012] In one aspect, the present invention relates to a droplet or emulsion PGR comprising an aqueous droplet. In one embodiment, the aqueous droplet includes a polymerase chain reaction mixture, an oil phase, and a surfactant having Formula I:
R — (OCH₂CH₂)ₙ — OH,

[0013] wherein R is an alkyl group consisting of 12 to 18 carbons and n is 2 to 25.

[0014] In some embodiments, the polymerase chain reaction mixture may comprise a polymerase, a primer, nucleotides, and a template nucleic acid. In some embodiments, the surfactant can be one selected from the group consisting of laureth-4, laureth-23, ceteth-2, ceteth-20, ceteareth-12, ceteareth-20, ceteareth-25, steareth-2, steareth-10, steareth-20, steareth-21, oleth-2, oleth-5, oleth-10, and oleth-20.

[0015] In another aspect, the present invention relates to methods for preparing a mixture for droplet or emulsion PGR. In one embodiment, the method includes forming an aqueous droplet comprising a polymerase chain reaction mixture. In another embodiment, the method includes surrounding the aqueous droplet with an oil phase. In another embodiment, the method includes adding a surfactant having Formula 1:

R — (OCH₂CH₂)ₙ — OH,

[0016] wherein R is an alkyl group consisting of 12 to 18 carbons and n is 2 to 25. In one embodiment, the polymerase chain reaction mixture may comprise a polymerase, a primer, nucleotides, and a template nucleic acid. In some embodiments, the added surfactant can be selected from the group consisting of laureth-4, laureth-23, ceteth-2, ceteth-20, ceteareth-12, ceteareth-20, ceteareth-25, steareth-2, steareth-10, steareth-20, steareth-21, oleth-2, oleth-5, oleth-10, and oleth-20.

[0017] In yet another aspect, the present invention also relates to methods for determining the capability of a surfactant for droplet or emulsion PGR. In one embodiment, the method includes preparing an aqueous droplet comprising Taq polymerase. In another embodiment, the method includes suspending the aqueous droplet in an oil phase comprising a dissolved surfactant. In a
further embodiment, the method includes forming a hanging drop of the aqueous droplet suspended in the oil phase. In an additional embodiment, the method includes measuring a property of the hanging drop. In some embodiments, the measured property is the interfacial tension of the hanging drop, which is determined using equation: $\gamma = 3A7gApD_e^{-0.68} O_s^{2.68}$, wherein $D_e$ is the largest width of the drop, $3\delta$ is the width of the drop at the distance $D_e$, $g$ is the acceleration due to gravity, 9.8 m/s$^2$, and $\Delta \rho$ is the difference in density between the aqueous droplet and the oil phase.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0018] The accompanying drawings, which are incorporated herein and form part of the specification, illustrate various embodiments of the present disclosure and, together with the description, further serve to explain the principles of the disclosure and to enable a person skilled in the pertinent art to make and use the embodiments disclosed herein. In the drawings, like reference numbers indicate identical or functionally similar elements.

[0019] FIG. 1 is a flow chart of a method for preparing a mixture for droplet or emulsion PGR in accordance with an embodiment of the present invention.

[0020] FIG. 2 is a flow chart of a method for determining the capability of a surfactant for droplet or emulsion PGR in accordance with an embodiment of the present invention.

[0021] FIG. 3 illustrates an exemplary hanging droplet in accordance with an embodiment of the present invention.

[0022] FIG. 4 is a graph of interfacial tension (dyne/cm) versus time (min) of solutions comprising mineral oil, Taq Pol, ABIL EM90, and Brij L4® in accordance with an embodiment of the present invention.
FIG. 5 is a graph of dynamic pseudo surface pressure measurements with surfactant and Taq as the deviation from the steady state surface tension with surfactant and without Taq for a 1.5% ABIL EM90 solution (dyne/cm) as a function of time (min) in accordance with an embodiment of the present invention.

FIG. 6 is a graph of dynamic pseudo surface pressure measurements with surfactant and Taq as the deviation from the steady state surface tension with surfactant and without Taq for a 0.5% Brij L4® solution (dyne/cm) as a function of time (min) in accordance with an embodiment of the present invention.

FIG. 7 is a graph of \( \ln(\frac{dT}{dt}) \) as a function of surface pressure (dyne/cm) of a Taq solution in accordance with an embodiment of the present invention.

FIG. 8 is a graph of surface pressure (dyne/cm) as a function of Taq Pol concentration (X) of a 1.5% ABIL EM90 solution and a 0.5% Brij L4® solution in accordance with an embodiment of the present invention.

FIG. 9 is a graph of the cycle threshold as a function of the Taq Pol concentration (X) of PGR reaction mixtures comprising 1.5% ABIL EM90 and 0.5% Brij L4® in accordance with an embodiment of the present invention.

**DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS**

The present invention has several embodiments and relies on patents, patent applications and other references for details known to those of the art. Therefore, when a patent, patent application, or other reference is cited or repeated herein, it should be understood that it is incorporated by reference in its entirety for all purposes as well as for the proposition that is recited.
The present invention relates to droplet and emulsion PCR. As understood by a person of ordinary skill in the art, droplet and emulsion PCR are commonly used techniques for the amplification and detection of nucleic acid sequences in integrated microsystems. In one aspect, the present invention relates to a droplet or emulsion PCR mixture. The droplet or emulsion PCR mixture is a mixture in which the PCR reaction is carried out. The aqueous droplet can be on the order of nanoliters to picoliters in size. For example, the aqueous droplets can have a diameter of, in a non-limiting example, $20 \pm 2 \mu m$, resulting in an aqueous droplet that is approximately 4 picoliters. Other droplet sizes may also be used.

In certain embodiments of the present invention, the aqueous droplet comprises a PCR mixture. In some embodiments, the PCR mixture may include one or more of a polymerase, a primer, nucleotides, a template nucleic acid, and a fluorescent die. In some embodiments, the PCR mixture includes Taq polymerase (Taq Pol) as the polymerase.

In some embodiments, the droplet or emulsion PCR mixtures of the present invention further comprise an oil phase. In certain embodiments of the present invention, the oil phase consists of a mineral oil solution. In exemplary embodiments of the present invention, the oil phase surrounds the aqueous droplet.

In conventional droplet or emulsion PCR, hydrophobic components of proteins bind to the oil phase at the surface of the aqueous droplet, resulting in the removal of hydrophobic components. During droplet formation, the adsorption of proteins to the interface can begin immediately. Initially, the adsorption is diffusion controlled, wherein a clean interface adsorbs surfactants and proteins as they diffuse from within the droplet or surrounding continuous phase. Interfacial tension between the continuous phase and droplet phase may decrease proportionally to the number of amphiphiles bound to the interface. Once an appreciable film of proteins is
formed, an energy barrier to create space within the interface can resist adsorption. The decrease in interfacial tension at this stage is a result of the adsorption of proteins, as well as the molecular rearrangement within the film (e.g., proteins denaturing at the surface).

[0033] In certain embodiments of the present invention, a surfactant is included in the droplet or emulsion PCR mixture. In some embodiments, the surfactant can be added to the oil phase and can surround the surface of the aqueous droplet. In one embodiment, the surfactant can be added to the oil phase before the aqueous droplet is surrounded by or suspended in the oil phase. In another embodiment, the surfactant can be added to the oil phase after the aqueous droplet is surrounded by or suspended in the oil phase. The surfactant can act to prevent the interaction of hydrophobic components with the oil phase. Further, the surfactant can act to stabilize the aqueous droplet. The use of the surfactants described herein result in more efficient amplification during droplet or emulsion PCR.

[0034] In one embodiment, the surfactant has Formula 1:

\[
R - (\text{OCH}_2\text{CH}_2)_n - \text{OH},
\]

[0035] wherein R is an alkyl group consisting of 12 to 18 carbons and n is 2 to 25. In some embodiments, suitable surfactants include laureth-4, laurate-23, ceteth-2, ceteth-20, ceteareth-12, ceteareth-20, ceteareth-25, steareth-2, steareth-10, steareth-20, steareth-21, oleth-2, oleth-5, oleth-10, and oleth-20. In one embodiment, the surfactant is laureth-4. These surfactants are available under the tradename Brij (e.g., Brij L, Brij C, Brij CS, Brij S, and Brij O) from Croda Europe Ltd. (East Yorkshire, England). The above-described surfactants are alkyl polyglycol ethers derived from a variety of straight and branched chain alcohols produced by reacting ethylene oxide with fatty alcohols.
In another aspect, the present invention relates to methods for preparing droplet or emulsion PGR mixtures. FIG. 1 is a flow chart of method 100 for preparing a mixture for droplet or emulsion PGR in accordance with one embodiment of the present invention. Method 100 includes forming an aqueous droplet comprising a PGR mixture (step 101). The droplet or emulsion PGR mixture is a mixture in which the PGR reaction is carried out. In some embodiments, the aqueous droplet comprises a PGR mixture, which may include one or more of a polymerase, a primer, nucleotides, a template nucleic acid, and a fluorescent die. In some embodiments, the PGR mixture includes Taq Pol as the polymerase.

In a non-limiting embodiment, the aqueous droplets can be formed in a polydimethylsiloxane (PDMS) microfluidic chip using flow focusing geometry. When preparing the aqueous droplets, the aqueous droplets can be captured as a single droplet within a well. The well can be one suitable for carrying out droplet or emulsion PGR.

In some embodiments, method 100 further includes surrounding the aqueous droplet with an oil phase (step 102). In some embodiments, the oil phase comprises a mineral oil solution. In one embodiment, the oil phase can be present in a well in which an aqueous droplet is introduced. Alternatively, in a second embodiment, the oil phase can be added to an aqueous droplet resulting in the oil phase surrounding the aqueous droplet.

In other embodiments, method 100 further includes adding a surfactant having the Formula 1:

\[ R - (OCH_2CH_2)_n - OH, \]

wherein R is an alkyl group consisting of 12 to 18 carbons and n is 2 to 25 (step 103). In some embodiments, the surfactant is one selected from the group consisting of laureth-4, laureth-23, ceteth-2, ceteth-20, ceteareth-12, ceteareth-20, ceteareth-25, steareth-2, steareth-10, steareth-
20, steareth-21, oleth-2, oleth-5, oleth-10, and oleth-20. In one embodiment, the surfactant is laureth-4. In one embodiment, the surfactant can be added to the oil phase prior to the introduction of the aqueous droplet. Alternatively, in a second embodiment, the surfactant can be added to the oil phase after the aqueous droplet has been surrounded by the oil phase.

[0041] In certain embodiments, the aqueous droplets can be used for emulsion or droplet PGR. In some embodiments, a plurality of aqueous droplets is formed. In some embodiments, only a portion of the plurality of aqueous droplets comprises a template. In some embodiments, the template is randomly distributed in certain aqueous droplets, while other aqueous droplets do not comprise a template.

[0042] In certain embodiments, the method can further include steps involved in droplet or emulsion PGR. For example, the droplet or emulsion PGR mixture can be hot started. In one embodiment, the mixtures are hot started at 95 °C for a period of time. The droplet or emulsion PGR mixture can then be thermocycled for a number of cycles. In some embodiments, each thermocycle can an annealing step, an extension step, and a melting step. For example, in one embodiment, each thermocycle can consist of 30 seconds at 55 °C for annealing, 30 seconds at 72 °C for extension, and 30 seconds at 95 °C for melting. In certain exemplary embodiments, real time amplification results are obtained. For example, real time amplification results can be obtained by exciting droplets using a light emitting diode (LED). In some embodiments, the fluorescence emission can be captured with a charged couple device (CCD). In some embodiments, certain aqueous droplets will produce a difference in fluorescence emission than other aqueous droplets. In some embodiments wherein a plurality of aqueous droplets are formed, some with a template and others without a template, the fluorescence emission for the
aqueous droplets with the template can differ from the fluorescence emission for the aqueous droplets without the template.

[0043] In another aspect, the present invention includes a method of measuring adsorption and loss of polymerase at a droplet surface for surfactant-oil combinations. FIG. 2 is a flow chart of method 200 for determining the capability of a surfactant for droplet or emulsion PGR in accordance with one embodiment of the present invention. The method allows for identification of superior surfactants for use in emulsion and droplet PGR. In contrast, traditional surfactant selection for emulsion and droplet PGR relies on trial and error.

[0044] In certain embodiments, the pendant drop technique is used to determine the ability of a surfactant to prevent polymerase loss from the PGR reaction. Method 200 includes forming an aqueous droplet comprising a polymerase (step 201). In certain embodiments, the polymerase is Taq Pol. The concentration of Taq Pol is non-limiting and can vary. In some embodiments, the aqueous droplet comprises a concentration of Taq Pol in the amount of 0 to 8X, wherein IX is 0.025 μL. In other embodiments, the aqueous droplet comprises one or more of a PGR buffer, a primer, nucleotides, a template nucleic acid, and a fluorescent die. In certain embodiments, the aqueous droplet is stored on ice.

[0045] In some embodiments, method 200 further includes preparing an oil phase comprising a surfactant (step 202). In one embodiment, the oil phase comprises mineral oil. For example, the oil phase can comprise a stock mineral oil solution of the surfactant cetyl PEG / PPG-10/1 dimethicone sold under the name ABIL EM90 by Evonik Industries (Germany). In another embodiment, the oil phase can comprise a stock mineral oil solution of a surfactant having Formula I described herein. In one embodiment, the surfactant is laureth-4. In one embodiment, the oil phase can comprise a surfactant in varying concentrations. In some embodiments, the oil
phase can comprise a stock mineral oil solution of 1.5% (w/w) ABIL EM90 or 0.5% (w/w) laureth-4. In some embodiments, the mineral oil solutions can be preheated and maintained at a temperature using a water bath. In one embodiment, the mineral oil solutions can be preheated and maintained at a temperature of about 55°C.

[0046] In some embodiments, method 200 further includes forming a hanging drop of the aqueous droplet in the oil phase (step 203). In certain embodiments, the hanging drop is the largest non-spherical drop that can be formed without pinching off the aqueous droplet suspended in the oil phase. FIG. 3 illustrates an exemplary hanging drop 300. In one embodiment, the hanging drop can be a single droplet formed from the end or tip of an object. In another embodiment, the hanging drop can be formed at the tip of a syringe. In certain embodiments, the hanging drop is formed by using a 16, 18, or 26 gauge syringe needle. In some embodiments, the syringe can be rinsed prior to use with ultrapure deionized water and dried with nitrogen.

[0047] In some embodiments, method 200 further includes measuring a property of the hanging drop (step 204). In certain embodiments, the property is the interfacial tension. In one embodiment, the interfacial tension can be determined by analyzing the geometry of the hanging drop. The adsorption of the polymerase at the droplet interface can be measured, thereby indicating the capability of the surfactant to reduce the loss of polymerase in a droplet or emulsion PGR reaction.

[0048] FIG. 3 illustrates an exemplary hanging droplet 300 in accordance with certain non-limiting embodiments of the present invention. Hanging drop 300 comprises $D_3301$, which is the largest equatorial width of hanging drop 300. Hanging drop 300 further comprises $D_3302$, which is defined as the width of hanging drop 300 at a distance of $D_3$ from the bottom of hanging...
drop 300. The shape, $S$, of the hanging drop can be determined based on the largest equatorial width, $D_e$, and the width $D_s$. The shape, $S$, of hanging drop 300 can be measured as $D_s/D_e$.

[0049] In one embodiment, the interfacial tension can be determined using the above described measurements. The interfacial tension can be determined using the following Equation 1:

$$\gamma = 3.17 g \Delta \rho D_e^{-0.68} D_s^{2.68}.$$

[0050] In Equation 1, $D_e$ is the largest equatorial width of the hanging drop, $O_s$ is the width of the hanging drop at a distance $D_e$ from the bottom of the hanging drop, $g$ is the acceleration due to gravity, 9.8 m/s$^2$, and $\Delta \rho$ is the difference in density between the aqueous droplet and the oil phase.

[0051] In some embodiments, the interfacial tension can be measured over time. In one embodiment, the interfacial tension is measured immediately after formation of the hanging drop. In some embodiments, the interfacial tension is measured at subsequent points in time. In some embodiments, a steady state interfacial tension is measured. A steady state interfacial tension can be an interfacial tension, which no longer changes over time within a certain degree of error. By measuring the interfacial tension of a hanging drop over time, the adsorption of surfactants and proteins to a surface can be indirectly examined. Interfacial tension between the continuous phase and droplet phase decreases proportionally to the number of amphiphiles bound to the surface. Thus, a decrease in interfacial tension over time is indicative of an increase in the adsorption of surfactants and proteins. In a PCR reaction mixture, surfactants and proteins can compete for the surface. A continuous decrease in interfacial tension over time can be indicative of an increase in protein adsorption, which has a negative effect on PCR. A small decrease (or absence of a decrease) in interfacial tension over time is indicative of a surfactant
that has a higher capability and/or efficiency for droplet or emulsion PGR, Thus, in some embodiments of the present invention, the use of a surfactant that results in a small decrease (or the absence of a decrease) in interfacial tension over time is desirable. In some embodiments, a difference in interfacial tension between the initial interfacial tension measurement and the steady state interfacial tension measurement of about 1.0 dyne/cm is indicative of a low amount of protein adsorption, which is preferential for droplet or emulsion PCR. In certain embodiments, the difference in interfacial tension is about 0.75 dyne/cm, about 0.50 dyne/cm, about 0.25 dyne/cm, or less than about 0.25 dyne/cm.

[0052] In some embodiments, the measured property of the hanging drop is the surface pressure. In one embodiment, the surface pressure can be measured using Equation II:

\[
\frac{dn}{dt} = k_\gamma V C e^{-\frac{T}{k_\gamma V C}} - k_\lambda V C^* e^{\frac{\Pi \Delta A}{kT}}.
\]

[0053] In Equation II, \(k_\gamma\) and \(k_\lambda\) are the adsorption and desorption rate constant, \(V\) is the number of adsorbing groups, \(C\) and \(C^*\) are the bulk and surface concentration of the species in the same units, \(\Delta A\) is the area created in the film to adsorb the species, and \(\Pi \Delta A\) is the work required to create space of size \(\Delta A\) in a film under surface pressure \(\Pi\).

[0054] When the adsorption of the species is irreversible, the surface pressure can be measured using Equation III:

\[
\ln(\frac{dn}{dt}) = \ln(k_\gamma V C) - \frac{\Pi \Delta A}{kT}.
\]

[0055] In Equation III, \(k_\gamma\) is the adsorption and desorption rate constant, \(V\) is the number of adsorbing groups, \(C\) is the bulk concentration of the species, \(\Delta A\) is the area created in the film to adsorb the species, and \(\Pi \Delta A\) is the work required to create space of size \(\Delta A\) in a film under surface pressure \(\Pi\).
In another aspect, the present invention provides a kit for performing droplet or emulsion PGR. In some embodiments, the kit comprises a surfactant having Formula 1:

\[
R - (OCH_2CH_2)_n - OH,
\]

wherein R is an alkyl group consisting of 12 to 18 carbons and n is 2 to 25. In other embodiments, the kit includes instructions for performing a test. In certain embodiments, the kit can also contain common reagents necessary for PGR such as polymerases, primers, dNTPs, buffers, salts, etc. Such reagents are known to persons of ordinary skill in the art.

EXAMPLES

The present invention is described by reference to the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Materials and Chemicals: Mineral oil and Brij L4® purchased from Sigma-Aldrich Corporation (St. Louis, MO) and ABIL EM90 obtained from Evonik Industries (Germany). Microfluidic chips and PGR wells were fabricated from Sylgard 184 PDMS. PGR master mix consisted of Taq polymerase (Bio-Rad, Hercules, CA), PGR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), magnesium chloride, an equimolar deoxynucleotide mix, and LC Green (BioFire Diagnostics, Inc., Salt Lake City, UT). pUC 19 plasmid (Thermo, Waltham, MA) was utilized as the DNA template for PGR. The plasmid was amplified using a forward primer with sequence ACA GAG TTC TTG AAG TGG TGG (SEQ ID NO. 1), and a reverse primer with sequence TGG TTT GTT TGC CGG GAT CAA (SEQ ID NO. 2), where were purchased from Integrated DNA Technologies (Coralville, LA).
EXAMPLE 2

Interfacial Tension Measurement: Interfacial tension measurements were taken using the pendant drop technique. Drop shape factors, $D_e$, taken as the largest width of the drop and, $D_s$, taken as the width of the drop at a distance of $D_s$ from the bottom of the drop as shown in FIG. 3, were empirically correlated to the interfacial tension $\gamma$ using Equation 1:

$$\gamma = 3.17g\Delta pD_e^{-0.68}D_s^{2.68},$$

where $g$ is the acceleration due to gravity, 9.8 m/s$^2$, and $\Delta p$ is the difference in density between the aqueous droplet and the oil phases. See A.W. Adamson, Physical Chemistry of Surfaces, 3rd ed., Wiley, New York, 1976.

Stock mineral oil solutions of 1.5% (w/w) AB1L EM90, 0.5% (w/w) Brij L4, and unaltered mineral oil were stored at room temperature. Aqueous solutions of Taq consisting of 20 mM Tris-HCl, 50 mM KCl, and 1.5 mM MgCl$_2$ were made the day of experiments and stored on ice. The concentration of Taq Pol in the aqueous droplet phase ranged from 0 to 8X, wherein 1X was 0.025 U/µL. Hanging aqueous droplets were formed in mineral oil solutions using 16, 18, or 26 gauge syringe needles to form the largest non-spherical droplets without pinching off. No difference was seen in $\gamma$ by using different sized syringe needles. Syringes were rinsed out thoroughly with ultrapure deionized water (18.2 MΩ) and dried with nitrogen between experiments. Mineral oil solutions were preheated and maintained at 55 °C with a water bath during all experiments to obtain measurements at relevant elevated PGR temperatures.

Interfacial tensions for each time point were calculated from 5 images of the pendant droplet using a Pulnix TM-1405GE CCD. See P.E. Rueger et al., Chem. Eng. Res. Des. All of the equipment was setup on a vibration isolation table to minimize droplet movement, with the exception of the CCD, which was stabilized on a tripod. Each experimental condition was
observed for at least 1 hour and conducted in triplicate. The maximum error in $\gamma$ at each time point was 0.1 dyne/cm, and the maximum error from drop to drop was 0.5 dyne/cm.

[0064] The adsorption of Taq Pol, ABIL EM90, and Brij L4® were studied indirectly by measuring the interfacial tension over time using the pendant drop technique. FIG. 4 depicts the interfacial tension (dyne/cm) as a function of time (minutes). In FIG. 4, the upward triangles represent an unaltered mineral oil and aqueous solution without Taq Pol (oil control), the downward triangles represent an unaltered mineral oil and IX Taq Pol (Taq control), the circles represent a 1.5% ABIL EM90 mineral oil and aqueous solution (ABIL control), and the squares represent a 0.5% Brij L4® mineral oil and aqueous solution (Brij control). The inset in FIG. 4 depicts the interfacial tension of the ABIL control and Brij control over the first ten minutes.

[0065] As depicted in FIG. 4, the interfacial tension of (i) unaltered mineral oil and aqueous solution without Taq Pol (oil control), (ii) 1.5% ABIL EM90 mineral oil and aqueous solution (ABIL control), and (iii) unaltered mineral oil and IX Taq Pol (Taq control) solution all decreased as a function of time. In contrast, the interfacial tension of 0.5% Brij L4® mineral oil and aqueous solution (Brij control) was constant over time.

[0066] The steady state interfacial tension of the oil control, $\gamma_0$, was equal to 36.4 ± 0.5 dyne/cm and was used as the reference for surface pressure, $\Pi$, which is equal to $\gamma_0 - \gamma$. Initially, $\gamma$ of the ABIL Control was 3.8 ± 0.7 dyne/cm and reached a steady state interfacial tension, $\gamma_{AO}$, of 2.17 ± 0.07 dyne/cm within 45 minutes. The Taq control was initially 15.3 ± 0.7 dyne/cm and decreased to a steady state of 9.6 ± 0.1 dyne/cm within 45 minutes. The steady state interfacial tension of the Brij control, $\gamma_{BO}$, was measured to be 1.82 ± 0.02 dyne/cm.

[0067] Adsorption dynamics of surfactants and Taq Pol at relevant PGR concentrations and time scales are not diffusion controlled. Diffusion kinetics are only valid at low surface
pressures, which were never observed on the timescale of the experiments disclosed herein. During droplet formation, a surfactant or protein film was formed within seconds to milliseconds, whereby the interfacial tension drastically decreased immediately. The initial surface pressure of the Taq Control was \(21.1 \pm 0.9\) dyne/cm. Over time the surface pressure increased to \(26.8 \pm 0.5\) dyne/cm, indicating that Taq irreversibly denatures and adsorbs to the surface. The initial surface pressure of the ABIL Control was \(32.6 \pm 0.9\) dyne/cm. In this case, the surface pressure increases to \(34.2 \pm 0.5\) dyne/cm, indicating that the interface was not completely saturated and ABIL EM90 continued to bind to open sites within the surfactant film. The surface pressure of the Brij control remained constant through the experiment at \(34.6 \pm 0.5\) dyne/cm. This signifies that the surface was saturated immediately, and binding sites within the film were not available.

[0068] FIG. 5 and FIG. 6 depict dynamic pseudo surface pressure measurements for 2 phase systems with surfactant and Taq are shown as the deviation from the steady state surface tension with surfactant and without Taq for 1.5% ABIL EM90 (\(\gamma_A^0 - \gamma\)) and 0.5% Brij L4® (\(\gamma_B^0 - \gamma\)), respectively. In FIG. 5 and FIG. 6, the circles represent solutions comprising 0.1X (0.0025 U/µL) Taq Pol, the squares represent solutions comprising 1.4X (0.035 U/µL) Taq Pol, and the triangles represent solutions comprising 8.0X (0.20 U/µL) Taq Pol. Initial pseudo surface pressures were negative for Taq concentrations less than 0.035 U/µL in 1.5 wt% ABIL EM90 but reached a stable plateau greater than or equal to zero within 45 minutes. At higher Taq concentrations in 1.5 wt% ABIL EM90, initial pseudo surface pressures were positive and also reached a steady state within 45 minutes. No change in pseudo surface pressures was seen in 0.5 wt% Brij L4® with all measured concentrations of Taq Pol and were stable over time, suggesting that the surface was immediately saturated, and no further adsorption occurred over time.
The kinetics of adsorption at liquid-liquid interfaces with significant excess surface concentrations of an amphiphile have been studied by Ward and Tordai. See A.F.H Ward and L. Tordai, Reel. Trav. Chim. Pays-Bas 1952, 71, 572-584. The Ward and Tordai rate equation is set forth in Equation II:

\[ \frac{dn}{dt} = k_v C e^{-\Pi \Delta A/kT} - k_v C^* e^{-\Pi \Delta A/kT}. \]

Equation II captures the energetic barrier of binding to a crowded interface in the case of adsorption limited kinetics where \( k_v \) and \( k_v \) are the adsorption and desorption rate constant, \( v \) is the number of adsorbing groups, \( C \) and \( C^* \) are the bulk and surface concentration of the species in the same units, \( \Delta A \) is the area created in the film to adsorb the species, and \( \Pi \Delta A \) is the work required to create space of size \( \Delta A \) in a film under surface pressure \( \Pi \).

When the adsorption of the species is irreversible, Equation II simplifies to Equation III:

\[ \ln(\frac{dn}{dt}) = \ln(k_v C) - \frac{\Pi \Delta A}{kT}. \]

FIG. 7 depicts the term \( \ln(\Pi/dt) \) for IX Taq and mineral oil as a function of \( \Pi \). In FIG. 7, the circles represent the dynamic interfacial measurements of the IX Taq and mineral oil solution. Linear portions in the curve with different slopes are representative of the different areas required for making a small space in the film for absorption and for making a larger space in the film for a protein to surface denature. See E. Tornberg, J. Colloid Interface Sri. 1978, 64, 391-402. In FIG. 7, the black line represents the initial linear portion of the curve at the lower surface pressure, which was fitted to Equation III where \( \Delta A \) was found to be \( 4 \pm 1 \text{ nm}^2 \) and corresponds to penetration of Taq into the film. This penetration area was comparable to that of \( \beta \)-lactoglobulin, a much smaller protein. The smaller penetration area relative to the size of Taq Pol was most likely due to its large hydrophobic content. Once a small portion of Taq Pol was bound, the entire protein interacted with the interface quickly. The linear portion at higher
surface pressures corresponds with the area required for denaturing and spreading of Taq in the film, where \( \Delta A \) was found to be \( 55 \pm 30 \) nm\(^2\). The area \( \beta \)-lactoglobulin required for molecular rearrangement was an order of magnitude smaller. A sizeable portion of Taq Pol is hydrophobic, which would have a larger footprint when denatured than proteins of similar or smaller size.

[0073] Since adsorption begins during droplet formation, it is desirable to block the hydrophobic surface immediately to prevent proteins from binding at the interface. Using ABIL EM90, the interface does not saturate quickly, which would allow protein adsorption to occur during droplet formation and well downstream of droplet formation. A cleared area of only \( 4 \pm 1 \) ran\(^2\) in the film would be required for Taq to penetrate and adsorb in the film, which is similar to the \( 2 \) nm\(^2\) required by BSA - a smaller protein. See E. Tornburg supra. Brij L4® would be a superior surfactant, minimizing protein adsorption during droplet formation, because it completely saturates the surface immediately.

[0074] Brij L4® was shown to be a better surfactant than ABIL EM90 to immediately block the interface by the steady state surface pressure measurements with Taq Pol in the system. FIG. 8 depicts the change of surface of pressure, \( \Pi \), with increasing Taq Pol concentration. In FIG. 8, the circles represent the surface pressure in dyne/cm as a function of the Taq Pol concentration (X) of the ABIL control. In FIG. 8, the squares represent the surface pressure in dyne/cm as a function of the Taq Pol concentration (X) of the Brij control. The initial positive slope of the ABIL EM90 curve of surface pressure with Taq Pol concentration indicated that upon droplet formation, the fresh interface adsorbed both Taq Pol and ABIL EM90, and therefore the initial amount of protein adsorbed increased as Taq concentration increased. It was not until 4X Taq Pol was in solution that the interface began to fully saturate at \( \Pi \) of \( 35.23 \pm 0.03 \) dyne/cm. In picoliter droplets, however, at a concentration of 4X Taq Pol, much of the enzyme would be
adsorbed and non-functional, and thus PCR could not be applied. In the case of Brij L4®, II remained constant at 34.59 ± 0.02 dyne/cm over all measured Taq concentrations, indicating significant protein adsorption was prevented. Initial Taq adsorption onto a fresh droplet interface was minimized by the immediate saturating surface coverage of Brij L4®. As a result, PCR would be expected to operate successfully, even at standard Taq concentrations.

[0075] **EXAMPLE 3**

[0076] **DROPLET PCR:** The PCR master mix consisted of 20 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTPs, 1X LC Green, and varying concentrations of Taq Pol. The initial concentration of pUC-19 was 5 x 10⁶ copies/μL. Wells were fabricated by creating holes in 2.5 mm thick cured PDMS using a 5 mm biopsy punch. Individual wells were separated and bonded to cover glass slips; bonding of the PDMS to glass was performed by spinning PDMS at 3000 rpm for 30 s on the glass to form a thin layer that served as an adhesive. Aqueous droplets 20 ± 2 μm in diameter (approximately 4 pL) were formed in the PDMS microfluidic chips using a flow focusing geometry and then captured in wells filled with mineral oil solutions. Care was taken to capture single droplets in well when using Brij L4® as the surfactant due to droplet coalescence during thermocycling. Wells were sealed with cover glass slips before thermocycling on an aluminum plate heated and cooled with a peltier.

[0077] Aqueous droplets were initially hot started at 95 °C for 3 minutes, then thermocycled for 35 cycles. Each thermocycle consisted of 30 seconds at 55 °C for annealing, 30 seconds at 72 °C for extension, and 30 seconds at 95 °C for melting. Real-time amplification results were obtained after every extension step by exciting droplets using a blue LED (Innovations in Optics, Woburn, MA) with a Brightline 424 - 438 nm bandpass filter, while capturing fluorescence emission with a CCD (Allied Vision, Stradtroda, Germany) fitted with a high pass filter (475 nm
The cycle threshold was defined as the cycle where the fluorescence during extension of a given cycle was greater than the fluorescence during the extension of the first cycle by 10 standard deviations.

[0078] Confirmation of Brij L4® as a superior surfactant for PGR was performed with 4 pL droplet thermocycling and real time fluorescence measurements. At the 4 pL droplet size, the number of initial copies of pUC-19 per droplet varies probabilistically but with an average of 25 initial plasmids. Therefore, the cycle threshold of real-time qPCR experiments would vary. Though initial plasmid copy numbers per droplet were low, fluorescence of droplets with LC Green at room temperature was clearly distinguishable from the background (though at 72 °C, the initial fluorescence was nearly indistinguishable from the background).

[0079] In each cycle of PGR, primers hybridize to template DNA to form a partially double stranded segment and are subsequently extended by Taq Pol. If the PGR is efficient, all bound primers are extended every cycle and the copies of the PGR product sequence defined by the primers (i.e., amplicons) roughly doubles every cycle. This is achieved if sufficient active Taq Pol is in solution to catalyze the extension of all bound primers. An excess of Taq Pol in solution would have no effect on the cycle threshold, whereas a depletion of Taq Pol would decrease the cycle efficiency of PGR and thus increase the cycle threshold.

[0080] FIG. 9 depicts the cycle threshold as a function of Taq Pol concentration. In FIG. 9, the circle represents a PGR mixture comprising 1.5% ABIL EM90 and the square represents a PGR mixture comprising 0.5% Brij L4®. As illustrated in FIG. 9, the PGR cycle efficiency of 0.5% Brij L4® was drastically better than the PGR cycle efficiency of 1.5 wt% ABIL EM90 in picoliter droplets. Using 1.5% ABIL EM90 and 8X Taq Pol, 16 ± 2 cycles were necessary to reach the fluorescence threshold. Blocking the hydrophobic interface with 0.5% Brij L4®
resulted in much more efficient PGR, with a cycle threshold of $7 \pm 1.5$ cycles at the same Taq Pol and initial pUC-19 concentration. While sufficient Taq Pol was in solution with ABIL EM90 to amplify DNA, much more was available using Brij 14®. Furthermore, using less than 8X Taq Pol with ABIL EM90 resulted in Taq Pol concentrations below a critical level sufficient to perform PGR. Efficient PGR was achieved with Taq Pol concentrations as low as 2X with Brij L4® with a cycle threshold of $7 \pm 1.5$ cycles. Though an efficient number of cycles were necessary to reach a fluorescence threshold using Brij L4®, copies of the DNA sequence defined by the primers would increase by a factor of approximately 60 if all bound primers were extended. DNA amplification was still achieved using 1X Taq Pol with 0.5% Brij L4®, 21 ± 5 cycles were necessary to reach the fluorescence threshold. The sharp increase indicates that in fact a fraction of Taq Pol is absorbed during droplet formation, but that using a concentration of 2X Taq Pol is sufficient to completely overcome this lost Taq Pol.

[0081] Successful PGR with Brij L4® also confirms that surfactant was not completely degraded during thermocycling. Although, droplet coalescence was observed when using Brij L4®, this would only be an issue in PGR emulsion experiments, and it could be circumvented by using a combination of ABIL EM90 and Brij L4®. With this combination, the interface would still be saturated immediately. The majority of the film would be composed of Brij 1.4® and Taq Pol adsorption would be minimized; ABIL EM90 would make up a lesser portion but still prevent droplet coalescence.

[0082] The surfactant Brij L4® was shown to be a surprisingly superior surfactant than conventional surfactants used in droplet or emulsion PGR, such as ABIL EM90. As little as 1X Taq Pol was used for amplification of droplet PGR mixtures with Brij L4®, which was much less than the concentration of Taq Pol required for amplification of droplet PGR mixtures with
conventional surfactants. It was observed that Brij L4® immediately saturates the interface, and therefore, minimizes Taq Pol adsorption during droplet formation.

[0083] While various embodiments have been described above, it should be understood that they have been presented by way of example only, and not limitation. Thus, the breadth and scope of the present disclosure should not be limited by any of the above-described exemplary embodiments. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the disclosure unless otherwise indicated herein or otherwise clearly contradicted by context.

[0084] Additionally, while the methods described above and illustrated in the drawings are shown as a sequence of steps, this was done solely for the sake of illustration. Accordingly, it is contemplated that some steps may be added, some steps may be omitted, the order of steps may be re-arranged, and some steps may be performed in parallel.
We claim:

1. A droplet or emulsion polymerase chain reaction (PCR) mixture comprising:
   an aqueous droplet comprising a polymerase chain reaction mixture;
   an oil phase; and
   a surfactant having Formula I: \[ R - (0 \text{C}n\text{H}_{2})n - \text{OH}, \]
   wherein \( R \) is an alkyl group consisting of 12 to 18 carbons and \( n \) is 2 to 25.

2. The droplet or emulsion PCR mixture of claim 1, wherein the polymerase chain reaction mixture comprises a polymerase, a primer, nucleotides, and a template nucleic acid.

3. The droplet or emulsion PCR mixture of claim 1, wherein the surfactant is selected from the group consisting of laureth-4, laureth-23, ceteth-2, ceteth-20, ceteareth-12, ceteareth-20, ceteareth-25, steareth-2, steareth-10, steareth-20, steareth-21, oleth-2, oleth-5, oleth-10, and oleth-20.

4. The droplet or emulsion PCR mixture of claim 3, wherein the surfactant is laureth-4.

5. The droplet or emulsion PCR mixture of claim 2, wherein the polymerase is Taq polymerase.

6. The droplet or emulsion PCR mixture of claim 1, wherein the oil phase comprises mineral oil.
7. The droplet or emulsion PGR mixture of claim 1, wherein the aqueous droplet is surrounded by the oil phase and the surfactant,

8. A method for preparing a mixture for droplet or emulsion polymerase chain reaction ("PGR") comprising:
   forming an aqueous droplet comprising a polymerase chain reaction mixture;
   surrounding the aqueous droplet with an oil phase;
   adding a surfactant having Formula 1: \( R - (\text{OCH}_2\text{CH}_2)_n - \text{OH} \),
wherein \( R \) is an alkyl group consisting of 12 to 18 carbons and \( n \) is 2 to 25.

9. The method of claim 8, wherein the polymerase chain reaction mixture comprises a polymerase, a primer, nucleotides, and a template nucleic acid.

10. The method of claim 9, wherein the surfactant is selected from the group consisting of laureth-4, laureth-23, ceteth-2, ceteth-20, ceteareth-12, ceteareth-20, ceteareth-25, steareth-2, steareth-10, steareth-20, \textbf{steareth-21}, oleth-2, oleth-5, oleth-10, and oleth-20.

11. The method of claim 10, wherein the surfactant is laureth-4.

12. The method of claim 9, wherein the polymerase is Taq polymerase.

13. The method of claim 8, wherein the oil phase comprises mineral oil.
14. The method of claim 8, wherein the surfactant is added to the oil phase,

15. The method of claim 8, wherein the surfactant is added to the oil phase before aqueous droplet is surrounded by the oil phase.

16. The method of claim 8, wherein the surfactant is added after the aqueous phase is surrounded by the oil phase.

17. A method for determining the capability of a surfactant for droplet or emulsion polymerase chain reaction ("PGR") comprising:

   preparing an aqueous solution comprising polymerase;
   preparing an oil phase comprising a surfactant;
   forming a hanging drop of the aqueous droplet in the oil phase; and
   measuring a property of the hanging drop.

18. The method of claim 17, wherein the property is the interfacial tension of the hanging drop.

19. The method of claim 18, wherein the interfacial tension is determined using the following equation: \( \gamma = 3.17gA\rho O_e^{0.68} D_s^{2.68} \), wherein \( D_e \) is the largest width of the drop, \( D_s \) is the width of the drop at the distance \( D_e \), \( g \) is the acceleration due to gravity, 9.8 m/s\(^2\), and \( \Delta \rho \) is the difference in density between the aqueous droplet and the oil phase.

20. The method of claim 15, wherein the oil phase comprises mineral oil.
21. The method of claim 17, wherein the aqueous droplet further comprises a primer, nucleotides, and a template nucleic acid.

22. The method of claim 17, wherein the measurement of the property is used to determine an appropriate surfactant for use with droplet or emulsion PGR.

23. The method of claim 19, wherein the interfacial tension is less than 2.17 dyne/cm.

24. The method of claim 17, wherein the polymerase is Taq polymerase.
Forming an aqueous droplet comprising a polymerase chain reaction mixture

Surrounding the aqueous droplet with an oil phase

Adding a surfactant having Formula I: R-(OCH₂CH₂)ₙ-OH, wherein R is an alkyl group consisting of 12 to 18 carbons and n is 2 to 25

FIG. 1
Preparation Flowchart:

1. Preparing an aqueous solution comprising polymerase

2. Preparing an oil phase comprising a surfactant

3. Forming a hanging drop of the aqueous droplet in the oil phase

4. Measuring a property of the hanging drop

FIG. 2
FIG. 4
FIG. 5
FIG. 6
FIG. 7
FIG. 8
FIG. 9
AClassification of Subject Matter

<table>
<thead>
<tr>
<th>IPC(8)</th>
<th>CPC</th>
</tr>
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<tbody>
<tr>
<td>C12Q 1/68, C12P 19/34 (2015.01)</td>
<td>C12Q 1/6883, C12Q 1/686</td>
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</table>

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)

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<td>C12Q 1/68, C12P 19/34 (2015.01)</td>
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</tbody>
</table>

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

USPC- 435/6.1, 435/91.2 (keyword search, terms below)

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

PatBase (PGPB, USPT, USOC, EPAB, JPAB, DWPI, TDBD), FreePatentsOnline (US Pat, PgPub, EPO, JPO, WIPO, NPL), GoogleScholar (PL, NPL); search terms: polymerase chain reaction droplet PCR oil phase surfactant laureth ceteth cetearth steareth oleth method device system emulsion per Taq polymerase laureth-4 mineral oil

C. Documents Considered to be Relevant

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tbody>
<tr>
<td>Y</td>
<td>US 2010/01/73394 A1 (Colston et al.) 8 July 2010 (08.07.2010) Abstract, para [0145]-[0147], [0166], [0216], [0820].</td>
<td>1-16</td>
</tr>
<tr>
<td>Y</td>
<td>US 5,783,525 A (Blanco et al.) 21 June 1998 (21.06.1998) col 1, in 55-67, claim 18</td>
<td>1-16</td>
</tr>
<tr>
<td>Y</td>
<td>US 201/0076751 A1 (Fabis et al.) 31 March 2011 (31.03.2011) para [0008], [0010], [0013], [0048]</td>
<td>1-16</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C.

* 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: 13 April 2015 (13.04.2015)
Date of mailing of the international search report: 15 May 2015

Name and mailing address of the ISA/US
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Facsimile No. 571-273-8300

Authorized officer: Lee W. Young
PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774
INTERNATIONAL SEARCH REPORT

Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
See extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☑ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos. 1-16

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)
Continuation of Box III:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-16, directed to a droplet or emulsion polymerase chain reaction (PCR) mixture and method of preparing said composition.

Group II, claims 17-24, directed to a method for determining the capability of a surfactant for droplet or emulsion polymerase chain reaction

The inventions listed as Group I-II do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

The special technical feature of Group I is a method for preparing a mixture for droplet or emulsion polymerase chain reaction, not required in Group II.

The special technical feature of Group II is a method for determining the capability of a surfactant for droplet or emulsion polymerase chain reaction, not required in Group I.

Common Technical Features

Groups I and II share the technical feature of a droplet or emulsion polymerase chain reaction (PCR) mixture comprising: an aqueous droplet comprising a polymerase chain reaction mixture; an oil phase; and a surfactant. However, this technical feature is not a unifying technical feature as being anticipated by US 2010/0173394 A1 to Colston, Jr., et al. (hereinafter Colston, Jr.), Colston, Jr. discloses a droplet or emulsion polymerase chain reaction (PCR) mixture (Abstract, para [0145]-[0147]: . . . a PCR-stable emulsion, which is an emulsion that resists coalescence throughout the thermal cycling of PCR . . . ) comprising: an aqueous droplet comprising a polymerase chain reaction mixture (para [0145]: water-in-oil (W/O) emulsion; [0164]: droplet-based assays disclosed herein may include the polymerase chain reaction (PCR); para [0396]: . . . an emulsion including droplets disposed in a carrier fluid, each droplet containing a partition of a sample prepared as a reaction mixture for amplification of a nucleic acid target . . . ); an oil phase (para [0139]: . . . the droplets are suspended in an immiscible carrier fluid, such as oil, to form an emulsion); and a surfactant having (para [0150]: a surfactant comprising TWEEN).

As said technical feature was known in the art at the time of the invention, this cannot be considered special technical feature that would otherwise unify the groups.

Therefore, Groups I and II lack unity under PCT Rule 13.