



(51) International Patent Classification:

C12M 1/00 (2006.01)

C12Q 1/68 (2018.01)

C12Q 1/00 (2006.01)

(21) International Application Number:

PCT/US2019/023234

(22) International Filing Date:

20 March 2019 (20.03.2019)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/645,449

20 March 2018 (20.03.2018)

US

(71) Applicants: **GODX, INC.** [US/US]; 510 Charmany Drive, Suite 257 & 265, Madison, WI 53719 (US). **THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH & HUMAN SERVICES** [US/US]; 6011 Executive Blvd., Suite 325, MSC 7660, Bethesda, MD 20892-7660 (US).

(72) Inventors: **KIM, Chang, Hee**; 849 Walter Run, Waunakee, WI 53597 (US). **XIANG, Lichen**; 6433 Rock Forest Dr., Apt. 114, Bethesda, MD 20817 (US). **HENDERSON, Wendy, A.**; 5612 Southwick St., Bethesda, MD 20817 (US). **JIANG, Xiao**; 7634 Mid Town Rd. Apt. 106, Madison, WI 53719 (US).

(74) Agent: **GULMEN, Tolga, S.**; Quarles & Brady LLP, 411 East Wisconsin Avenue, Milwaukee, WI 53202 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

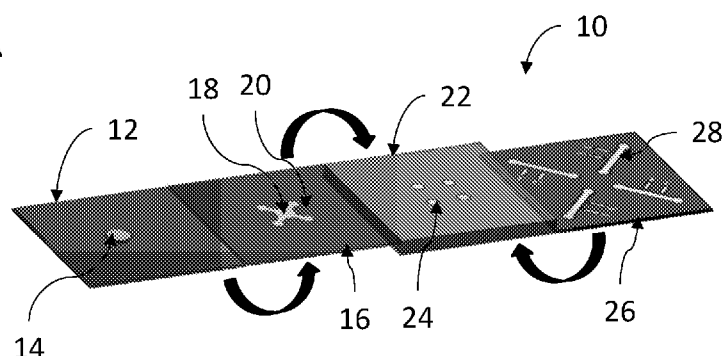
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU,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

Published:

- with international search report (Art. 21(3))
- 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))

(54) Title: INSTRUMENTATION-FREE PAPER ORIGAMIRAPID MOLECULAR DIAGNOSTIC DEVICE

Fig. 1A



(57) Abstract: Described herein are microfluidic devices for rapidly amplifying and detecting nucleic acids suitable for point-of-need (PON) or point-of-care (POC) portable settings. The device comprising a sample loading panel, the sample loading panel comprising a sample loading area, and one or more amplification panels, the amplification panel comprising an amplification reaction area; wherein each of the panels comprises an absorbent material configured to wick an aqueous sample across a superimposed stack of the panels. Also disclosed are methods of amplifying and detecting target nucleic acids.

INSTRUMENTATION-FREE PAPER ORIGAMI RAPID MOLECULAR DIAGNOSTIC DEVICE

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. Provisional Patent Application No.
5 62/645,449 that was filed March 20, 2018, the entire contents of which are hereby incorporated by
reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was created in the performance of a Cooperative Research and Development
Agreement with the National Institutes of Health, an Agency of the Department of Health and
10 Human Services and with government support under R44TR001912 awarded by National
Institutes of Health and under 1ZIANR000018 awarded by the National Institutes of Health. The
Government of the United States has certain rights in this invention.

REFERENCE TO A SEQUENCE LISTING

This application is being filed electronically via EFS-Web and includes an electronically
15 submitted Sequence Listing in .txt format. The .txt file contains a sequence listing entitled
"165369.00003_Seq_Listing.txt" created on March 20, 2019 and is 10,340 bytes in size. The
Sequence Listing contained in this .txt file is part of the specification and is hereby incorporated
by reference herein in its entirety.

BACKGROUND OF THE INVENTION

20 For point-of-need (PON) medical testing, sample preparation and detection must have
rapid turnaround time, be easy to operate, be easy to port to the point of need, and be low-cost.
PON devices bring diagnostic testing close to the healthcare providers so they can treat patients in
real time. Nitrocellulose-based lateral flow rapid test strips are widely used for testing a variety of
diseases and biomarkers, such as the pregnancy test strip. Multiplex lateral flow test strips have
25 also been developed as to immobilize several biological probes specific to different biomarkers
and cell types. Paper-based microfluidic analytical pads or microPADS have been described in a
2-D or 3-D configuration to detect analytes such as proteins, sugars, nucleic acids and other
metabolites. Such pads have been created using photolithography or wax ink printing. Specifically,

in the latter case, a wax printer was used to produce patterns of microfluidic channels within hydrophobic wax ink printed areas on paper. The usefulness of PON devices is sometimes limited by the limits of detection. Thus, there is a need for developing PON devices that are not only rapid, easy to operate, easy to port, and low cost but also capable of detecting analytes at low concentrations.

BRIEF SUMMARY OF THE INVENTION

Described herein are microfluidic devices for rapidly amplifying and detecting nucleic acids suitable for point-of-need (PON) or point-of-care (POC) portable settings. One aspect of the invention is a microfluidic amplification device for amplifying a target nucleic acid sequence in a biological sample. The amplification device comprises a sample loading panel and one or more amplification panels. The sample loading pane comprises a sample loading area. The one or more amplification panels comprise an amplification reaction area. Each of the panels of the amplification device comprise an absorbent material configured to wick an aqueous sample across a superimposed stack of the panels.

In some embodiments, the amplification reaction area may comprise an amplification medium therein. The amplification medium may comprise primers specific for the target nucleic acid sequence. Suitably, the amplification reaction area has a soaking volume effective in retaining the amplification medium and a biological sample contained therein.

In some embodiments, the device may further comprise a control panel. The control panel may comprise a control volume extending from the amplification reaction area. In some embodiments, the amplification reaction area and the control volume together have a soaking volume effective in retaining the amplification medium and a biological sample contained therein.

In some embodiments, the amplification device comprises more than one amplification area. Suitably the amplification device may further comprise a distribution panel. The distribution panel may comprise a sample loading hub and distribution arms extending from the sample loading hub to each of the amplification reaction areas. When the device comprises more than one amplification area, each of the different amplification areas may have the same amplification medium or different amplification mediums contained therein. Suitably, the different amplification mediums may comprise different primers specific for different target nucleic acid sequences.

Another aspect of the invention is a device for detecting the presence of a target nucleic acid sequence in a sample. The device comprises any of the amplification devices described herein

and further comprising a diagnostic device. Preferably, the diagnostic device comprises a detectably labelled probe specific for the target nucleic acid sequence. Suitably, the diagnostic device comprises at least one lateral flow device. In some embodiments, the amplification device further comprises a diagnostic panel comprising the diagnostic device. In other embodiments, the diagnostic device comprises a cassette housing the lateral flow device.

The lateral flow device may comprise a diagnostic loading area positioned at one end of the lateral flow device. The lateral flow device may also comprise an area comprising the detectably labelled probe specific for the target nucleic acid sequence, wherein said detectably labelled probe is not bound to the lateral flow device and is capable of wicking across at least a portion of the lateral flow device. The lateral flow device may also comprise an area comprising a capture probe for the target nucleic acid sequence, wherein said capture probe for the target nucleic acid sequence is immobilized on the lateral flow device. The lateral flow device may also comprise an absorbent material, wherein the absorbent material wicks the biological sample across the lateral flow device when the aqueous sample is added to the diagnostic loading area. The lateral flow device may also comprise an area comprising a second capture probe for a control nucleic acid sequence, wherein said control nucleic acid sequence is complementary to a sequence of the probe specific for the target nucleic acid sequence, and wherein said second capture probe for a control nucleic acid sequence is attached to the lateral flow device. Suitably, the detectably labelled probe specific for the target nucleic acid sequence is labeled with a moiety selected from a gold nanoparticle, a protein binding ligand, a hapten, an antigen, a fluorescent compound, a dye, a radioactive isotope and an enzyme.

In some embodiments, the target nucleic acid sequence is indicative of a pathogen or a cell present in the sample. Another aspect of the invention comprises a method for amplifying a target nucleic acid in a sample. The method comprising providing any of the amplification devices described herein, adding a lysed sample to the sample loading area; heating the amplification reaction area to an effective temperature for an effective amount of time to produce amplicons of the target nucleic acid sequence. The method may further comprise adding an amplification medium to the amplification reaction area and/or adding a washing buffer medium to the sample loading area after the amplification reaction area is heated.

In some embodiments adding the lysed sample to the sample loading area comprises contacting a sample lysate with a plurality of buoyant, inorganic, nucleic-acid-capture

microspheres; contacting the sample loading area with the plurality of buoyant, inorganic, nucleic-acid-capture microspheres; and adding an eluent to the sample loading area having the plurality of buoyant, inorganic, nucleic-acid-capture microspheres absorbed thereon.

Another aspect of the invention is a method for detecting a target nucleic acid in a biological sample. The method comprises providing any of the devices described herein. The method may further comprise adding a lysed sample to the sample loading area; heating the amplification reaction area to an effective temperature for an effective amount of time to produce amplicons of the target nucleic acid sequence; and detecting the presence of the target nucleic acid sequence. Suitably the presence of the target nucleic acid sequence is detected by a trimolecular hybridization of the target nucleic acid sequence or an amplicon thereof. The method may further comprise adding an amplification medium to the amplification reaction area and/or adding a washing medium to the sample loading area after the amplification reaction area is heated.

BRIEF DESCRIPTION OF THE DRAWINGS

Non-limiting embodiments of the present invention will be described by way of example with reference to the accompanying figures, which are schematic and are not intended to be drawn to scale. In the figures, each identical or nearly identical component illustrated is typically represented by a single numeral. For purposes of clarity, not every component is labeled in every figure, nor is every component of each embodiment of the invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention.

Figures 1A-1C illustrate an exemplary microfluidic device. Fig. 1A illustrates an extended microfluidic device. Fig. 1B illustrates the microfluidic device of Fig. 1A in a partial folded state. Fig. 1C illustrates the microfluidic device of Fig. 1C in a superimposed stack of panels.

Figure 2 illustrates an exemplary lateral flow device.

Figures 3A-3D illustrate an exemplary microfluidic device and diagnostic device. Fig. 3A illustrates an extended microfluidic device. Fig. 3B illustrates a diagnostic device for use with the microfluidic device. Fig. 3C illustrates the microfluidic device of Fig. 3A in a superimposed stack associated with the diagnostic device of Fig. 3B. Fig. 3D illustrates a cross-sectional view of the microfluidic device of Fig. 3A in a superimposed stack associated with the diagnostic device of Fig. 3C.

Figure 4 illustrates the use of microspheres for the detection of nucleic acids obtained from a sample.

Figures 5 illustrates schematically the use of microspheres for the separation of nucleic acids by absorbing the lysate particulate phase.

DETAILED DESCRIPTION OF THE INVENTION

Described herein are microfluidic devices for rapidly amplifying and detecting nucleic acids suitable for point-of-need (PON) or point-of-care (POC) portable settings. These microfluidic devices are rapid, easy to operate, easy to port, low cost, and, also, integrate an amplification reaction that can boost the copies of a target nucleic acid sequence. This allows for the limits of detection to be increased.

The present invention is capable of amplifying and/or detecting a multiplex of nucleic acids from a biological sample using a three-dimensional, folded, paper-based microfluidic device with channels created by hydrophobic and hydrophilic patterns. The device may be readily used in the PON or POC. A sample may be obtained by an individual, a patient, a nurse, a technician, a doctor, or a healthcare provider and the test performed on-site by lysing the sample, adding the lysed sample comprising the target DNA or RNA onto the sample loading area of the microfluidic device, amplifying target DNA or RNA within the microfluidic device, and reading results, such as visible dots, lines, or other shaped patterns using colorimetric methods (*e.g.*, gold nanoparticles, horseradish peroxidase or other nanoparticles, read by eye or a scanner or a smartphone scanner), or fluorescent methods (*e.g.*, SYBR green read with a portable long wave UV light source or a fluorescent reader) without the need for expensive or scarce instrumentation. The entire DNA or RNA collection, amplification, and detection protocol can be finished rapidly without using any professional laboratory instrumentation, such as centrifuges or refrigeration, which will greatly improve the cost of sample preparation in both time and economical ways.

Suitably the devices and methods described herein are capable of detecting target nucleic acids in a sample at a concentration of 1 pg/mL or greater, such as 5, 10, 50, or 100 pg/mL.

In addition to the ease of use, the microfluidic devices are easy to prepare without the need for specialized facilities or a clean room. The devices may be prepared by patterning a hydrophobic sealing material onto a panel, which may also be referred to as a layer, of absorbent material such that the hydrophobic sealing material diffuses through the absorbent material, thereby forming hydrophilic area capable of wicking aqueous materials laterally along the long dimensions of a panel or layer or vertically through the short dimension of the panel or layer. This allows for the aqueous sample to wick across a superimposed stack of the panels.

The absorbent material which wicks an aqueous sample across the lateral flow device may comprise cellulose. Suitably the cellulose is selected from filter paper, chromatographic paper, nitrocellulose, and/or cellulose acetate. As used herein, a material that “wicks” an aqueous sample refers to any structure, material, and/or device, etc., that permits movement and/or transportation of an aqueous sample and at least some of its contents, or that permits the aqueous sample to migrate through one or more panels or diagnostic devices.

Suitably the microfluidic devices may be prepared by patterning the absorbent material with a wax printer. To prepare the device, the absorbent material may be printed with a wax using a printer then heated to a sufficient temperature for a sufficient time to melt the printed wax, which diffuses through the absorbent to form hydrophobic and hydrophilic patterns of channels on both sides of the panel and the form a hydrophobic solid ink channel across the entire thickness of the absorbent. For example, the device may be prepared by printing a black wax onto an absorbent material, such as filter paper, chromatographic paper, nitrocellulose, or cellulose acetate, using a solid-ink printer and then heating the absorbent material at 100 °C for about 2 min in an oven to melt the printed wax. The hydrophobic areas block the migration of an aqueous sample, resulting in the controllable flow of the aqueous sample through the hydrophilic areas.

The patterned absorbent material comprising hydrophobic and hydrophilic areas may be segmented into superimposable panels. As used herein, a "panel" means a separate or distinct part of a surface. Suitably some or all of the panels are flexibly interconnected such that panels may be folded into a superimposed stack of the panels. When the panels are superimposed, adjacent panels may allow for fluid communication where hydrophilic areas of the respective panels are in contact when the panels are superimposed. As a result, three-dimensional microfluidic devices may be prepared, including three-dimensional microfluidic devices having areas or chambers of controlled volume spanning more than one panel.

The microfluidic devices described herein may be referred to as a "microfluidic amplification device for amplifying a target nucleic acid sequence in a biological sample" or, simply "amplification device", for (i) microfluidic devices comprising one or more sample handling panels (e.g., sample loading, distribution, and/or control panels), one or more amplification panels, and a diagnostic panel but excluding the diagnostic panel or (ii) microfluidic devices comprising one or more sample handling panels and one or more amplification panels but lacking a diagnostic panel. The microfluidic devices described herein may be referred to as a

"device for detecting the presence of a target nucleic acid sequence in a sample" for (i) microfluidic devices comprising one or more sample handling panels (e.g., sample loading, distribution, and/or control panels), one or more amplification panels, and a diagnostic panel and including the diagnostic panel or (ii) microfluidic devices comprising one or more sample handling panels and
 5 one or more amplification panels in combination with a diagnostic device, such as a lateral flow device or a cassette comprising a lateral flow device.

An exemplary microfluidic device is illustrated in Figs. 1A-1C from an extended microfluidic device (Fig. 1A), through partial folding of the device (Fig. 1B), and ending into a superimposed stack of panels (Fig. 1C). When the panels are superimposed, each of the panels are
 10 in fluid communication from sample loading through detection.

The device 10 comprises a sample loading panel 12 comprising a sample loading area 14. The sample loading panel 12 and sample loading area 14 are configured to receive a sample. The sample loading area 14 is a hydrophilic area suitable for depositing an aqueous sample. The sample loading area may comprise a material that traps debris. The debris may comprise a component of
 15 a lysed or unlysed biological sample, an eluate, or a microsphere. In some embodiments, the material comprises glass fiber. In some embodiments, the material comprises polyester and/or cellulose. In some embodiments, the material that traps debris is any commercially available microporous material. As used herein, "traps" or "trapping" refers to immobilizing, delaying movement, capturing (temporarily or permanently), impeding movement, or hindering movement.
 20 As used herein, "debris" means any particulate matter other than the components of the disclosed assays or devices. In some embodiments, "debris" includes tissue, food particles, clumped cells, cell walls, microspheres, and the like.

As used herein, a "sample" is a substance that comprises or may comprise nucleic acids. The sample may be a biological sample obtained from a subject. Suitably, the biological sample
 25 may comprise peripheral blood, sera, plasma, ascites, urine, cerebrospinal fluid (CSF), sputum, saliva, bone marrow, synovial fluid, aqueous humor, amniotic fluid, cerumen, breast milk, bronchoalveolar lavage fluid, semen, prostatic fluid, cowper's fluid or pre-ejaculatory fluid, female ejaculate, sweat, fecal matter, hair, tears, cyst fluid, pleural and peritoneal fluid, pericardial fluid, lymph, chyme, chyle, bile, interstitial fluid, menses, pus, sebum, vomit, vaginal secretions,
 30 mammary secretions, mucosal secretion, stool, stool water, pancreatic juice, lavage fluids from sinus cavities, bronchopulmonary aspirates, breath or expiratory aerosolized matter, blastocyl

cavity fluid, and umbilical cord blood. In some embodiments, the sample is a gastrointestinal fluid. In some embodiments, the biological sample is stool. In some embodiments, the biological sample is selected from a skin swab sample, a throat swab sample, a genital swab sample and an anal swab sample.

5 As used herein, “nucleic acids” mean unmodified or modified DNA or unmodified or modified RNA. The DNA may be genomic DNA (e.g., DNA encoding a protein, open reading frames, or regulatory sequences), mitochondrial DNA, extracellular DNA, plasmid DNA, or cell-free fetal DNA. The RNA may be involved in protein synthesis, involved in post-transcriptional modification, DNA replication, or regulation. RNAs involved in protein synthesis may include, without limitation, mRNAs, rRNAs, tRNAs, or SRP RNAs. RNAs involved in post-transcriptional
10 modification may include, without limitation, snRNAs, snoRNAs, or Y RNAs. Regulatory RNAs may include, without limitation, antisense RNAs, CRISPR RNAs, guide RNAs, long noncoding RNAs, microRNAs, siRNAs, piRNAs, tasiRNAs, 5'UTR sequences, 3'UTR sequences, RNA splicing regulatory sequences, IRES sequences, or polyA signal sequences.

15 In some embodiments, the sample is a tissue sample such as a biopsy sample. The tissue sample may comprise cells indicative of a disease or condition. Exemplary diseases include cancers, such as bladder, breast, colorectal, kidney, lung, lymphoma, melanoma, oral or oropharyngeal, pancreatic, prostate, thyroid, or uterine cancer.

 In other embodiments, the sample may be an environmental sample from a source other
20 than a subject. Suitably, the environmental sample may be a water sample such as from a drinking or cooking water source. Such drinking or cooking water sources include, without limitation, municipal water sources, wells, lakes, rivers, or reservoirs. In other embodiments, the environmental sample may be a food sample or other consumable sample. In other embodiments, the environmental sample is a surface sample such as may be obtained from swabbing a surface or
25 gathering air or aerosol samples.

 Suitably the sample may be sample lysate. As used herein, a “sample lysate” comprises the material formed by the lysis of cells, including nucleic acids and other biomolecules such as proteins, lipids, or carbohydrates. The sample lysate may further comprise one or more of the following: a lysis or denaturing agent, a nucleic acid preservation agent, a buffering agent, and a
30 solvent. Combinations of a lysis or denaturing agent, a nucleic acid preservation agent, a buffering agent, and a solvent may be referred to a “lysis buffer” or “lysis medium”.

As used herein, a “lysis or denaturing agent” is a composition capable of breaking down or disrupting a cellular membrane. The lysis or denaturing agent may be a chaotropic salt, a lytic enzyme, a detergent, or any combination thereof. Suitably, the lysis or denaturing agent is present in an amount sufficient to break down or disrupt cellular membranes.

In some embodiments, the chaotropic salt is selected from guanidium thiocyanate, alkali metal perchlorates, alkali metal iodides, alkali metal trifluoroacetates, alkali metal trichloroacetates, and alkali metal thiocyanates. In some embodiments, the chaotropic salt is selected from urea, guanidine HCl, guanidinium thiocyanate, guanidium thiosulfate, thiourea, or any combination thereof. In some embodiments, the lysis or denaturing agent is a lytic enzyme.

In some embodiments, the lytic enzyme is selected from the group consisting of beta glucuronidase, glucanase, glucosylase, lysozyme, lyticase, mannanase, mutanolysin, zymolase, cellulase, lysostaphin, pectolyase, streptolysin O, and various combinations thereof.

In some embodiments, the lysis or denaturing agent is a detergent. In some embodiments, the detergent is Tween. In some embodiments, the detergent is selected from 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, octyl- β -thioglucopyranoside, octyl-glucopyranoside, 3-(4-heptyl) phenyl 3-hydroxy propyl dimethylammonio propane sulfonate, 3-[N,N-dimethyl(3-myristoylamino)propyl]ammonio]propanesulfonate, 3-(decyldimethylammonio)propanesulfonate inner salt, 3-(dodecyldimethylammonio)propanesulfonate inner salt, 3-(N,N-dimethylmyristylammonio)propanesulfonate, n-dodecyl α -D-maltoside and combinations thereof.

As used herein, a “nucleic acid preservation agent” is a composition capable of retarding the degradation of nucleic acids in the sample lysate. Nucleic acid preservation agents often act through the inhibition of nucleases. The nucleic acid preservation agent may be an enzyme inhibitor, a metabolic inhibitor, or any combination thereof. The one or more nucleic acid preservation agent may include a formaldehyde releaser such as one selected from the group consisting of: diazolidinyl urea, imidazolidinyl urea, dimethoyl-5,5-dimethylhydantoin, dimethylol urea, 2-bromo-2-nitropropane-1,3-diol, oxazolidines, sodium hydroxymethyl glycinate, 5-hydroxymethoxymethyl-1-laza-3,7-dioxabicyclo [3.3.0]octane, 5-hydroxymethyl-1-laza-3,7-dioxabicyclo[3.3.0]octane, 5-hydroxypoly[methyleneoxy]methyl-1-laza-3,7-dioxabicyclo[3.3.0]octane, quaternary adamantine and any combination thereof. The one or more enzyme inhibitors may be selected from the group consisting of: diethyl pyrocarbonate,

ethanol, aurintricarboxylic acid (ATA), glyceraldehydes, sodium fluoride, ethylenediamine tetraacetic acid (EDTA), formamide, vanadyl-ribonucleoside complexes, macaloid, heparin, hydroxylamine-oxygen-cupric ion, bentonite, ammonium sulfate, dithiothreitol (DTT), beta-mercaptoethanol, cysteine, dithioerythritol, tris(2-carboxyethyl)phosphene hydrochloride, a
 5 divalent cation such as Mg^{+2} , Mn^{+2} , Zn^{+2} , Fe^{+2} , Ca^{+2} , Cu^{+2} , and a chaotropic salt such as guanidinium thiocyanate, and any combination thereof. The one or more metabolic inhibitors may be selected from the group consisting of: glyceraldehyde, dihydroxyacetone phosphate, glyceraldehyde 3-phosphate, 1,3-bisphosphoglycerate, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, pyruvate and glycerate dihydroxyacetate, sodium fluoride, $K_2C_2O_4$ and any
 10 combination thereof. Suitably, the nucleic acid preservation agent is present in an amount sufficient to retard the degradation of nucleic acids in the sample lysate.

Buffering agents may include one or more of the following: N-(2-acetamido)-aminoethanesulfonic acid; Cary-Blair medium; acetate; N-(2-acetamido)-iminodiacetic acid; 2-aminoethanesulfonic acid; ammonia; 2-amino-2-methyl-1-propanol; 2-amino-2-methyl-1,3-propanediol; N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid; N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid; carbonate; N,N'-bis(2-hydroxyethyl)-glycine; [bis-(2-hydroxyethyl)-imino]-tris-(hydroxymethylmethane); 1,3-bis[tris(hydroxymethyl)-methylamino]propane; boric acid; dimethylarsinic acid; 3-(cyclohexylamino)-propanesulfonic acid; 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid; cyclohexylaminoethanesulfonic
 15 acid; citrate; 3-[N-bis(hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; formate; glycine; glycyglycine; N-(2-hydroxyethyl)-piperazine-N'-ethanesulfonic acid; N-(2-hydroxyethyl)-piperazine-N'-3-propanesulfonic acid; N-(2-hydroxyethyl)-piperazine-N'-2-hydroxypropanesulfonic acid; imidazole; malate; maleate; 2-(N-morpholino)-ethanesulfonic acid; 3-(N-morpholino)-propanesulfonic acid; 3-(N-morpholino)-2-hydroxypropanesulfonic acid; phosphate; piperazine-N,N'-bis(2-ethanesulfonic acid); piperazine-N,N'-bis(2-hydroxypropanesulfonic acid); pyridine; succinate; 3-{[tris(hydroxymethyl)-methyl]-amino}-propanesulfonic acid; 3-[N-tris(hydroxymethyl)-methylamino]-2-hydroxypropanesulfonic acid; triethanolamine; 2-[tris(hydroxymethyl)-methylamino]-ethanesulfonic acid; N-[tris(hydroxymethyl)-methyl]-glycine; and tris(hydroxymethyl)-aminomethane. The buffering
 25 agent may be added to the lysis medium as a salt comprising the buffering agent and a counter ion.

Suitably, the buffering agent is present in an amount sufficient to maintain the acidity of a solution near the chosen value in the sample lysate.

The solvent may be any suitable solvent for the lysis or denaturing agent, a nucleic acid preservation agent, or a buffering agent. The solvent may be suitably selected from water, an alcohol (*e.g.*, such as ethanol or isopropanol), or a combination thereof.

In some embodiments of the invention, one or more compositions may perform the same function of a lysis or denaturing agent and a nucleic acid preservation agent, a lysis or denaturing agent and a buffering agent, or a nucleic acid preservation agent and a buffering agent. By way of example, guanidinium thiocyanate may be both a lysis or denaturing agent and a nuclear preservation agent because it can both break down or disrupt a cellular membrane and also denature a nuclease.

Suitably, the lysate sample has an ionic strength sufficiently high to allow for the formation of the salt bridges between the nucleic acids and microspheres. The positively charged ions bridging the nucleic acids and the microspheres may be present as a result of the addition of a lysis or denaturing agent, a nucleic acid preservation agent, a buffering agent, a salt thereof, or any combination thereof. The positively charged ion is selected from a monovalent ion such as Na^+ or K^+ , a divalent cation such as Mg^{+2} , Mn^{+2} , Zn^{+2} , Fe^{+2} , Ca^{+2} , Cu^{+2} , or positively charged, nitrogen containing ion such as guanidinium. The positively charged ion may be present in a concentration greater than or equal to 1M, 2M, 3M, or 4M.

Suitably the sample comprises a target sequence. As used herein, a “target sequence” or “target nucleic acid sequence” is a nucleic acid sequence indicative of an origin or source. Suitably, the target sequence is indicative of the presence of a particular organism such as a pathogen. In other embodiments, the target sequence is indicative of the presence or absence of a disease or condition, such as the presence or absence of a genetic mutation associated with the disease or condition as may be the case with a cancer. In yet other embodiments, the target sequence is indicative of the prognosis, progression, or response to treatment for a disease or condition, such as the presence or absence of a genetic mutation or genetic marker associated with the prognosis, progression, or response to treatment for a disease or condition such as cancer. As used herein, “indicative” or “indicates” means to point to or be a sign of an origin or source, whether alone or in combination with additional target sequences or other information.

The sample may comprise a pathogen. As used herein, a “pathogen” is any microorganism capable of causing disease in a subject. A “subject” may be interchangeable with “patient” or “individual” and means an animal, which may be a human or non-human animal, in need of treatment. A “subject in need of treatment” may include a subject having a disease caused by a pathogen.

In some embodiments, the target nucleic acid sequence is a nucleic acid sequence from a eukaryotic source. In some embodiments, the eukaryotic source is selected from algae, protozoa, fungi, slime molds and/or mammalian cells.

In some embodiments, the target nucleic acid sequence is a nucleic acid sequence from a microorganism or virus. Suitably, the microorganism or virus may be *Escherichia*, *Campylobacter*, *Clostridium difficile*, Enterotoxigenic *E.coli* (ETEC), Enteroaggregative *E.coli* (EAggEC), Shiga-like Toxin producing *E.coli*, *Salmonella*, *Shigella*, *Vibrio cholera*, *Yersinia enterocolitica*, Adenovirus, Norovirus, Rotavirus A, *Cryptosporidium parvum*, *Entamoeba histolytica*, *Giardia lamblia*, *Clostridia*, Methicillin-resistant *Staphylococcus aureus* MRSA, *Klebsiella pneumoniae* flu, Zika, dengue, chikungunya, West Nile virus, Japanese encephalitis, malaria, HIV, H1N1, and *Clostridium difficile* resistant organisms. In some embodiments, the target nucleic acid sequence is from a microorganism or virus selected from L DENV-1, DENV-2, DENV-3, DENV-4 RNA (dengue), tcdA and tcdB (C diff toxin genes), ZIKV RNA (Zika), CHIKV RNA (chikungunya), Giar-4, Giar-6 (*Giardia lamblia*), invasion antigen loci (ial), invasion plasmid antigen H (ipa H) (*Shigella*), GARV, VP7, NSP3 (rotavirus), and HuNoV (norovirus). In some embodiments, the pathogen is associated with sepsis such as Group B *Streptococcus* (GBS), *E. coli*, *Staphylococcus aureus*, Coagulase-negative *Staphylococcus* (CoNS), *Listeria monocytogenes*, *Enterococcus* sp, *Klebsiella* sp., and *Pseudomonas aeruginosa*.

In some embodiments, the target nucleic acid sequence is an rDNA or rRNA sequence from an organism. In some embodiments, the target nucleic acid sequence is an rRNA. In some embodiments, the rRNA is selected from 5s, 16s and 23s rRNA. In some embodiments, the target nucleic acid sequence is selected from 5s, 5.8s, 28s, and 18s rRNA. In some embodiments, any embodiment listed herein is specifically excluded from the devices and methods disclosed herein.

In some embodiments, the target nucleic acid sequence is anywhere on the genome of a specific organism or virus that is specific to said organism or virus.

The present technology may utilize nucleic-acid-capture microspheres and the sample may comprise nucleic-acid-capture microspheres. As used herein, “nucleic-acid-capture microspheres” comprise microspheres capable of binding nucleic acids in a complex matrix and releasing them when contacted with an eluent. The nucleic-acid-capture microspheres may be referred to as “glass bubbles”, “hollow microspheres”, or, simply, “microspheres”. Nucleic-acid-capture microspheres are typically unicellular but may contain some microspheres having a plurality of internal voids separated by extremely thin veils. The microspheres may vary in diameter from a few microns to hundreds of microns, *e.g.*, approximately 5-300 microns, 5-200 microns, or 10-100 microns. The exterior wall thickness of the microspheres varies, usually from approximately 5% to about 20% of the diameter of a complete microsphere or a fraction of a micron (*e.g.*, 0.5 microns) to several microns (*e.g.*, 5 microns). The microspheres are typically buoyant. As used herein, “buoyant” means that the majority of the microspheres have an average true density lower than water, typically from about 0.05-0.60 grams/cm³, 0.10-0.40 grams/cm³, or about 0.15-0.30 grams/cm³. An “average true density” is determined by placing microspheres in a chamber which is filled with air under compression. The air volume in that chamber is compared with the air volume in an identical sized chamber in which air is under equal compression. The difference in air volume is recorded; and the true volume occupied by the bubbles is calculated. The average true particle density is obtained by dividing the true volume occupied by the bubble sample into the weight of the sample. Additional details of such microspheres can be found in International Patent Application Serial No. PCT/US2018/063663, incorporated by reference herein.

As used herein, an “eluent” is a material used to extract nucleic acids adsorbed or bound onto the surface of microspheres from the surface. The “eluate” is the composition comprising the extracted nucleic acids. The eluent may be any suitable material for extracting the nucleic acids from the microspheres. Exemplary eluents include, without limitation, water, 50mM NaCl, TE buffer (10 mM Tris brought to pH 8.0 with HCl, 1mM EDTA), or any combination thereof.

The compositions of the microspheres may vary but are typically inorganic. As used herein, “inorganic” means that the microspheres are substantially free of carbon. Suitably the ingredients used to prepare the microspheres include at least some SiO₂, a fixing ingredient such as an alkali metal oxide, and one or more bivalent, trivalent, quadrivalent, or pentavalent oxides so that the inorganic components provide a composition which melts to form a glass at a temperature between approximately 1200°C-1500°C. Suitably, soda-lime-silica or soda-lime-borosilicate glasses may

be used to prepare the microspheres. In some embodiments, the microspheres for use in practicing the invention have a compositional analysis within the approximate ranges set forth in Table 1.

TABLE 1: Exemplary compositional analysis of nucleic-acid-capture microspheres

Ingredient	Weight percent
SiO ₂	60-80
Na ₂ O	5-16
CaO	5-25
K ₂ O + Li ₂ O	0-10
Na ₂ O + K ₂ O + Li ₂ O	5-16
RO (other than CaO)	0-15
RO ₂	0-10
R ₂ O ₃	0-20
R ₂ O ₅	0-25
F	0-5

- 5 Suitably, RO is selected from alkaline earth oxides (*e.g.*, BaO, MgO, and SrO) as well as bivalent oxides such as ZnO and PbO; RO₂ is selected TiO₂, MnO₂, and ZrO₂; R₂O₃ is selected from B₂O₃, Al₂O₃, Fe₂O₃, and Sb₂O₃; R₂O₅ is selected from P₂O₅ and V₂O₅; or any combination thereof.

10 In some embodiments, the microspheres comprise a silica shell completely or partially surrounding the microsphere compositions described above. Suitably, the silica shell may comprise between about 1-20 weight percent, 1-10 weight percent, or about 1-5 weight percent of the microsphere. The silica shell may be amorphous but need not be.

Exemplary microspheres include glass bubbles from 3M™ such as the glass bubbles described in Table 2.

15

TABLE 2: Exemplary nucleic-acid-capture microspheres

		Typical particle size (microns, by volume)
Target crush strength	True density	Distribution:

	(90% survival, psi)	(g/cc)	10th%	50th%	90th%
K1	250	0.125	30	65	115
K15	300	0.15	30	60	105
S15	300	0.15	25	55	90
S22	400	0.22	20	35	65
K20	500	0.2	25	55	95
K25	750	0.25	25	55	90
S32	2000	0.32	20	40	70
S35	3000	0.35	10	40	75
K37	3000	0.37	20	45	80
XLD3000	3000	0.23	15	30	40
S38	4000	0.38	15	40	75
S38HS	5500	0.38	15	40	75
S38XHS	5500	0.38	15	40	70
K46	6000	0.46	15	40	70
K42HS	7500	0.42	11	22	37
S60	10000	0.6	15	30	55
S60HS	18000	0.6	11	30	50
IM16K	16000	0.46	12	20	30
IM30K	28000	0.6	9	16	25

As demonstrated in the examples that follow, K20 and XLD3000 were successfully used to separate nucleic acids from complex samples. The chemical composition of both K20 and XLD3000 glass bubbles is 97% soda lime borosilicate glass and 3% synthetic amorphous crystalline free silica at the bubble surface.

Advantageously, the microspheres nonspecifically bind nucleic acids. This allows for the microspheres to be used to separate a variety of nucleic acids without having to be tailored for a specific target. As a result, the microspheres of the present invention do not require target-specific binding moieties, such as nucleic acids or proteins, to be bound to the surface of the microspheres.

The device 10 also comprises one or more amplification panels 22. The amplification panel 22 comprises an amplification reaction area 24. The amplification reaction area 24 is a hydrophilic

area configured to amplify the number of copies of a target nucleic acid sequence when heated to an effective temperature for an effective amount of time to produce amplicons of the target nucleic acid sequence. Suitably the effective temperature is a temperature above room temperature, e.g., at least 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, or 65 °C. Suitably the effective amount of time to produce amplicons is at least 1 min, e.g., at least 5 min, 10 min, 15 min, 20 min, 30 min, 40 min, 50 min, or 60 min.

Suitably the amplification reaction area 24 comprises an amplification medium. Suitably the amplification medium may be preimpregnated into the amplification reaction area 24 or added to the amplification reaction area 24 in temporal proximity to the addition of a sample. As used herein, an “amplification medium” is a composition for use in the production of amplicons of a target nucleic acid sequence by nucleic acid amplification. As used herein, an “amplicon” means a piece of DNA or RNA that is the source and/or product of nucleic acid amplification. The amplicon may be produced by any amplification technique, including a polymerase chain reaction (PCR) technique or an isothermal amplification technique. Exemplary isothermal amplification techniques include, without limitation, Loop-mediated isothermal amplification (LAMP), Reverse-transcriptase loop-mediated isothermal amplification (RT-LAMP), Recombinase polymerase amplification (RPA), Strand displacement amplification (SDA), Helicase-dependent amplification (HDA), Nucleic acid sequence based amplification (NASBA), Nicking enzyme amplification reaction (NEAR), and transcription-mediated amplification (TMA). Details of such isothermal amplification techniques can be found in Zhao *et al.* Chem. Rev. 2015, 115, 12491–12545 and Craw and Balachandran Lab Chip, 2012, 12, 2469-2486.

Suitably the amplification panel 22 may comprise more than one amplification reaction areas 24. For example, the amplification panel may have 1, 2, 3, 4, 5, 6, 7, 8, or more than 8 amplification reaction areas. In some embodiments, each of the amplification reaction areas may comprise the same amplification medium. In other embodiments, each of the amplification reaction areas may comprise different amplification media. In yet other embodiments, at least two of the amplification reaction areas may comprise the different amplification media from each other. Having more than one amplification reaction area allows for multiplexed and/or replicated nucleic acid amplification or detection.

The amplification medium may comprise a polymerase, a primer, nucleoside triphosphates, a cofactor, a buffering agent, a solvent, an amplification enhancer, or any combination thereof.

When the device 10 comprises different amplification mediums any of the medium components may be different. In some embodiments, the different amplification mediums comprise different primers. Suitably the different primers are indicative of different pathogens.

As used herein, a “polymerase” is an enzyme capable of catalyzing the formation of nucleic acids. The polymerase may be a DNA polymerase or an RNA polymerase. Suitably, the polymerase may be selected from a Taq polymerase or a Bst polymerase.

As used herein, a “primer” means a nucleic acid designed to bind via sequence complementarity to sequences that flank the target sequence in the template nucleic acid. During amplification, polymerases extend the primers. As such, the primer’s binding site should be unique to the vicinity of the target sequence with minimal homology to other sequences to ensure specific amplification of the intended target sequence. Suitably the primer is suitable for use with a polymerase chain reaction (PCR) technique or any of the isothermal amplification techniques described above.

In some embodiments, the isothermal amplification technique is a LAMP technique. Exemplary primers for amplifying a target nucleic acid sequence for each of *E. coli*, *C. diff.*, and Enteropathogenic *E. coli* (EPEC) are provided in Table 3.

TABLE 3: LAMP primers sequences

Oligo Name	5' Mod	Sequence
<i>E. coli</i>		
E-F3		GCCATCTCCTGATGACGC (SEQ ID NO: 25)
E-B3		ATTTACCGCAGCCAGACG (SEQ ID NO: 26)
E-BIP		CTGGGGCGAGGTCGTGGTATTCCGACAAACACCACGAATT (SEQ ID NO: 27)
E-FIP		CATTTTGCAGCTGTACGCTCGCAGCCCATCATGAATGTTGC T (SEQ ID NO: 28)
E-LF		CTTTGTAACAACCTGTCATCGACA (SEQ ID NO: 29)
E-LB		ATCAATCTCGATATCCATGAAGGTG (SEQ ID NO: 30)
<i>C. diff</i>		
CD-F3		GTATCAACTGCATTAGATGAAAC (SEQ ID NO: 31)

CD-B3		CCAAAGATGAAGTAATGATTGC (SEQ ID NO: 32)
CD-FIB		CTGCACCTAAACTTACACCATCTATCTTCCTACATTATCTGA AGGATT (SEQ ID NO: 33)
CD-BIP		GAGCTAAGTGAAACGAGTGACCCGCTGTTGTAAATTTACT GCC (SEQ ID NO: 34)
CD-FL- FAM	[6FAM]	AATAGTTGCAATTATAGG (SEQ ID NO: 35)
CD-BL- BIO	[Btm]	AGACAAGAAATAGAAGCTAAGATAGG (SEQ ID NO: 36)
EPEC		
EPEC-F3		CGACGATTTGGTCGTTGAA (SEQ ID NO: 41)
EPEC-B3		TGTCATCGGTCATGTTGC (SEQ ID NO: 42)
EPEC-FIP- FAM	[6FAM]	CAAAATGATCTGCTGACCAGGCTTTTTAAGCATTATACAG TTCTGAAAGC (SEQ ID NO: 43)
EPEC- BIP-BIO	[Btm]	ACAGTGCACTACCACTTTTAGGTTTTTCATTTTAGTCAGTT TATTCGTGT GA (SEQ ID NO: 44)

In some embodiments, the isothermal amplification technique is a RT-LAMP technique. Exemplary primers for amplifying a target nucleic acid sequence for each of Norovirus G1 and Norovirus G2 are provided in Table 4.

5

TABLE 4: RT-LAMP primers sequences

Oligo Name	Sequence
Norovirus G1	
FIP	AGCGTCCTTAGACGCCATCATCACCTCGGATTGTGGACAGG (SEQ ID NO: 37)
BIP	GGCGCTGGTCAGTTGGTACCCGCTACAGGATCCATTGCA (SEQ ID NO: 38)
F3	YATGTTCCGYTGGATGCG (SEQ ID NO: 39)
B3	AACTTGCCCAGCAGTTGC (SEQ ID NO: 40)

Norovirus G2	
Nov-G2-FIP	ATAGCGGCACCAACAACGGCCTCGTCCCAGAGGTCAAC (SEQ ID NO: 45)
Nov-G2-BIP	ACCTGTAGCGGGCCAACAACCTCTCCACCAGGGGCTT (SEQ ID NO: 46)
Nov-G2-F3*	CCCATCTGATGGGTCCRCA (SEQ ID NO: 47)
Nov-G2-B3	CACCTGGAGCGTTTCTAGG (SEQ ID NO: 48)

*Note: Y is a mixture of C or T; R is a mixture of A or G

In some embodiments, the isothermal amplification technique is a RPA technique. Exemplary primers for amplifying a target nucleic acid sequence for each of Enterotoxigenic *E. Coli* (ETEC) and *C. diff.* are provided in Table 6.

TABLE 6. RPA primers sequences

Oligo Name	5' Mod	Sequence	3' Mod
ETEC			
ETEC-F		GCCATTATATGCAAATGGCGACAAATTATACCGTGC (SEQ ID NO: 49)	
ETEC-R	[Btm]	CCTGCTAAGTGAGCACTTCTC AACTAAGA (SEQ ID NO: 50)	
ETEC-P	[6FAM]	CCCAGAGGGCATAATGAGTACTTCGATAGA/idSp/GAA CTCAAATGAATA (SEQ ID NO: 51)	[Spc3]
<i>C. diff</i>			
CD-F		ATATCAGAGACTGATGAGGGATTAGTATA (SEQ ID NO: 52)	
CD-R	[Btm]	TATATGATTAGCATATTCAGAGAATATTGT (SEQ ID NO: 53)	

Nucleoside triphosphates are present for the formation of nucleic acids. The nucleoside triphosphates may include deoxynucleoside triphosphates (dNTPs), *e.g.*, dATP, dCTP, dGTP, and dTTP.

As used herein, a “cofactor” means a substance other than the substrate that is essential for the activity of an enzyme. Suitably, the cofactor may be Mg^{2+} which functions as a cofactor for the activity of a variety of polymerases, enabling the formation of nucleic acids during polymerization. The cofactor may be introduced to the amplification medium as a salt, *e.g.*, $MgSO_4$, $Mg(CH_3COO)_2$, or $MgCl_2$.

As used herein, a “buffering agent” comprises a weak acid or base used to maintain the acidity (pH) of a solution near a chosen value after the addition of another acid or base. Suitably, the buffering agent may be selected from Tris-HCl, $(NH_4)_2SO_4$, or KCl.

The solvent may be selected from any suitable solvent or combination of solvents that allow for application. Suitably, the solvent is water. An amplification medium without a solvent may be referred to as a “dry amplification reagent.”

As used herein, an “amplification enhancer” is a substance that may enhance amplification specificity, efficiency, consistency, and/or yield. Suitably, the amplification enhancer comprises dimethyl sulfoxide, glycerol, formamide, polyethylene glycol, N,N,N-trimethylglycine (betaine), bovine serum albumin, tetramethylammonium chloride, a detergent, or combinations thereof. Suitably, the detergent is a nonionic detergent such as Tween 20 or Triton X-100.

Suitably the amplification medium is tailored to the desired amplification technique. In some embodiments, the amplification medium is a LAMP amplification medium. An exemplary LAMP amplification medium is provided in Table 7.

TABLE 7: LAMP Amplification Medium

Amplification reagents	Final Conc
Bst DNA polymerase buffer	10x
Tris-HCl (pH 8.8)	20 mM
$(NH_4)_2SO_4$	10 mM
KCl	10 mM
$MgSO_4$	2 mM
Tween 20	0.10%

dNTPs (100 mM)	1 mM
Betaine (5 M)	200 mM
F3 primer (10 pmol/ μ L)	7.5 pmol
B3 primer (10 pmol/ μ L)	7.5 pmol
FIP primer (10 pmol/ μ L)	75 pmol
BIP primer (10 pmol/ μ L)	75 pmol
LF primer (10 pmol/ μ L)	30 pmol
LB primer (10 pmol/ μ L)	30 pmol
Bst DNA polymerase	8 U
H ₂ O	q.s.
MM total	23 μl

In some embodiments, the amplification medium is a RPA amplification medium. An exemplary RPA amplification medium is provided in Table 8.

5 TABLE 8: RPA reaction master mix composition

Master mix reagents	Volume
Rehydration buffer	29.5 μ l
Forward primer (10 μ M)	1.05 μ l
Reverse primer (10 μ M)	1.05 μ l
Nfo probe (10 μ M)	0.6 μ l
Magnesium acetate (280 mM)	2.5 μ l
Water	13.2 μ l
Dried reagent in tube	-
MM total	50 μl

The microfluidic device may comprise a diagnostic panel 26 comprising a diagnostic device 28 that allows for the detection of a target nucleic acid sequence. Suitably the diagnostic device is a lateral flow device. As used herein, a “lateral flow device” is a porous device capable of detecting the presence of a target sequence traversing a series of beds. Lateral flow devices comprise (a) a diagnostic loading area at one end of the lateral flow device; (b) an area comprising

a detectably labelled probe specific for a target nucleic acid sequence, wherein said detectably labelled probe is not bound to the lateral flow device and is capable of wicking across the lateral flow device; (c) an area comprising a capture probe for the target nucleic acid sequence, wherein said capture probe for the target nucleic acid sequence is immobilized on the lateral flow device; and (d) absorbent material, wherein the absorbent material wicks an aqueous sample across the lateral flow device when the aqueous sample is added to the sample loading area. In some embodiments, the capture probe is capable of moving toward the area comprising the detectably labelled probe either by movement of the capture probe itself (*i.e.*, the capture probe is not immobilized), or by movement of the area comprising the capture probe. Details of such a method can be found in United States Patent Publication No. 2018/0148774, incorporated by reference herein.

The diagnostic loading area may comprise a material that traps debris. The debris may comprise a component of a lysed or unlysed biological sample, an eluate, or a microsphere. In some embodiments, the material comprises glass fiber. In some embodiments, the material comprises polyester and/or cellulose. In some embodiments, the material that traps debris is any commercially available microporous material. As used herein, “traps” or “trapping” refers to immobilizing, delaying movement, capturing (temporarily or permanently), impeding movement, or hindering movement. As used herein, “debris” means any particulate matter other than the components of the disclosed assays or devices. In some embodiments, “debris” includes tissue, food particles, clumped cells, cell walls, microspheres, and the like.

In some embodiments, the diagnostic loading area is a microsphere-loading area. As used herein, a “microsphere-loading area” comprises material that traps microspheres transferred onto or into the microsphere-loading area and also allows for an eluate to traverse the microsphere-loading area when an eluent contacts trapped microspheres having nucleic acids adsorbed thereto. When the lateral flow device comprises a microsphere-loading area, the lateral flow device may also comprise an amplification area for amplifying nucleic acids within the eluate extracted from the microspheres trapped on or in the microsphere-loading area.

The lateral flow device may comprise a solid support such as a paper. Suitably, the solid support comprises cellulose, such as filter paper, chromatographic paper, nitrocellulose, and cellulose acetate. In some embodiments, the solid support comprises materials such as glass fibers,

nylon, dacron, PVC, polyacrylamide, cross-linked dextran, agarose, polyacrylate, ceramic materials, and the like.

The lateral flow device may comprise an absorbent sample pad infused with the gold conjugated detection probe, a lateral flow channel which contains the spotted streptavidin fixed biotinylated capture probe on the test area and spotted streptavidin fixed biotinylated control probe on the control area.

The conjugation area comprises a detectably labelled probe specific for a target nucleic acid sequence, wherein said detectably labelled probe is not bound to the lateral flow device and is capable of wicking across the lateral flow device.

The detectably labelled probe specific for a target nucleic acid sequence may be labeled with a moiety selected from a gold nanoparticle, a protein binding ligand, a hapten, an antigen, a fluorescent compound, a dye, a radioactive isotope and an enzyme. In some embodiments, the detectably labelled probe is labelled with a gold nanoparticle. In some embodiments, the detectably labelled probe is labelled with latex beads, latex microspheres and/or magnetic beads.

Choosing and designing the sequence of the probe specific for a target nucleic acid sequence is based on the nature of the source of the target nucleic acid sequence. Generally, the probe specific for the target nucleic acid that will be detectably labelled is capable of specifically hybridizing to part of the target nucleic sequence, separate from the sequence to which the capture probe will specifically hybridize.

Exemplary nucleic acid detection probes, control probes, capture probes have been designed for each target pathogens including *E. coli*, *C. diff*, *Campylobacter*, *Cryptosporidium*, *Giardia*, *Norovirus*, *ETEC*, and *EPEC*. The exemplary probe sequences are listed in Table 9.

TABLE 9: DNA probes of eight pathogens for lateral flow diagnostic device

Oligo Name	5' Mod	Sequence	3' Mod
<i>E. coli</i>			
E-DC		GAG CGT TCT GTA AGC CTG CGA AAA AAA AA (SEQ ID NO: 1)	[BtnTg]
E-C	[Btn]	AA AAA AAA TAC CTC CAG CAT GCC TCA CAG (SEQ ID NO: 2)	
E-D		TCG CAG GCT TAC AGA ACG CTC AAA AAA AA (SEQ ID NO: 3)	[ThiC3]

<i>C. diff</i>		
CD-DC		CCA CAT GTC CTT ACG GTC ATG AAA AAA AA (SEQ ID NO: 4) [BtnTg]
CD-C	[Btn]	AA AAA AAA GTA GGG GAG CTT CCC ATA CGG (SEQ ID NO: 5)
CD-D		CAT GAC CGT AAG GAC ATG TGG AAA AAA AA (SEQ ID NO: 6) [ThiC3]
<i>Cryptosporidium</i>		
CRF-DC		AGC CTG AGA AAC GGC TAC CAC ATC AAA AAA AA (SEQ ID NO: 7) [BtnTg]
CRF-C	[Btn]	AA AAA AAA GTA ATT TGC GCG CCT GCT GCC (SEQ ID NO: 8)
CRF-D		GAT GTG GTA GCC GTT TCT CAG GCT AAA AAA AA (SEQ ID NO: 9) [ThiC3]
<i>Giardia</i>		
GRD-DC		GTC AAG CTC AGC AAC ATG AAC AAA AAA AA (SEQ ID NO: 10) [BtnTg]
GRD-C	[Btn]	AA AAA AAA TCT TGT CGT GGA ACC TGC TGA (SEQ ID NO: 11)
GRD-D		GTT CAT GTT GCT GAG CTT GAC AAA AAA AA (SEQ ID NO: 12) [ThiC3]
<i>Campylobacter</i>		
Camp-DC		CAC AAG TTG AGT AGG GAA AGT AAA AAA AA (SEQ ID NO: 13) [BtnTg]
Camp-C	[Btn]	AA AAA AAA ACT ATA TAG TCT CAT CCT ACA (SEQ ID NO: 14)
Camp-D		ACT TTC CCT ACT CAA CTT GTG AAA AAA AA (SEQ ID NO: 15) [ThiC3]
<i>Norovirus</i>		
Noro-DC		ATG ATG CAG ACT ACT CTC GTT AAA AAA AA (SEQ ID NO: 16) [BtnTg]
Noro-C	[Btn]	AA AAA AAA AGT ACT GCC CTC TGT TGT GTT (SEQ ID NO: 17)
Noro-D		AAC GAG AGT AGT CTG CAT CAT AAA AAA AA (SEQ ID NO: 18) [ThiC3]
<i>ETEC</i>		
ETEC TOX-DC		ATC TTT CCC CTC TTT TAG TCA AAA AAA AA (SEQ ID NO: 19) [BtnTg]
ETEC TOX-C	[Btn]	AA AAA AAA TTT TGA AGA GTC AAG TGA TTC (SEQ ID NO: 20)
ETEC TOX-D		TGA CTA AAA GAG GGG AAA GAT AAA AAA AA (SEQ ID NO: 21) [ThiC3]
<i>EPEC</i>		
EPEC-DC		CAG CCC GGA GGG CTG CAT TAC AAA AAA AA (SEQ ID NO: 22) [BtnTg]

EPEC-C	[Btn]	AA AAA AAA GCT CGG CTT TCA GCC CTC TTG (SEQ ID NO: 23) GTA ATG CAG CCC TCC GGG CTG AAA AAA AA (SEQ ID NO: 24)	[ThiC3]
EPEC-D			

The test area comprises a capture probe for the target nucleic acid sequence, wherein said capture probe for the target nucleic acid sequence is immobilized on the lateral flow device, is also called the test probe area. The test area can be in any form with well-defined boundaries, such as a dot, or a strip. The capture probe may be immobilized on the lateral flow device by covalent coupling or affinity binding. Suitably, the capture probe is attached to the lateral flow device by biotin: streptavidin affinity binding. Generally, the capture probe is capable of specifically hybridizing to part of the target nucleic acid sequence, separate from the sequence to which the detectably labelled probe will bind.

The lateral flow device may comprise an area comprising a control probe, wherein said control probe is immobilized on the lateral flow device. This area is also called the control area, or the control probe area. The control probe may comprise a sequence complementary to the detectably labelled probe. The control probe may be immobilized on the lateral flow device by covalent coupling or affinity binding. Suitably, the control probe is attached to the lateral flow device by biotin: streptavidin affinity binding.

The absorbent material which wicks an aqueous sample across the lateral flow device may comprise cellulose. Suitably the cellulose is selected from filter paper, chromatographic paper, nitrocellulose, and/or cellulose acetate. In some embodiments, the absorbent material is in the form of an absorbent pad at the end of lateral flow device opposite of the sample loading area. In other embodiments, the absorbent material runs the length of the lateral flow device.

The lateral flow device may be capable of multiplex nucleic acid detection (*i.e.*, the point of need testing device comprises at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, at least twelve, at least thirteen, at least fourteen, at least fifteen, at least twenty, or at least twenty five lateral flow devices). In some embodiments, each lateral flow device comprises a probe specific for a different target nucleic acid (*e.g.*, a different microorganism or virus).

In some embodiments, the plurality of lateral flow devices are arranged in a radial manner (*i.e.*, similar to a star). In some embodiments, the lateral flow devices are arranged in a radial

manner around a central sample loading area. For example, in one embodiment the point of need device is a star-shaped multiplex paper strip. In some embodiments, thin sheets of porous nitrocellulose membranes are cut into star shapes using a computer-controlled X-Y knife plotter cutter. This device incorporates a knife in place of the traditional ink pen. The knife rotates freely
5 on a turret, enabling precise cutting of various features. The control lines (containing the control probe) and test lines (containing the detection probe) will be spotted on each of the arms. The lateral flow paper strips are spotted using a BioDot AD1520 tabletop aspirating/dispensing workstation outfitted with two BioJet™ Elite dispensers capable of generating overlapping spots within nitrocellulose (minimal volumes of 20 - 50 nL) or continuous reagent lines (1 µL/cm).
10 Dispensing protocols are custom written for the snowflake nitrocellulose design and optimized for buffer conditions, dispense volume, and spatial separation.

In some embodiments, the plurality of lateral flow devices are arranged in a lateral manner.

In some embodiments, the plurality of lateral flow devices are micropatterned onto the point of care device by a method of patterning a porous, hydrophilic substrate into hydrophobic
15 and hydrophilic regions. In some embodiments, such a method comprises disposing a wax material onto the hydrophilic substrate in a predetermined pattern; and heating the substrate to a temperature sufficient to melt the wax material, the melted wax material substantially permeating the thickness of the substrate and defining a pattern of one or more hydrophobic regions. Details of such a method can be found in International Patent Publication No. WO2010/102294,
20 incorporated by reference herein.

An exemplary lateral flow strip having an antiFITC and biotin printed at the test line and control line, respectively, as schematically illustrated in Fig. 2. The streptavidin gold nanoparticles may be preloaded at the conjugation pad. Once the target sequences are successfully amplified, the amplicon would form a biotin-DNA-FAM structure, where the biotin functional group will
25 bind to the streptavidin-gold and the FAM tag will be captured by the anti-FITC at the test line. In the absence of amplification, the biotin-DNA-FAM sequence would not form and the test line would not show color. Regardless of the presence or absence of the amplicon, the streptavidin-gold will move to the top of the lateral flow assay and bind with the biotin at the control line area.

The device 10 may further comprise a distribution panel 16 comprising a sample loading
30 hub 18 and distribution arms 20 extending from the sample loading hub to the amplification reaction areas 24. Both the sample loading hub 18 and the distribution arms 20 comprising

hydrophilic areas that fluidly connects a sample loading panel 12 and an amplification panel 22 and allows for the distribution of a single sample into at least two different amplification reaction areas 24.

Figures 3A-3D illustrate another exemplary microfluidic device 110 for the amplification of a target nucleic acid and associated diagnostic device 140 for detecting the target nucleic acid. The device 110 comprises a sample loading panel 112 comprising a sample loading area 114. The sample loading panel 112 and sample loading area 114 are configured to receive a sample. The sample loading area 114 is a hydrophilic area suitable for depositing an aqueous sample.

The device 110 also comprises more than one amplification panel 122A-122D. The amplification panels 122A-122D each comprise amplification reaction areas 124A-124D associated with panels 122A-122D, respectively. The amplification reaction areas 124A-124D are hydrophilic areas configured to amplify the number of copies of a target nucleic acid sequence when heated to an effective temperature for an effective amount of time to produce amplicons of the target nucleic acid sequence. The amplification reaction areas 124A-124D together form a soaking volume.

As used herein, a "soaking volume" is hydrophilic volume effective in retaining the amplification medium and a biological sample added to the microfluidic device. Suitably the device may have any number of amplification panels to provide an appropriate soaking volume, *e.g.*, the device may comprise 1, 2, 3, 4, 5, 6, or more than 6 amplification panels. The soaking volume allows a superimposed stack of panels to be effectively heated for an effective amount of time to amplify the target nucleic acid sequence. Without an appropriately sized control volume, the sample may migrate through the amplification reaction area before the amplification reaction can sufficiently amplify the target nucleic acid.

After the device is heated to an effective temperature for an effective amount of time to produce amplicons of the target nucleic acid sequence, the sample and any amplicons may be displaced from the soaking volume by the addition of a washing medium. As used herein, a "washing medium" is a substance capable of diluting the sample and any amplicons contained therein. The washing medium may also be a substance capable of removing impurities adsorbed onto the surface of the microspheres or diluting residual lysate continuous phase associated with the particulate phase after separating the phases from one another. Suitably, the washing medium

may be selected from water, an alcohol such as ethanol, medium salt buffer such as 100mM or 200mM NaCl, or combinations thereof.

The washing medium is typically added in excess of the sample added to the device. Suitably, the washing medium may be added to the device at a volume at least 5 times greater than a volume of sample added to the device. In some embodiments, the washing medium is added at a volume at least 10, 15, or 20 times greater volume than the sample. Suitably the sample may be added at a volume of at least 10 μ L. For example, the sample may be added at a volume of 10 - 50 μ L. Suitably the washing medium may be added at a volume of 50 - 500 μ L.

The device 110 may further comprise a distribution panel 116 comprising a sample loading hub 118 and distribution arms 120 extending from the sample loading hub to the amplification reaction areas 124A. Both the sample loading hub 118 and the distribution arms 120 comprising hydrophilic areas that fluidly connects a sample loading panel 112 and an amplification panel 122A and allows for the distribution of a single sample into at least two different amplification reaction areas.

The device 110 may further comprise a control panel 130. The control panel 130 comprising a control volume 132 extending from the application reaction area 124D. The control panel 130 provides additional soaking volume. In some instances the soaking volume may be defined by the amplification reaction area and the control volume 132. The control volume may also facilitate the dilution of the amplicons by the washing medium. In some instances the amplification reaction may result in a sufficiently high amplicon concentration to interfere with the diagnostic device. The volume of washing medium added to the device may be selected to not only facilitate migration of the sample to the diagnostic device but also to appropriately dilute the sample so as not to interfere with the diagnostic device.

As illustrated in Figs. 3A-3D, the diagnostic device 140 is associable with the 110. In other embodiments, the diagnostic device may be incorporated as a diagnostic panel of the device. The diagnostic device 140 may comprise a cassette for housing one or more lateral flow devices 142A and 142B as described above. The diagnostic device 140 and/or the device 110 may comprise one or more markings or mechanisms for associating the two with proper alignment to have the sample migrate to a diagnostic loading area of the diagnostic device 140.

The devices described herein may be loaded with the use of nucleic-acid-capture microspheres. Figure 4 illustrates a method of separating a nucleic acid from a sample for its

eventual detection. The method comprises contacting a sample lysate with a plurality of nucleic-acid-capture microspheres 204. As a result of the contact between a sample lysate and the microspheres, the nucleic acids are adsorbed or bound onto the surface of the microsphere and a lysate dispersion is formed. The lysate dispersion comprises a lysate continuous phase and a lysate particulate phase. The particulate phase comprises the plurality of microspheres and adsorbed nucleic acids obtained from the sample. The particulate phase comprises the sample lysate less the nucleic acids adsorbed to the microspheres, *i.e.*, biomolecules other than adsorbed nucleic acids such as proteins, lipids, or carbohydrates, lysis or denaturing agents, nucleic acid preservation agents, buffering agents, or solvent that is not adsorbed onto the microspheres. Additional details of such a method can be found in International Patent Application Serial No. PCT/US2018/063663, incorporated by reference herein.

The method further comprises separating the lysate continuous phase from the particulate phase 206. Because the dispersion is unstable, the particulate phase with adsorbed nucleic acids spontaneously aggregates at the surface of continuous phase. The aggregation of the particulate phase allows for separation of the continuous phases. The separation may be accomplished, for example, by extracting the continuous phase, expelling the continuous phase, mechanically separating the continuous and particulate phases, or absorbing the particulate phase. Optionally the separation may employ a microsphere separation device such as a syringe, pipette, a microsphere-retaining mesh, a semi-permeable container, a absorption pad, or any combination thereof. Suitably at least some lysate continuous phase is separated from the particulate phase and, in some cases, a majority or substantially all of the lysate continuous phase is separated from the particulate phase.

The method also comprises contacting the particulate phase with an eluent 210. The eluent extracts the adsorbed nucleic acids from the surface of the microspheres, resulting in an eluate comprising nucleic acids obtained from the sample. Suitably the particulate phase is contacted with a sufficient amount of the eluent to extract nucleic acids bound onto the surface of the microspheres and, in some cases, a majority or substantially all of the nucleic acids bound onto the surface of the microspheres. In some embodiments, contacting the particulate phase with an eluent forms a eluate dispersion comprising a continuous phase and a particulate phase. The continuous phase of the eluate dispersion comprises the eluate and the particulate phase comprises the microspheres. The separation of the continuous and particulate phases may be accomplished by any method

suitable for separating the continuous and particulate phases of a lysate dispersion. Such methods suitably include extracting the continuous phase, expelling the continuous phase, mechanically separating the continuous and particulate phases, or absorbing the particulate phase.

The entire nucleic acid separation protocol may typically be finished within 20 minutes without using any professional laboratory instrumentation such as centrifuges or refrigeration.

The method may further comprise washing the particulate phase prior to contacting the particulate phase with an eluent. Washing the particulate phase may comprise contacting the particulate phase with a washing medium to form a washing dispersion and separating the continuous and particulate phases of the washing dispersion. The washing medium should be selected to remove impurities more weakly adsorbed onto the surface of the microspheres than the adsorbed nucleic acids without extracting substantially all of the nucleic acids or dilute any residual lysate continuous phase associated with the particulate phase. Suitably the particulate phase is contacted with a sufficient amount of the washing medium to move some or all of the impurities adsorbed onto the surface of the microspheres or to dilute any residual lysate continuous phase associated with the particulate phase. The separation of the continuous and particulate phases may be accomplished by any method suitable for separating the continuous and particulate phases of a lysate dispersion, Such methods suitably include extracting the continuous phase, expelling the continuous phase, mechanically separating the continuous and particulate phases, or absorbing the particulate phase.

The method may further comprise providing a sample. Providing the same may comprise contacting a sample with a lysis or denaturing agent to prepare the sample lysate. Suitably the sample may be contacted with a lysis medium comprising the lysis medium comprising the lysis or denaturing agent.

Another aspect of the invention is a method for amplifying a nucleic acid obtained from a sample. The method comprises separating nucleic acids from a sample as described above and further comprising amplifying the nucleic acid separated from the sample.

Figure 5 illustrates the separation of the particulate phase by absorption of the particulate phase of a lysate dispersion onto a sample loading area or absorption pad. Suitably the absorption pad is a microsphere loading pad. The lysate dispersion in a sample collection vessel can be augmented, such as with a washing medium, to increase the volume of the continuous phase. As the volume of the continuous phase, the aggregated particulate phase will rise with the rising level

of the surface of the continuous phase. A sufficient amount of augmentation, a convex meniscus may form and rise above the top of the vessel. The microspheres within the meniscus may be adsorbed onto the absorption pad by dabbing the absorption pad onto the meniscus, separating the particulate phase and nucleic acids adsorbed thereon from the continuous phase.

5 Unless otherwise specified or indicated by context, the terms “a”, “an”, and “the” mean “one or more.” For example, “a molecule” should be interpreted to mean “one or more molecules.”

As used herein, “about”, “approximately,” “substantially,” and “significantly” will be understood by persons of ordinary skill in the art and will vary to some extent on the context in which they are used. If there are uses of the term which are not clear to persons of ordinary skill
10 in the art given the context in which it is used, “about” and “approximately” will mean plus or minus $\leq 10\%$ of the particular term and “substantially” and “significantly” will mean plus or minus $>10\%$ of the particular term.

As used herein, the terms “include” and “including” have the same meaning as the terms “comprise” and “comprising.” The terms “comprise” and “comprising” should be interpreted as
15 being “open” transitional terms that permit the inclusion of additional components further to those components recited in the claims. The terms “consist” and “consisting of” should be interpreted as being “closed” transitional terms that do not permit the inclusion additional components other than the components recited in the claims. The term “consisting essentially of” should be interpreted to be partially closed and allowing the inclusion only of additional components that do not
20 fundamentally alter the nature of the claimed subject matter.

All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed.
25 No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

30 Preferred aspects of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred aspects may become

apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect a person having ordinary skill in the art to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited
5 in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

EXAMPLES

EXAMPLE 1: Preparation of a microfluidic device

10 A microfluidic device as schematically illustrated in Figure 3A was designed in Adobe Illustrator then printed on chromatography paper (GE Whatman™ 3001-816) by Xerox Solid ink printer (Xerox Colorcube 8580). The printed device was heated at 100 °C for 5 min. This heating process is to allow the printed solid ink to be melted again then soak into the chromatography paper to form a hydrophobic channel wall through the paper. Therefore, the liquid sample flow is
15 directed by the design of the solid ink channel to the desired destinations. After the heating process, two lateral flow assay strips were incorporated into the bottom origami layer through double-sided adhesive to form a lateral flow detection layer. The design of the origami pattern is shown in Figure 3A, where the device comprises a sample loading panel 112, a sample distribution panel 116, a multiplicity of amplification panels 122A-122D, and a control panel. There are four individual
20 wax-patterned paper layers stacked together to provide a soaking volume adequate enough to hold the amplification buffer and the distributed liquid sample.

EXAMPLE 2: Impregnating the device of Example 1 with amplification mediums comprising ETEC and *C. diff.* primers

25 The device of Example 1 was impregnated with RPA amplification mediums as described in Table 8. In one set of amplification reaction areas 124A-124B, the ETEC RPA primers as described in Table 6 were added. In the remaining amplification reaction areas, the *C. diff.* RPA primers as described in Table 6 was added. The device is folded and sealed with single-sided acetate films to prevent the evaporation of the amplification reagents.

EXAMPLE 3: Amplifying a sample comprising *C. diff* genomic DNA

20 ul of *C. diff* genomic DNA solution was added to the preimpregnated device of Example 2. After the *C. diff* genomic DNA sample was added to the device, the device was sealed with single-sided acetate films and incubated at 40 °C for 15 min using a hotplate.

5 EXAMPLE 4: Detecting *C. diff* genomic DNA

After sample is amplified according to Example 3, 200 ul washing buffer was added to the device. The washing buffer carried the amplicons to the lateral flow strips. The *C. diff* test strip developed the control and the test band, in contrast, the ETEC test strip only developed the control band, which indicated that the origami-lateral flow combination device has a good pathogen
10 specificity without cross-talk between individual amplification chambers.

CLAIMS

1. A microfluidic amplification device for amplifying a target nucleic acid sequence in a biological sample, the device comprising:
a sample loading panel, the sample loading panel comprising a sample loading area; and
5 one or more amplification panels, the amplification panel comprising an amplification reaction area;
wherein each of the panels comprises an absorbent material configured to wick an aqueous sample across a superimposed stack of the panels.
2. The device of claim 1, wherein the amplification reaction area comprises an amplification
10 medium therein.
3. The device of claim 2, wherein the amplification reaction area has a soaking volume effective in retaining the amplification medium and a biological sample contained therein.
4. The device of claim 2 further comprising a control panel, the control panel comprising a
15 control volume extending from the amplification reaction area,
wherein the amplification reaction area and the control volume together have a soaking volume effective in retaining the amplification medium and a biological sample contained therein.
5. The device of any one of claims 1-4, wherein the device comprises more than one amplification panel.
- 20 6. The device of claim 1 further comprising a distribution panel,
wherein the device comprises more than one amplification reaction area and
wherein the distribution panel comprises a sample loading hub and distribution arms extending from the sample loading hub to each of the amplification reaction areas.
7. The device of claim 6, wherein each of the more than one amplification reaction areas
25 comprise an amplification medium therein.
8. The device of claim 7, wherein the device comprises at least two different amplification mediums in two different amplification reaction areas.
9. The device of claim 8, wherein the different amplification mediums comprise different primers specific for different target nucleic acid sequences.

10. The device of any one of claims 6-9, wherein the amplification reaction area has a soaking volume effective in retaining the amplification medium and a biological sample contained therein.
- 5 11. The device of any one of claims 6-9 further comprising a control panel, the control panel comprising a control volume extending from each of the amplification reaction areas, wherein the amplification reaction areas and the control volume together have a soaking volume effective in retaining the amplification medium and a biological sample contained therein.
- 10 12. The device of any one of claims 6-11, wherein the device comprises more than one amplification panel.
13. A device for detecting the presence of a target nucleic acid sequence in a sample, the device comprising:
the amplification device of claim 1 and further comprising a diagnostic device comprising a detectably labelled probe specific for the target nucleic acid sequence.
- 15 14. The device of claim 13, wherein the diagnostic device comprises at least one lateral flow device comprising:
a diagnostic loading area positioned at one end of the lateral flow device;
an area comprising the detectably labelled probe specific for the target nucleic acid sequence, wherein said detectably labelled probe is not bound to the lateral flow device
20 and is capable of wicking across at least a portion of the lateral flow device;
an area comprising a capture probe for the target nucleic acid sequence, wherein said capture probe for the target nucleic acid sequence is immobilized on the lateral flow device;
and
an absorbent material, wherein the absorbent material wicks the biological sample across
25 the lateral flow device when the aqueous sample is added to the diagnostic loading area.
15. The device of claim 14, wherein amplification device further comprises a diagnostic panel comprising the diagnostic device.
16. The device of claim 14, wherein the diagnostic device comprises a cassette housing the lateral flow device.
- 30 17. The device of any one of claims 14-16, wherein said at least one lateral flow device further comprises an area comprising a second capture probe for a control nucleic acid sequence,

wherein said control nucleic acid sequence is complementary to a sequence of the probe specific for the target nucleic acid sequence, and wherein said second capture probe for a control nucleic acid sequence is attached to the lateral flow device.

18. The device of any one of claims 13-17, wherein the detectably labelled probe specific for the target nucleic acid sequence is labeled with a moiety selected from a gold nanoparticle, a protein binding ligand, a hapten, an antigen, a fluorescent compound, a dye, a radioactive isotope and an enzyme.

19. The device of any one of claims 13-18, wherein the target nucleic acid sequence is indicative of a pathogen or a cell present in the sample.

20. The device of any one of claims 13-19, wherein the amplification device is the amplification device of any one of claims 2-12.

21. A method for amplifying a target nucleic acid in a sample, the method comprising: providing the amplification device of claim 1;

adding a lysed sample to the sample loading area; and

heating the amplification reaction area to an effective temperature for an effective amount of time to produce amplicons of the target nucleic acid sequence.

22. The method of claim 21 further comprising adding an amplification medium to the amplification reaction area and/or adding a washing medium to the sample loading area after the amplification reaction area is heated.

23. The method of claim 22, wherein the method comprises adding an amplification medium to the amplification reaction area and adding a washing medium to the sample loading area after the amplification reaction area is heated.

24. The method of claim 21, the amplification reaction area comprises an amplification medium therein.

25. The method of claim 24 further comprising adding a washing buffer to the sample loading area after the amplification reaction area is heated.

26. The method of any one of claims 22-25, wherein the device has a soaking volume effective in retaining the amplification medium and the sample contained therein.

27. The method of claim 26, wherein adding a washing buffer is effective in washing the sample through the amplification device.

28. The method of claim 27, wherein the washing medium added to the amplification device is at a volume least 5 times greater than a volume of the sample added to the amplification device.
29. The method of any one of claims 21-29, wherein the lysed sample is added to the sample loading area by:
5 contacting a sample lysate with a plurality of buoyant, inorganic, nucleic-acid-capture microspheres;
contacting the sample loading area with the plurality of buoyant, inorganic, nucleic-acid-capture microspheres; and
10 adding an eluent to the sample loading area having the plurality of buoyant, inorganic, nucleic-acid-capture microspheres absorbed thereon.
30. The method of any one of claims 21-29, wherein the amplification device is the amplification device of any one of claim 2-12.
31. A method for detecting a target nucleic acid in a biological sample, the method comprising
15 providing the device for detecting the presence of a target nucleic acid sequence in a sample of claim 13
adding a lysed sample to the sample loading area;
heating the amplification reaction area to an effective temperature for an effective amount of time to produce amplicons of the target nucleic acid sequence; and
20 detecting the presence of the target nucleic acid sequence.
32. The method of claim 31, wherein the presence of the target nucleic acid sequence is detected by a trimolecular hybridization of the target nucleic acid sequence or an amplicon thereof.
33. The method of any one of claims 31 or 32 further comprising adding an amplification medium to the amplification reaction area and/or adding a washing medium to the sample loading are after the amplification reaction area is heated.
25
34. The method of claim 33, wherein the method comprises adding an amplification medium to the amplification reaction area and adding a washing medium to the sample loading are after the amplification reaction area is heated.
- 30 35. The method of any one of claims 31 or 32, the amplification reaction area comprising an amplification medium therein.

36. The method of claim 35 further comprising adding a washing medium to the sample loading area after the amplification reaction area is heated.
37. The method of any one of claims 31-36, wherein the amplification device has a soaking volume effective in retaining an amplification medium and the biological sample contained therein.
38. The method of claim 37, wherein adding a washing medium is effective is washing the sample through the amplification device and onto the diagnostic device.
39. The method of claim 38, wherein the washing medium added to the amplification device is at a volume least 5 times greater than a volume of the sample added to the amplification device.
40. The method of any one of claims 31-39, wherein the lysed sample is added to the sample loading area by:
contacting a sample lysate with a plurality of buoyant, inorganic, nucleic-acid-capture microspheres;
contacting the sample loading area with the plurality of buoyant, inorganic, nucleic-acid-capture microspheres; and
adding an eluent to the sample loading area having the plurality of buoyant, inorganic, nucleic-acid-capture microspheres absorbed thereon.
41. The method of any one of claims 31-48, wherein the device for detecting the presence of a target nucleic acid sequence in a sample is the device for detecting the presence of a target nucleic acid sequence in a sample of any one of claims 14-20.

Fig. 1A

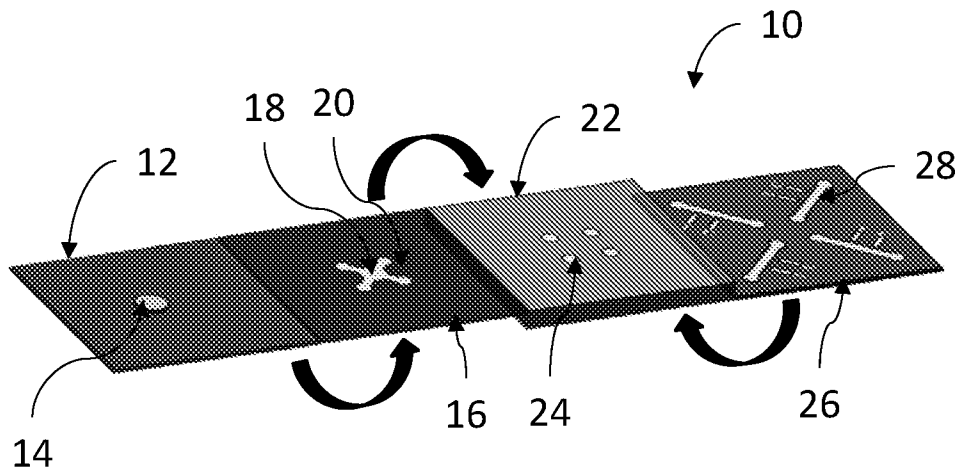


Fig. 1B

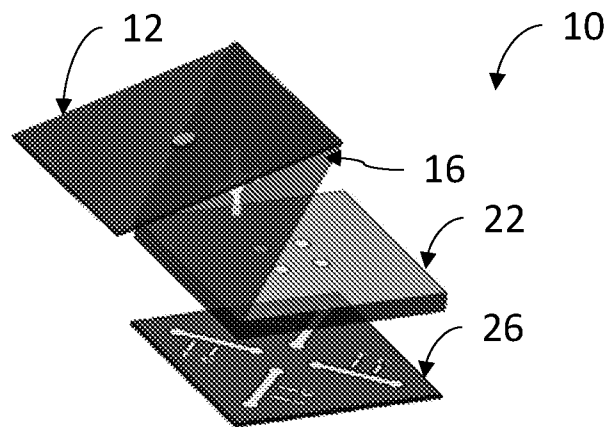


Fig. 1C

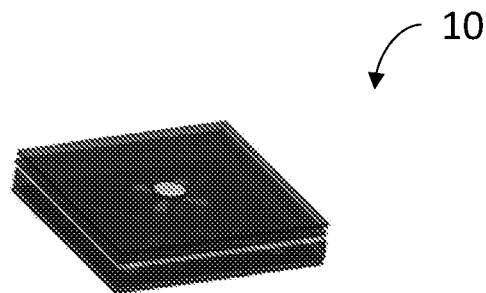


Fig. 2

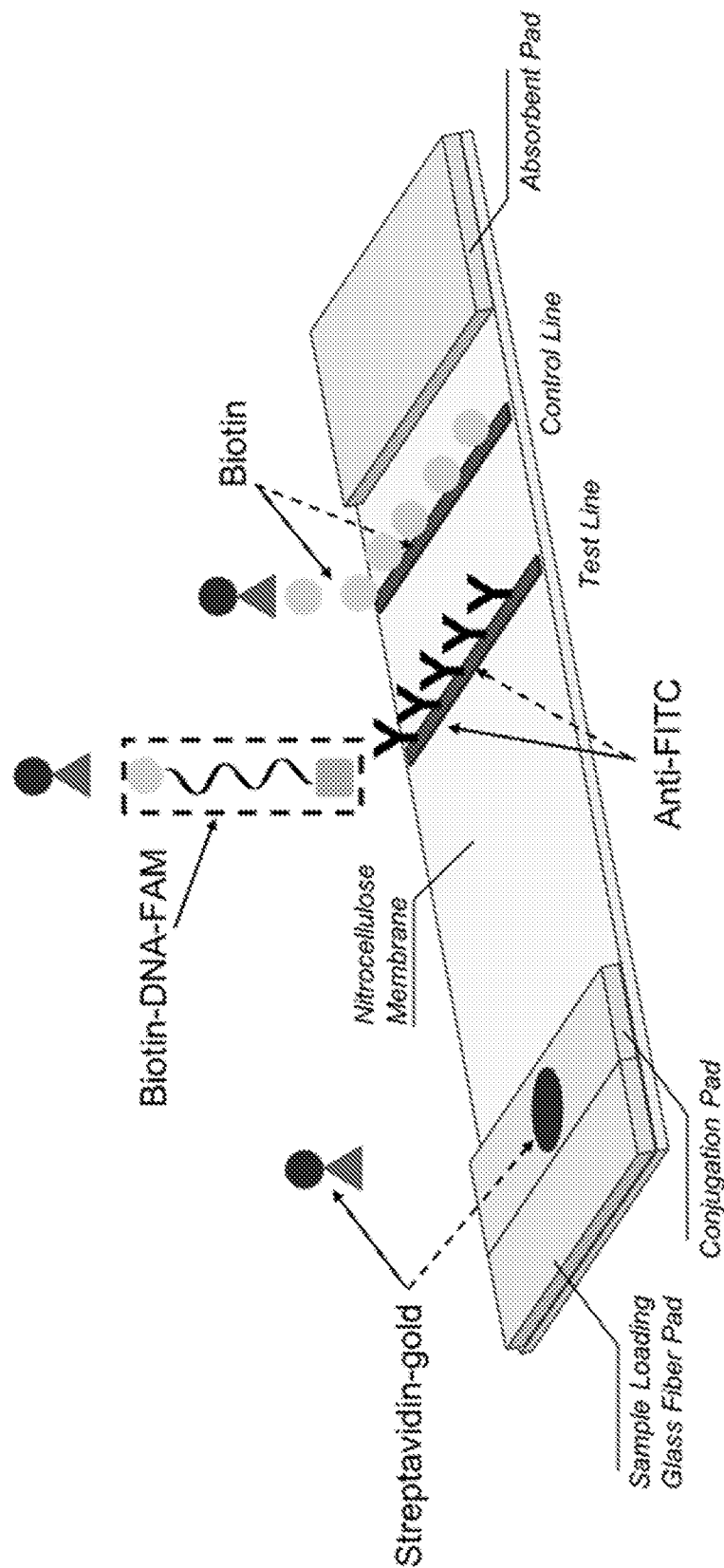


Fig. 3A

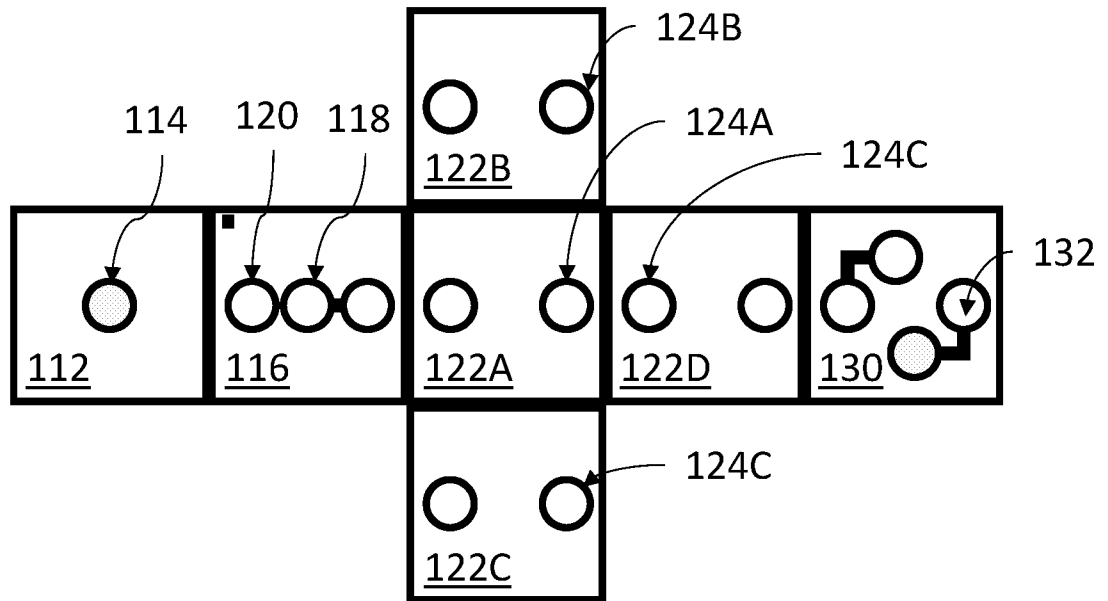


Fig. 3B

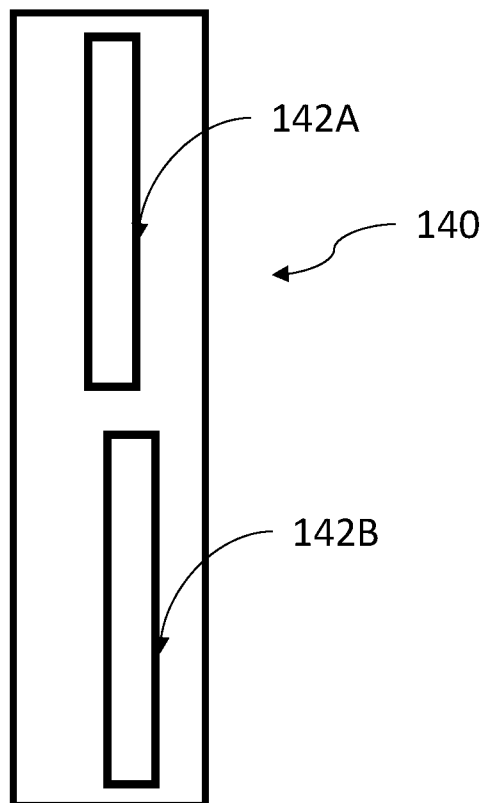


Fig. 3C

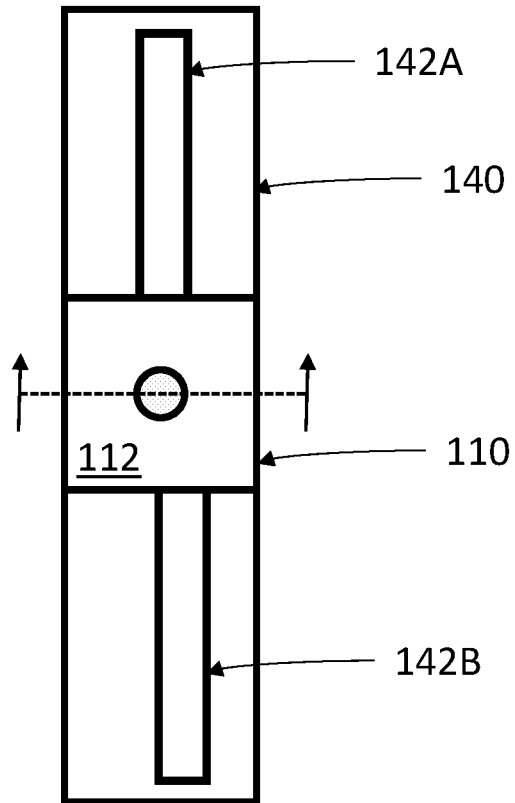


Fig. 3D

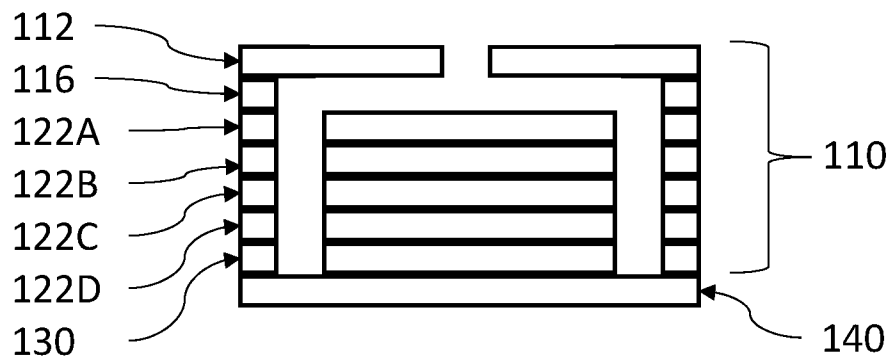


Fig. 4

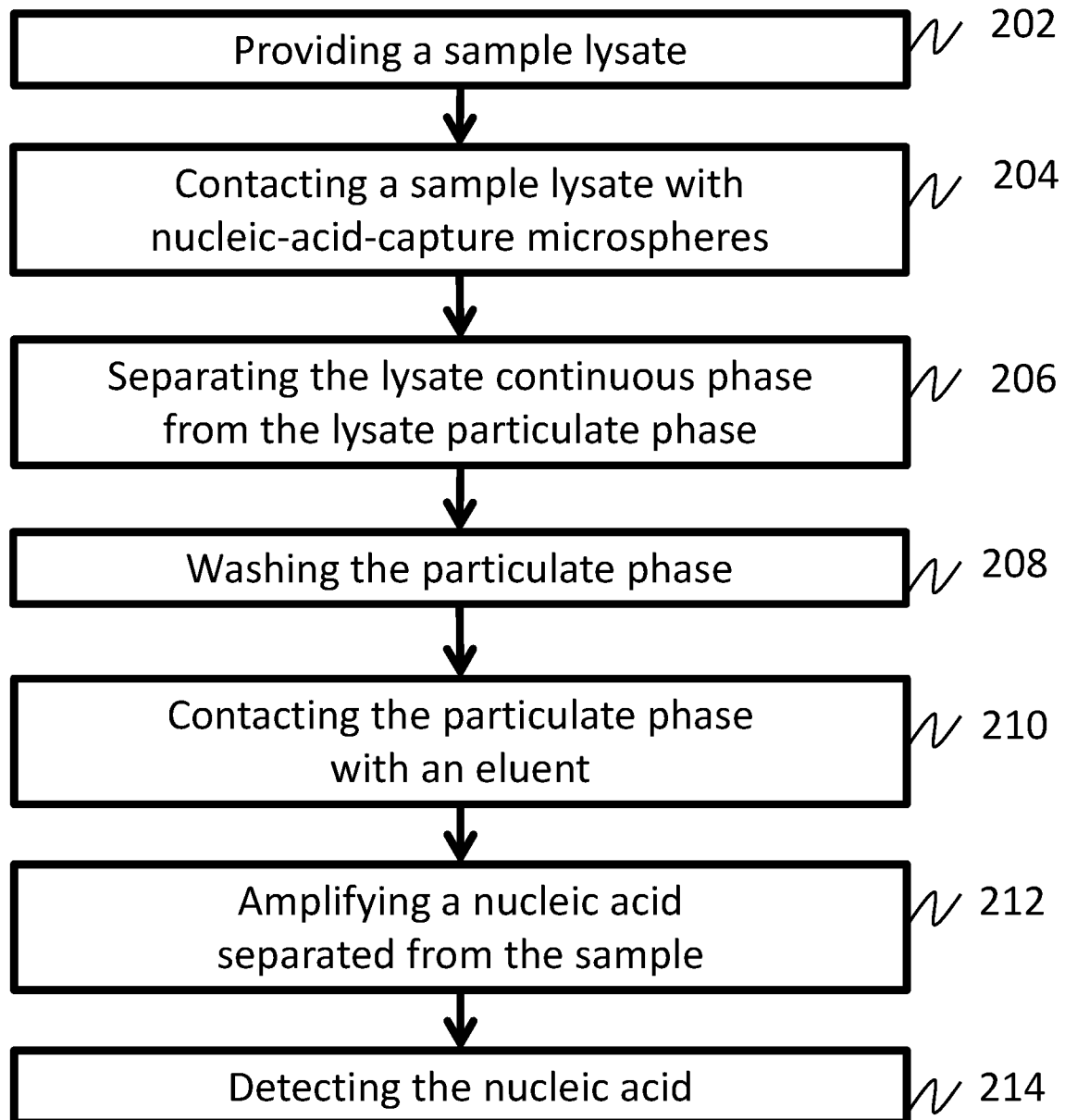
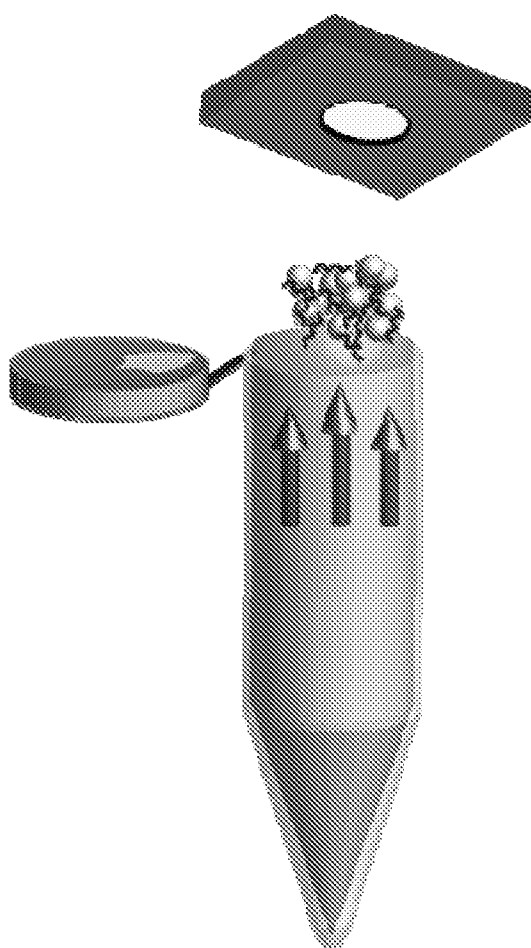


Fig. 5



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/23234

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

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

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/23234

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.: 12, 18-20, 29, 30, 37-41
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:
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.

Group I, claims 1-11, 13-17, directed to a microfluidic amplification device for amplifying a target nucleic acid sequence in a biological sample.

Group II, claims 21-28, 31-36, directed to a method for amplifying a target nucleic acid in a sample.

The inventions listed as Group I and Group 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:

***** See Supplemental Sheet to continue *****

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.: 1-11, 13-17

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.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/23234

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12M 1/00, C12Q 1/00, C12Q 1/68 (2019.01)

CPC - B01L 3/5023, C12Q 1/00, C12Q 1/6816, C12Q 1/6825, C12Q 1/6834, C12Q 1/6888, C12Q 1/689, C12Q 1/701, G01N 15/06, G01N 15/1056, G01N 15/1463, G01N 15/1484, G01N 33/487, B01L 2200/141, B01L 2300/069, B01L 2400/0406, G01N 2015/0693, G01N 2015/1006, Y02 A50/51, Y02A 50/52, Y02A 50/53, Y02A 50/58

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	SEOK, et al. A Paper-Based Device for Performing Loop-Mediated Isothermal Amplification with Real-Time Simultaneous Detection of Multiple DNA Targets. Theranostics. 2017, Vol 7, No 8, pages 2220-2230; Abstract; pg 2222, col 1; pg 2223; pg 2224, Fig 1 and its legend; pg 2226, col 2	1-11 ----- 13-17
X	WO 2016/187160 A1 (GODX INC.) 24 November 2016 (24.11.2016) para [0009], [00014], [00054]	1
Y	MORI, et al. Sequence specific visual detection of LAMP reactions by addition of cationic polymers. BMC Biotechnol. 10 January 2006, Vol 6, pages 3 (pp 1-10); Abstract; pg 4, Fig 2 and its legend; pg 5, Fig 3 and its legend	13-17

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

22 July 2019

Date of mailing of the international search report

05 AUG 2019

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/23234

In Continuation of Box III. Observations where unity of invention is lacking:

The inventions of Group II include the special technical feature of a method for amplifying a target nucleic acid in a sample that is not required by Group I.

Some inventions of Group I and Group II share the technical features of a microfluidic amplification device of claim 1. However, this shared technical feature does not represent an improvement over prior art as being anticipated by a paper titled "A Paper-Based Device for Performing Loop-Mediated Isothermal Amplification with Real-Time Simultaneous Detection of Multiple DNA Targets" by Seok, et al. (Theranostics, 1 June 2017, Vol 7, No 8, pp 2220-2230) (hereinafter "Seok").

Seok discloses a microfluidic amplification device for amplifying a target nucleic acid sequence in a biological sample (Abstract, "we propose a paper-based device for performing loop-mediated isothermal amplification (LAMP) with real-time simultaneous detection of multiple DNA targets"; pg 2224, Figure 1 and its legend, "Scheme and images of a paper-based device for performing loop-mediated isothermal amplification (LAMP) with real-time simultaneous detection of multiple DNA targets. (a) Schematic illustration of structure and sample flow"), the device comprising:

a sample loading panel, the sample loading panel comprising a sample loading area (pg 2224, Figure 1 and its legend, "(c) Image of fabrication process... ii) Fluidic channel pad stacked on the transfer pad. It is patterned by wax printing, resulting in four channels shown as blue circles. Blue aqueous ink was used for visualization of the fluidic channels. The smaller white circle in the center is the area for the sample injection hole"); and

one or more amplification panels, the amplification panel comprising an amplification reaction area (pg 2224, Figure 1 and its legend, "(b) Analysis principle of the reaction pad. The four reaction pads contain target-specific LAMP primers and reagents for the LAMP reaction in the dry condition. When sample solution contacts the reaction pad, the dried reagents are activated. The LAMP reaction is performed by activating the dried reagents"; pg 2223, col 2, "the paper device was designed with sample injection and the LAMP zone on the same side, which is advantageous for adding instruments that perform heating and fluorescence analysis"); wherein each of the panels comprises an absorbent material configured to wick an aqueous sample across a superimposed stack of the panels (pg 2224, Figure 1 and its legend, "(a) Schematic illustration of structure and sample flow. The structure of the paper device is fabricated by stacking of three functional layers including the transfer pad, fluidic channel pad, and reaction pad"; pg 2223, col 2, "The fabrication process is shown in Figure 1 (c). The device was fabricated by simple sequential stacking of functional layers (transfer pad, fluidic channel pad, reaction pad, and sealing tape), and is a small and compact structure suitable for POC testing in resource-limited settings"). As the technical features were known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the inventions.

Some inventions of Group I and Group II share the technical features of a device for detecting the presence of a target nucleic acid sequence in a sample (claim 13). However, these shared technical features do not represent an improvement over prior art as being obvious over Seok, as above, in view of a paper titled "Sequence specific visual detection of LAMP reactions by addition of cationic polymers" by Mori, et al. (BMC Biotechnol. 2006, Vol 6, p 3) (hereinafter "Mori").

Seok further discloses a device for detecting the presence of a target nucleic acid sequence in a sample, the device comprising: the amplification device of claim 1 (Abstract, "This proposed platform allowed analysis of three different meningitis DNA samples in a single device with single-step operation") and further comprising a diagnostic device comprising means for detection of the amplified nucleic acid sequence (pg 2224, Figure 1 and its legend, "(b) Analysis principle of the reaction pad... The fluorescence intensity of HNB [Hydroxynaphthol blue] is reduced during the LAMP reaction, which indicates the result of the analysis. (c) Image of fabrication process"; pg 2226, col 2, "In the optimized paper device, dried LAMP reagents and HNB were activated automatically and simultaneously. Figure 3 (b) shows that real-time LAMP analysis for three DNA targets was successfully performed in real time; pg 2228, Figure 3. LAMP analysis of the paper device with real-time simultaneous detection of multiple DNA targets. Target 1 (T1): 700 pg of *Streptococcus agalactiae* DNA. T2: 700 pg of *Streptococcus pneumoniae* DNA. T3: 700 pg of *Staphylococcus aureus* DNA. (a) Simultaneous detection of three DNA targets in the paper device. The fluorescence of HNB in the paper device was analyzed after a 60-min LAMP reaction. Each set of data is labeled with the type of injected target DNA"), but does not specifically disclose that said means for detection of the amplified nucleic acid sequence is a detectably labelled probe specific for the target nucleic acid sequence.

Mori discloses using detectably labelled probe specific for the target nucleic acid sequence (Abstract, "Oligo DNA probes labeled with different fluorescent dyes were prepared for multiple nucleic acid templates, and the templates were amplified by the LAMP reactions under the existence of the probes. At completion of the LAMP reaction, an optimal amount of low molecular weight polyethylenimine (PEI) was added, resulting in the precipitation of the insoluble LAMP amplicon-PEI complex. The fluorescently labeled Oligo DNA probes hybridized to the LAMP product were incorporated into the precipitation, and the precipitate emitted fluorescence corresponding to the amplified nucleic acid templates. The color of emitted fluorescence can be detected easily by naked eye on a conventional UV illuminator. Conclusion: The presence or absence of minute amount of nucleic acid templates could be detected in a simple manner through visual assessment for the color of the LAMP amplicon-PEI complex precipitate"). It would have been obvious to one of ordinary skill in the art at the time of the invention to combine, in the course of routine experimentation and with a reasonable expectation of success, Seok and Mori by substituting the HNB [Hydroxynaphthol blue]-based sequence non-specific nucleic acid detection disclosed by Seok with the sequence specific fluorescently labeled oligo DNA probes disclosed by Mori (Mori, pg 4, Figure 2 and its legend, "Sequence-specific visual detection method that utilizes precipitation titration of LAMP products by adding PEI Sequence-specific visual detection method that utilizes precipitation titration of LAMP products by adding PEI. (A) Results of sequence-specific visual detection after adding PEI to LAMP reaction solution. After LAMP reaction in the presence of both FITC-labeled HBV probes and ROX-labeled HCV probes followed by addition of the prescribed amount... of PEI (Mw = 600), it was centrifuged for several seconds using a desk-top, low-speed centrifuge. The tube was then visually observed as is on a UV illuminator (365 nm). It was possible to differentiate the LAMP reaction by visualizing the presence of precipitate fluorescence and the color of the fluorescence") in the device disclosed by Seok, to improve accuracy of the amplified nucleic acid detection. As the technical feature would have been obvious to one of ordinary skill in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the inventions.

Therefore, inventions of Group I and Group II lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.