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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 November 2016 (17.11.2016)

(10) International Publication Number
**WO 2016/183282 A1**

(51) International Patent Classification: *C12Q 1/68* (2006.01)

(21) International Application Number: PCT/US2016/032011

(22) International Filing Date: 12 May 2016 (12.05.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/160,591 12 May 2015 (12.05.2015) US

(71) Applicant: **GEN-PROBE INCORPORATED** [US/US]; 10210 Genetic Center Drive, San Diego, California 92121 (US).

(72) Inventors: **LINNEN, Jeffrey M.**; 13302 Bronco Way, Poway, California 92064 (US). **CHELLISERRY, Jijumon**; 13369 Cooper Greens Way, San Diego, California 92129 (US).

(74) Agent: **LANDES, Jeffrey E.**; Gen-probe Incorporated, 10210 Genetic Center Drive, San Diego, California 92121 (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, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, 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))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2016/183282 A1

**(54) Title**: METHOD OF POOLING BLOOD SAMPLES

**(57) Abstract**: The disclosure provides methods of pooled analysis of samples containing red blood cells, such as whole blood cell samples. The methods are particularly useful for screening whole blood samples collected from many individuals to be used for transfusions and the like. Aliquots of such samples are individually contacted with a lysis reagent, further described below, before pooling to lyse red blood cells in the samples. After pooling, the lysates, the combined lysate is tested for presence of target(s) characteristic of pathogen(s). The method are particularly useful for identifying pathogens of red blood cells, but can also be used to run a complete panel of testing including for all of the typical targets.

# METHOD OF POOLING BLOOD SAMPLES

## CROSS REFERENCE TO RELATED APPLICATIONS

**[0001]** This application claims the benefit of priority of U.S. provisional application no. 62/160,591, which was filed on May 12, 2015, and which is incorporated herein by reference.

## BACKGROUND

**[0002]** Blood transfusion has an essential role in patient management. Blood used for transfusion is often collected from many volunteers. Such samples are screened for transmissible infections, such as HIV-1, HIV-2, hepatatis B virus, hepatitis C virus, dengue virus, west nile virus, treponema pallidum, Anaplasma phagocytophilum, and depending on local prevalence, Trypanosoma cruzi and Plasmodium species among others.

**[0003]** Screening for additional pathogens would be desirable. For example, human Babesiosis is an emerging infectious disease which is a tick-borne intraerythrocytic infection. *Babesia microti*, the most common cause for human Babesiosis in the U.S is widely endemic in the Northeastern and upper Midwestern United States. This species has been implicated in majority of the transfusion-transmitted babesiosis (TTB) cases in the United States and is currently the most reported transfusion transmitted disease to the FDA. 159 well-documented cases of *B. microti* TTB were reported between 1979 and 2009 in the US (Herwaldt et al., Ann Intern Med 2011). 87% of transmitted infusions occurred in seven endemic US states. Twelve transfusion-associated fatalities associated with TTB have occurred since 2005.

**[0004]** Because the proportion of infected samples is low, screening can be simplified by pooling samples. If a pool sample is infected, the pool can be deconvoluted to identify the source of infection in an individual sample, but if the pool is not infected, all samples contributing to the pool can be assumed free of infection without individual testing.

**[0005]** Nucleic acid testing in minipools (of 6 to 24) is routinely used for screening for viruses (e.g., HBV, HIV) in donated blood after separating the whole blood to plasma or serum. However, plasma and serum cannot be used to detect pathogens of red blood cells. Additionally, whole blood cannot be pooled directly due to propensity for clotting when samples from different patients are mixed. Clots can also intefere with subsquent steps in the assay by altering the consistency of samples and clogging automated equipment.

## SUMMARY

**[0006]** Disclosed herein are methods for detecting a target in a sample aliquot comprising the steps of: providing a sample aliquot from each of a plurality of blood cell samples, thereby providing a plurality of sample aliquots; separately performing a lysing reaction on each of the sample aliquots, wherein the lysing step comprises contacting each of the sample aliquots with a lysis reagent comprising: a buffer, lithium lauryl sulfate (LLS), and at least one of a chloride containing salt and an anticoagulant selected from the group consisting of: EDTA, EDTA-$Na_2$, EGTA, and combinations thereof; wherein the reagent has a pH that is greater than 5.5, and whereby at least a portion of the blood cells in each of the sample aliquots lyse; combining the lysed sample aliquots into a single pooled sample to form a pooled lysate; separating a target from the pooled lysate; and performing a reaction to detect the separated target from the pooled lysate, wherein detection of the presence of the target in the pooled lysate indicates the presence of the target in at least one of the initially provided sample aliquots. In some embodiments, the methods further comprise, when the target is detected in the pooled lysate, individually testing the separate blood samples to identify which of the blood cell samples contain(s) the target. In some aspects, it is desired that the blood cell samples are free of the target, and as such samples determined to contain the target are discarded. In a non-limiting example of this aspect, a blood cell sample for use in blood banking is discarded if it is determined that a target is present in the sample. In some aspects, it is desired that the blood cell samples possess a target, and as such samples determined to contain the target are kept. In a non-limiting example of this aspect, a blood cell sample used for obtaining therapeutic targets is kept if it is determined that the target is present in the sample.

**[0007]** Disclosed herein are methods for separating a target from a plurality of sample aliquots, comprising the steps of: (a)providing a sample aliquot from each of a plurality of whole blood samples, thereby providing a plurality of sample aliquots; (b) separately performing a lysing reaction on each of the sample aliquots, wherein the lysing step comprises contacting each of the sample aliquots with a lysis reagent comprising (i) a buffer, (ii) lithium lauryl sulfate (LLS), and (iii) at least one of a chloride containing salt and an anticoagulant selected from the group consisting of: EDTA, EDTA-$Na_2$, EGTA, and combinations thereof; wherein the reagent has a pH that is greater than 5.5, and whereby at least a portion of the blood cells in each of the sample aliquots lyse; (c) combining the lysed sample aliquots into a single pooled sample to form a pooled lysate; and (d) separating a target from the pooled lysate. In some embodiments, the methods further comprise contacting the pooled lysate with a solid support configured to immobilize the target; and separating the immobilized target from the pooled lysate. In some embodiments, the target is a nucleic acid target and the solid support comprises an attached immobilized probe. In some embodiments wherein the target is a nucleic acid target, the methods further comprise contacting the pooled lysate with a

capture probe comprising a first segment complementary to the nucleic acid target, and a second segment complementary to the immobilized probe. In some aspects, the methods further comprise providing hybridization conditions that favor formation of a hybridization complex between the first segment of the capture probe and the nucleic acid target. In some aspects, the methods further comprise providing hybridization conditions that favor formation of a hybridization complex between the second segment of the capture probe and the immobilized probe attached to the solid support. In some embodiments, the solid support is a magnetic bead solid support. In some embodiments, the solid support is a silica solid support. in some aspects, the silica solid support is glass wool. In some aspects, the silica solid support is a bead. In some embodiments, the solid support is contained within a column.

**[0008]** In one embodiment of the methods, the target is a nucleic acid target, and wherein the pooled lysate is tested for the presence or absence of the nucleic acid target using a nucleic acid amplification reaction and a detection reaction. In one aspect, the amplification reaction is an isothermal amplification reaction. In one aspect, the amplification reaction comprises performing a transcription mediated amplification reaction to generate an amplification product and wherein the detection reaction comprises detecting the amplification product with a detection probe. In one aspect, the amplification reaction and the detection reaction are performed simultaneously. In one embodiment, one or more steps of the methods are performed on an automated device. In one aspect, the amplification reaction and the detection reaction are performed on an automated device. In one aspect, the step of separating the target from the pooled lysate is performed on an automated device. In one aspect, the lysing reaction, the pooling reaction, the target separating reaction, and the nucleic acid analysis reaction are all performed on an automated device. In one aspect, the automated device is an integrated device that performes all of the automated steps. In one aspect, the automated device is a collection of spatially separated devices that each perform one or more of the automated steps.

**[0009]** In one embodiment of methods, the lysing reaction is performed on at least 4 sample aliquots. In one embodiment of methods, the lysing reaction is performed on at least 16 sample aliquots. In one embodiment of the methods, the lysing reaction is performed on at least 20 sample aliquots. In one embodiment of the methods, the lysing reaction is performed on up to 200 sample aliquots. In one embodiment of the methods, the lysing reaction is performed on from about 4 sample aliquots to about 200 aliquots.

**[00010]** In one embodiment of the methods, the buffer is sodium bicarbonate. In one aspect, the buffer is sodium bicarbonate and the reagent comprises a chloride containing salt that is ammonium chloride. In one aspect, the buffer is sodium bicarbonate, the reagent comprises a chloride containing salt that is ammonium chloride, and the pH of the reagent is from about 7.0 to about 8.0. In one aspect, the ammonium chloride is present in the reagent at a concentration from about 100 mM to

about 500 mM, or at a concentration of about 250 mM. In one aspect, the sodium bicarbonate is present in the reagent at a concentration of about 5 mM to about 30 mM, or at a concentration of about 14 mM. In one aspect, the reagent comprises an anti-coagulant that is EDTA-$Na_2$ present in the reagent at a concentration of about 1 mM, EGTA present in the reagent at a concentration of about 1 mM, or is a combination of EDTA-$Na_2$ and EGTA, wherein the EDTA-$NA_2$ is present in the reagent at a concentration of about 1 mM and the EGTA is present in the reagent at a concentration of about 1 mM. In one aspect, the the lysis reagent comprises (i) about 14 mM sodium bicarbonate, (ii) about 5% (v/v) LLS, (iii) a chloride containing salt and an anticoagulant, wherein the chloride containing salt is ammonium chloride and the ammonium chloride is present in the reagent at a concentration of about 250 mM, and wherein the anti-coagulant is EDTA and the EDTA is present in the reagent at a concentration from about 0.1 mM to about 10 mM, and wherein the pH of the reagent is from about 7.2 to about 7.5. Ranges include all whole and partial numbers therein.

**[00011]** In one embodiment of the methods, the buffer is a TRIS buffer. In one embodiment, the reagent comprises a chloride salt that is magnesium chloride, and the magnesium chloride is present in the reagent at a concentration from about 20 mM to about 35 mM, or at a concentration of about 30 mM. In one aspect, the TRIS buffer is present in the reagent at a concentration from about 75 mM to about 150 mM, or at a concentration of about 100 mM. In one aspect, the reagent comprises a TRIS buffer and further comprises a chloride salt that is magnesium chloride, and the magnesium chloride is present in the reagent at a concentration from about 20 mM to about 35 mM, or at a concentration of about 30 mM. In one aspect, the lysis reagent comprises about 100 mM TRIS buffer and about 6% (v/v) LLS, and wherein the pH of the reagents is about 7.5. Ranges include all whole and partial numbers therein.

**[00012]** In one embodiment of the reagent, the LLS is present in the reagent at a concentration from about 4% (v/v) to about 15% (v/v), or at a concentration of about 10% (v/v), or at a concentration of about 8% (v/v), or at a concentration of about 6% (v/v), or at a concentration of about 5% (v/v). In one aspect, the buffer is sodium bicarbonate buffer. In one aspect, the buffer is TRIS buffer. Ranges include all whole or partial numbers therein.

**[00013]** In one embodiment, the reagent comprises a sodium phosphate buffer, about 10% (v/v) LLS, and an anti-coagulant, wherein the anti-coagulant is EDTA-$Na_2$ present in the reagent at a concentration of about 1 mM, EGTA present in the reagent at a concentration of about 1 mM, or is a combination of EDTA-$Na_2$ and EGTA, wherein the EDTA-$NA_2$ is present in the reagent at a concentration of about 1 mM and the EGTA is present in the reagent at a concentration of about 1 mM.

**[00014]** In one embodiment, the blood cell samples are whole blood samples. In one aspect, the blood cell samples are human whole blood samples. In one aspect, one or more of the blood cell samples are human whole blood samples and one or more of the blood cell samples are non-human whole blood samples. In one aspect, the blood cell samples contain red blood cells. In one aspect, the blood cell samples contain white blood cells.

**[00015]** In one embodiment, each sample aliquot is contacted with the lysis reagent at a volume ratio of sample aliquot to lysis reagent that is from about 1:2 to about 1:10 (v/v). In one aspect, the volume ratio of sample aliquot to lysis reagent is selected from the group consisting of: 1:2 (v/v), 1:3 (v/v), 1:4 (v/v), 1:5 (v/v), 1:6 (v/v), 1:7 (v/v), 1:8 (v/v), 1:9 (v/v), and 1:10 (v/v). In one aspect, the volume ratio of sample aliquot to lysis reagent is about 1:3 (v/v). In one aspect, the volume ratio of sample aliquot to lysis reagent is about 1:4 (v/v). Ranges include all whole and partial numbers therein.

**[00016]** In one embodiment, the target is a host-derived target. In one embodiment, the target is a pathogen-derived target. In one aspect, the target is released from a red blood cell. In one aspect, the target is a protein target. In one aspect, the target is a nucleic acid target. In one aspect, the target is an RNA target. In one aspect, the target is a ribosomal RNA target. In one aspect, the target is a pathogen derived target derived from a pathogen selected from the group consisting of: hepatitis viruses, human immunodeficiency viruses, dengue viruses, west nile viruses, flaviviruses, zika virus, and parasitic organisms. In one aspect, the target is a tpathogen-derived target derived from a parasitic organism selected from the group consisting of: parasites from the genus *Babesia*, parasites from the genus *Plasmodium*, parasites from the genus *Trypanosoma*, parasites from the genus *Leishmania*, parasites from the genus *Anaplasma*, parasites from the genus *Toxoplasma*, *Babesia microti, Babesia divergens, Babesia duncani*, *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, and Plasmodium knowlesi.*

**[00017]** In one embodiment of the methods, wherein the target is a nucleic acid target, the separating step comprises contacting the pooled lysate with a capture probe and an immobilized probe, the capture probe having a first segment complementary to the nucleic acid target, and a second segment complementary to the immobilized probe, wherein the nucleic acid target binds to the capture probe, and wherein the bound capture probe binds to the immobilized probe. In one aspect, the immobilized probe is attached to a solid support. In one aspect, the pooled lysate is contacted with a solid support configured to immobilize the target; and separating the immobilized target from the pooled lysate. In one aspect, there are provided hybridization conditions that favor formation of a hybridization complex between the first segment of the capture probe and the nucleic acid target. In one aspect there are provided hybridization conditions that favor formation of a hybridization complex between the second segment of the capture probe and the immobilized probe attached to the solid support. In one aspect, the solid support is a magnetic bead solid support. In one aspect, the solid support is a

silica solid support. In one aspect, the silica solid support is glass wool. In one aspect, the silica solid support is a bead. In one aspect, the solid support is contained within a column. In one aspect, the methods are performed without a centrifugation step.

**[00018]** In one embodiment, the entire volumes of the lysed sample aliquots are combined. In one aspect, 25% or less of volumes of the lysed sample aliquots are combined. In one aspect, at least 50% of the blood cells are lysed in five minutes or less after contacting the sample aliquot with the lysis reagent.

## DEFINITIONS

**[00019]** "Pathogens" include viruses, bacteria, protozoa, fungi, and other microorganisms responsible for disease in humans and other animals. Exemplary pathogens include, but are not limited to, hepatitis viruses, human immunodeficiency viruses, dengue viruses, west nile viruses, flaviviruses, zika virus, and parasitic organisms. Exemplary parasitic organisms include, but are not liminted to, parasites from the genus *Babesia*, parasites from the genus *Plasmodium*, parasites from the genus *Trypanosoma*, parasites from the genus *Leishmania*, parasites from the genus *Anaplasma*, parasites from the genus *Toxoplasma*, *Babesia microti, Babesia divergens, Babesia duncani, Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, and Plasmodium knowlesi*.

**[00020]** As used herein the term "target" can be a single type of molecule, such as a specific protein or nucleic acid from a pathogen or host cell. Alternatively, a target can be a class of molecules such as any protein target or nucleic acid target from a pathogen or human cell. For example, a target that is a single type of nucleic acid target from a pathogen can be a ribosomal RNA target from a parasite, such as 18S rRNA target from a *Babesia* organism, or a target that is a class of nucleic acid from a pathogen can be all RNA from a whole blood sample suspected of being infected by a pathogen. Targets from host cells can be therapeutic targets, such as, for example, an antibody or a protein (such as a clotting factor). Multiple distinct targets can also be analyzed, such as an RNA target and a protein target, or two distinct RNA targets, such as two different mRNA targets, or an mRNA target and an rRNA target. Targets include endogenous components of the host's blood cells (host-derived targets) and components arising as a result of pathogenic infection of infected red blood cells and are typically encoded by the infecting pathogen (i.e., "pathogenic" or "pathogen-derived" targets).

**[00021]** A "lysis reagent" as referred to herein, is a reagent, often provided in the form of a solution, effective for inducing lysis of blood cells in a sample, including whole blood samples, samples containing red blood cells, samples containing white blood cells and sample containing red blood cell products such as pelleted red blood cells. In some instances, the lysis reagent preferentially lyses red

blood cells. Preferential lysis of red blood cells over other cellular components of blood means that the percentage of red blood cells lysed is higher than that of other cellular components present in the sample being analyzed, the other cell types being assessed in the aggregate. A number of lysis reagents are useful for the lysate pooling methods described herein. Preferred lysis reagents are comprised of: a buffer, lithium lauryl sulfate (LLS), and at least one of a chloride containing salt and an anticoagulant selected from the group consisting of: EDTA, EDTA-$Na_2$, EGTA, and combinations thereof; wherein the reagent has a pH that is greater than 5.5.

**[00022]** As used herein, a "sample aliquot" refers to a smaller volume of a sample. Typically, the sample aliquot is removed from the larger sample volume for testing. Each of the sample aliquots are preferably separately lysed using a lysis reagent as described herein. Lysed sample aliquots are then combined to form a pooled lysate. The pooled lysate contains any target present in one or more of the now lysed sample aliquots, along with other components such as non-target material released from within the blood cells, and unlysed blood cells. As used herein, a plurality of sample aliquots refers to two or more sample aliquots, each of the sample aliquots being pulled from two or more samples. The sample aliquots are preferably not combined until after the sample aliquots are mixed with the lysis reagent under conditions to promote lysis of at least a portion of the blood cells in the sample aliquot.

**[00023]** Anionic detergents are compounds with a negatively charged, anionic head group and a long hydrocarbon tail, often provided as a salt with an alkali metal or ammonium ion.

**[00024]** Anti-coagulants inhibit clotting of whole blood. Anti-coagulants include heparins and calcium chelating agents. Heparins activate antithrombin III, which inhibits the activity of thrombin and other proteases involved in blood clotting. Calcium chelating agents, such as EDTA (Ethylenedinitrilo)tetraacetic acid), EDTA-Na2 (Disodium ethylenediaminetetraacetate dihydrate), EGTA (Ethylene-bis(oxyethylenenitrilo)tetraacetic acid), and citrate, bind calcium ions required for blood clotting.

**[00025]** A buffer refers to a weak acid or weak base used to maintain the pH of a solution. Preferred buffers for use herein include, but are not limited to sodium bicarbonate, TRIS (2-Amino-2-(hydroxymethyl)-1,3-propanediol), sodium phosphate dibasic, and sodium phosphate monobasic.

**[00026]** A nucleic acid refers to a multimeric compound comprising nucleotides or analogs that have nitrogenous heterocyclic bases or base analogs linked together to form a polymer, including conventional RNA, DNA, mixed RNA-DNA, and analogs thereof.

**[00027]** The nitrogenous heterocyclic bases can be referred to as nucleobases. Nucleobases can be conventional DNA or RNA bases (A, G, C, T, U), base analogs, and others (*See e.g.*, The

Biochemistry of the Nucleic Acids 5-36; Adams et al., ed., 11.sup.th ed., 1992; van Aerschott et al., 1995, Nucl. Acids Res. 23(21): 4363-70; Nair et al., 2001, Nucleosides Nucleotides Nucl. Acids, 20(4-7):735-8; Hill et al., 1998, Proc. Natl. Acad. Sci. USA 95(8):4258-63; Lin and Brown, 1992, Nucl. Acids Res. 20(19):5149-52; Okamoto et al., 2002, Bioorg. Med. Chem. Lett. 12(1):97-9; Nguyen et al., 1998, Nucl. Acids Res. 26(18):4249-58; Kiopffer & Engels, 2005, Nucleosides Nucleotides Nucl. Acids, 24(5-7) 651-4; Babu & Wengel, 2001, Chem. Commun. (Camb.) 20: 2114-5; Hrdlicka et al., 2005, J. Am. Chem. Soc. 127(38): 13293-9; U.S. Pat. No. 5,378,825; WO 93/13121; Gamper et al., 2004, Biochem. 43(31): 10224-36; *and* Berger et al., 2000, Nucl. Acids Res. 28(15): 2911-4). Many derivatized and modified nucleobases or analogues are commercially available (*e.g.*, Glen Research, Sterling, Va.).

**[00028]** A nucleobase unit attached to a sugar, can be referred to as a nucleobase unit, or monomer. Sugar moieties of a nucleic acid can be ribose, deoxyribose, or similar compounds, *e.g.*, with 2' methoxy or 2' halide substitutions. Nucleotides and nucleosides are examples of nucleobase units. The nucleobase units can be joined by a variety of linkages or conformations, including phosphodiester, phosphorothioate or methylphosphonate linkages, peptide-nucleic acid linkages (PNA; Nielsen et al., 1994, Bioconj. Chem. 5(1): 3-7; PCT No. WO 95/32305), and a locked nucleic acid (LNA) conformation in which nucleotide monomers with a bicyclic furanose unit are locked in an RNA mimicking sugar conformation (Vester et al., 2004, Biochemistry 43(42):13233-41; Hakansson & Wengel, 2001, Bioorg. Med. Chem. Lett. 11 (7):935-8), or combinations of such linkages in a nucleic acid strand. Nucleic acids may include one or more “abasic” residues, *i.e.*, the backbone includes no nitrogenous base for one or more positions (U.S. Pat. No. 5,585,481).

**[00029]** A nucleic acid may include only conventional RNA or DNA sugars, bases and linkages, or may include both conventional components and substitutions (*e.g.*, conventional RNA bases with 2'-O-methyl linkages, or a mixture of conventional bases and analogs). Inclusion of PNA, 2'-methoxy or 2'-fluoro substituted RNA, or structures that affect the overall charge, charge density, or steric associations of a hybridization complex, including oligomers that contain charged linkages (*e.g.*, phosphorothioates) or neutral groups (*e.g.*, methylphosphonates) may affect the stability of duplexes formed by nucleic acids.

**[00030]** An oligomer may contain a “random polymer” sequence that refers to a population of oligomers that are substantially the same in overall length and other characteristics, but in which at least a portion of the oligomer is synthesized by random incorporation of different bases for a specified length, *e.g.*, a random assortment of all four standard bases (A, T, G, and C) in a DNA oligomer, or a random assortment of a few bases (U or T and G) in a defined portion of a larger oligomer. The resulting oligomer is actually a population of oligomers whose finite number of members is determined by the length and number of bases making up the random portion (*e.g.*, $2^6$

oligomers in a population of oligomers that contains a 6-nt random sequence synthesized by using 2 different bases).

**[00031]** Complementarity of nucleic acids means that a nucleotide sequence in one strand of nucleic acid, due to orientation of its nucleobase groups, hydrogen bonds to another sequence on an opposing nucleic acid strand. The complementary bases typically are, in DNA, A with T and C with G, and, in RNA, C with G, and U with A. Complementarity can be perfect (*i.e.*, exact) or substantial/sufficient. Perfect complementarity between two nucleic acids means that the two nucleic acids can form a duplex in which every base in the duplex is bonded to a complementary base by Watson-Crick pairing. "Substantial" or "sufficient" complementary means that a sequence in one strand is not completely and/or perfectly complementary to a sequence in an opposing strand, but that sufficient bonding occurs between bases on the two strands to form a stable hybrid complex in set of hybridization conditions (*e.g.*, salt concentration and temperature). Such conditions can be predicted by using the sequences and standard mathematical calculations to predict the Tm of hybridized strands, or by empirical determination of Tm by using routine methods. Tm refers to the temperature at which a population of hybridization complexes formed between two nucleic acid strands are 50% denatured. At a temperature below the Tm, formation of a hybridization complex is favored, whereas at a temperature above the Tm, melting or separation of the strands in the hybridization complex is favored. Tm may be estimated for a nucleic acid having a known G+C content in an aqueous 1 M NaCl solution by using, *e.g.*, Tm=81.5+0.41(% G+C), although other known Tm computations take into account nucleic acid structural characteristics.

**[00032]** "Separating" or "isolating" or "purifying" refers to removing one or more components from a complex mixture, such as a sample. Preferably, a separating, isolating or purifying step removes at least 70%, preferably at least 90%, and more preferably at least 95% w/w of the target nucleic acids from other sample components. A separating, isolating or purifying step may optionally include additional washing steps to remove non-target sample components.

**[00033]** "Release" of a capture hybrid refers to separating one or more components of a capture hybrid from each other, such as separating a target nucleic acid from a capture probe, and/or a capture probe from an immobilized probe. Release of the target nucleic acid strand separates the target from other components of a capture hybrid and makes the target available for binding to a detection probe. Other components of the capture hybrid may remain bound, *e.g.*, the capture probe strand to the immobilized probe on a capture support, without affecting target detection.

**[00034]** A "label" refers to a molecular moiety that is detectable or produces a detectable response or signal directly or indirectly, *e.g.*, by catalyzing a reaction that produces a detectable signal. Labels include luminescent moieties (such as fluorescent, bioluminescent, or chemiluminescent compounds),

radioisotopes, members of specific binding pairs (*e.g.*, biotin and avidin), enzyme or enzyme substrate, reactive groups, or chromophores, such as a dye or particle that results in detectable color.

**[00035]** A "capture probe" includes a first segment including a target-complementary region of sequence and a second segment for attaching the capture probe, or a hybridization complex that includes the capture probe, to an immobilized probe. The first segment can be configured to substantially complementary to a specific target nucleic acid sequence so that a first segment and a target nucleic acid can hybridize to form a stable duplex (*i.e.*, having a detectable melting point) under hybridizing conditions, such as described in the Examples. Alternatively, the first segment can be configured to nonspecifically bind to nucleic acid sequences in a sample under hybridizing conditions (see WO 2008/016988). The second segment includes a region of sequence that is complementary to a sequence of an immobilized probe. Preferably, a chimeric capture probe includes a nucleic acid homopolymer (*e.g.*, poly-A or poly-T) that is covalently attached to the target-complementary region of the capture probe and that hybridizes under appropriate conditions to a complementary homopolymer of the immobilized probe (*e.g.*, poly-T or poly-A, respectively) as previously described (U.S. Pat. No. 6,110,678 to Weisburg et al.). Capture probes may further comprise a third segment that acts as a closing sequence to inactivate unbound target capture probes in a capture reaction. This third segment can flank the first segment opposite the second segment (*e.g.*, capture sequence:target hybridizing sequence:closing sequence) or it can flank the second segment opposite the first segment (*e.g.*, closing sequence:capture sequence:target hybridizing sequence). See WO 2006/007567 and US 2009-0286249.

**[00036]** An "immobilized probe" includes a nucleic acid joined directly or indirectly to a support. The nucleic acid is complementary to a nucleic acid in the capture probe, although may or may not be the same length (number of nucleobase units) as the in the capture probe. The nucleic acid in the immobilized probe preferably contains at least six contiguous nucleobase units and can contain for example 10-45 or 10-40 or 10-30 or 10-25 or 15-25, inclusively, L-nucleobase units. The nucleic acid is preferably a homopolymer, and more preferably a homopolymer of adenine or thymine. A preferred form of immobilized probe is or includes a homopolymer of 14 thymine residues for use in combination with a capture probe including a second segment with a homopolymer of adenine residues. The nucleic acid moiety of an immobilized probe is typically provided in single-stranded form, or if not, is denatured to single-stranded form before or during use.

**[00037]** As used herein, a "solid support" is any of a variety of materials that are useful as a support for the immobilized probes, *e.g.*, matrices or particles made of nitrocellulose, nylon, silica, polyacrylate, mixed polymers, polystyrene, silane polypropylene, and magnetically attractable materials. Solid supports in the shape of a sheet, a bead/sphere, awool/fiber and other common shapes are useful with the methods. Monodisperse magnetic beads are a preferred support because they are

relatively uniform in size and readily retrieved from solution by applying a magnetic force to the reaction container, preferably in an automated system. The solid supports may be broadly dispursed in a slurry or they may be packed into a column. An immobilized probe may be linked directly to the capture support, *e.g.*, by using any of a variety of covalent linkages, chelation, or ionic interaction, or may be linked indirectly via one or more linkers joined to the support. The linker can include one or more nucleotides not intended to hybridize to the capture probe but to act as a spacer between the nucleic acid of the immobilized probe and its support.

**[00038]** A "detection probe" is a nucleic acid or other molecule that binds specifically to a target sequence and which binding results, directly or indirectly, in a detectable signal to indicate the presence of the target sequence. A detection probe need not be labeled to produce a detectable signal, *e.g.*, an electrical impulse resulting from binding the probe to its target sequence may be the detectable signal. A "labeled probe" is a probe that contains or is linked, directly or indirectly, to a label (*e.g.*, Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd ed., Chapt. 10; U.S. Pat. No. 6,361,945, Becker et al.; U.S. Pat. No. 5,658,737, Nelson et al.; U.S. Pat. No. 5,656,207, Woodhead et al.; U.S. Pat. No. 5,547,842, Hogan et al.; U.S. Pat. No. 5,283,174, Arnold et al.; U.S. Pat. No. 4,581,333, Kourilsky et al.; U.S. Pat. No. 5,731,148, Becker et al.). For example, detection probes may include a non-nucleotide linker and a chemiluminescent label attached to the linker (U.S. Pat. Nos. 5,185,439, 5,585,481 and 5,639,604, Arnold et al.). Examples of detection probes include oligonucleotides of about 5 to 50 nucleotides in length having an attached label that is detected in a homogeneous reaction, *e.g.*, one that uses differential hydrolysis of a label on a bound or unbound probe.

**[00039]** Detection probes can have a nucleotide sequence that is of the same or opposite sense as a target sequence depending on the format of the assay. Detection probes can hybridize to the same or different segment of a target sequence as a capture probe. Some detection probes have an attached chemiluminescent marker, *e.g.*, an acridinium ester (AE) compound (U.S. Pat. Nos. 5,185,439, 5,639,604, 5,585,481, and 5,656,744). In some detection probes, an acridinium ester label is attached to a central region of the probe near a region of A and T/U base pairs by using a non-nucleotide linker (U.S. Pat. Nos. 5,585,481 and 5,656,744, Arnold, et al.) which restricts the amines of the nucleotide bases on both sides of the AE and provides a site for intercalation. Alternatively, an AE label may be attached to the 3' or 5' terminus of the detection probe which is used in conjunction with a second oligomer that hybridizes adjacent to the detection probe on the target nucleic acid to restrict the effects of nearby amine contributed by the target nucleic acid. In some detection probes, an AE label at or near the site of a mismatch with a related non-target polynucleotide sequence, to permit discrimination between the related sequence and the target sequence that may differ by only one nucleotide because the area of the duplex around the mismatch site is sufficiently destabilized to

render the AE on the probe hybridized to the related non-target sequence susceptible to hydrolysis degradation. HIV-1 and HCV may be detected using a modified form of the commercial PROCLEIX® ULTRIO assay from Grifols International, S.A. (Spain). The modification involves replacing the D-polyA and D-polyT sequences in capture and immobilized probes with L-poly A and L-poly-T, respectively.

**[00040]** "Hybridization condition" refers to the cumulative environment in which one nucleic acid strand bonds to a second nucleic acid strand by complementary strand interactions and hydrogen bonding to produce a hybridization complex. Such conditions include the chemical components and their concentrations (*e.g.*, salts, chelating agents, formamide) of an aqueous or organic solution containing the nucleic acids, and the temperature of the mixture. Other factors, such as the length of incubation time or reaction chamber dimensions may contribute to the environment (*e.g.*, Sambrook et al., Molecular Cloning, A Laboratory Manual, 2.sup.nd ed., pp. 1.90-1.91, 9.47-9.51, 11.47-11.57 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989)).

**[00041]** Specific binding of a target capture oligomer to a target nucleic or target nucleic acids means binding between a single defined sequence in the first segment of a target capture oligomer and an exactly or substantially complementary segment on target nucleic acid(s) to form a stable duplex. Such binding is detectably stronger (higher signal or melting temperature) than binding to other nucleic acids in the sample lacking a segment exactly or substantially complementary to the single defined target capture oligomer sequence. Non-specific binding of a target capture oligomer to target nucleic acids means that the target capture oligomer can bind to a population of target sequences that do not share a segment having exact or substantial complementarity to a single defined target capture oligomer sequence. Such can be achieved by for example using a randomized sequence in the first segment of the capture probe.

**[00042]** "Release" of a capture hybrid refers to separating one or more components of a capture hybrid from each other, such as separating a target nucleic acid from a capture probe, and/or a target capture oligomer from an immobilized probe. Release of the target nucleic acid strand separates the target from other components of a capture hybrid and makes the target available for binding to a detection probe. Other components of the capture hybrid may remain bound, *e.g.*, the target capture oligomer strand to the immobilized probe on a capture support, without affecting target detection.

**[00043]** "Sensitivity" is the proportion of true positives correctly identified as such (*e.g.* the percentage of infected blood samples as having the infection). Sensitivity can also be characterized by a lower limit of detection. For example, at pathogen concentration of 1 particle per mL blood before pooling, an assay detects at least 95% of infected samples. The lower the limit of detection, the higher the sensitivity of the assay. Specificity measures the proportion of true negatives which are

correctly identified (*e.g.* the percentage of uninfected blood samples correctly identified as not having the infection.)

**[00044]** Reference to a range of values also includes integers within the range and sub-ranges defined by integers in the range. Reference to any numerical value or range of numerical values should be understood as encompassing any such variation as is inherent in measuring that value other typical conditions of use.

## DETAILED DESCRIPTION

### I. General

**[00045]** The disclosure provides methods of pooled analysis of samples containing blood cells, such as whole blood cell samples. The methods are particularly useful for screening whole blood samples collected from many individuals to be used for transfusions and the like. Before pooling, aliquots of such samples are individually contacted with a lysis reagent, further described below, to lyse red blood cells in the samples. After pooling, the combined lysate or a specimen thereof is tested for presence of target(s) characteristic of pathogen(s). By lysing prior to pooling, multiple target molecules (e.g., RNA) can be released prior to pooling, increasing the probability that an infected sample will be detected against a background of uninfected samples with which it is pooled. The method are particularly useful for identifying pathogens of red blood cells, but can also be used to run a complete panel of testing including at least for all of the typical targets described in the Background.

### II. Lysis Reagents

**[00046]** The lysis reagent, which was first described in copending application WO 2016/064887 , filed October 20, 2015, incorporated by reference, includes at least a buffer, lithium lauryl sulfate (LLS), and at least one of a chloride containing salt and an anticoagulant selected from the group consisting of: EDTA, EDTA-Na2, EGTA, and combinations thereof.

**[00047]** In some embodiments, the buffer is a sodium bicarbonate buffer. In some embodiments, the buffer is a TRIS (2-Amino-2-(hydroxymethyl)-1,3-propanediol) buffer. In some embodiments, the buffer is a sodium bicarbonate buffer. In some embodiments, the buffer is a sodium phosphate buffer. In some embodiments, the buffer is a sodium bicarbonate buffer in the reagent in a concentration from about 5 mM to about 30 mM, from about 10 mM to about 20 mM, about 10 mM to about 15 mM, or from about 15 mM to about 20 mM. In some embodiments, the buffer is a TRIS buffer in the reagent at a concentration from about 75 mM to about 150 mM, from about 75 mM to about 125 mM, from about 100 mM to about 125 mM, or from about 90 mM to about 110 mM. In some embodiments, the buffer is a sodium phosphate ($Na_3PO_4$) buffer in the reagent at a concentration from about 5 mM to

about 30 mM, from about10 mM to about 20 mM, about 10 mM to about 15 mM, or from about 15 mM to about 20 mM. In some embodiments, the concentration of sodium phosphate in the reagent is from about 8 mM to about 40 mM, from about 10 mM to about 33 mM, from about 15 mM to about 30 mM, about 30 mM, or about 15 mM. In some embodiments, the concentration of sodium phosphate monobasic in the reagent is from about 8 mM to about 40 mM, from about 10 mM to about 33 mM, from about 15 mM to about 30 mM, about 30 mM, or about 15 mM. In some embodiments, the concentration of sodium phosphate dibasic in the reagent is from about 8 mM to about 40 mM, from about 10 mM to about 33 mM, from about 15 mM to about 30 mM, about 30 mM, or about 15 mM. Ranges include all whole and partial numbers therein.

**[00048]** In some embodiments, the anti-coagulant is one or more of EDTA ((Ethylenedinitrilo)tetraacetic acid), EDTA-$Na_2$ (Disodium ethylenediaminetetraacetate dihydrate), EGTA (Ethylene-bis(oxyethylenenitrilo)tetraacetic acid), heparin, or citrate. In some embodiments, the anti-coagulant comprises an EDTA in the reagent at a concentration from about 0.05 mM to about 15 mM, from about 0.1 mM to about 10 mM, or from about 0.5 mM to about 5 mM. In some embodiments, the anti-coagulant is EDTA in the reagent at a concentration from about 0.05 mM to about 15 mM, from about 0.1 mM to about 10 mM, or from about 0.5 mM to about 5 mM. In some embodiments, the anti-coagulant is EDTA-$Na_2$ in the reagent at a concentration from about 0.05 mM to about 15 mM, from about 0.1 mM to about 10 mM, or from about 0.5 mM to about 5 mM. In some embodiments, the anti-coagulant is EGTA in the reagent at a concentration from about 0.05 mM to about 15 mM, from about 0.1 mM to about 10 mM, or from about 0.5 mM to about 5 mM. Ranges include all whole and partial numbers therein.

**[00049]** In some embodiments, the salt comprises one or more of the following ions: a sodium ion, a potassium ion, an ammonium ion, a magnesium ion, a lithium ion, and a chloride ion. In some embodiments, the salt is magnesium chloride, ammonium chloride, potassium chloride, or sodium chloride. In some embodiments, the salt comprises a chloride ion and one of a magnesium ion, sodium ion or potassium ion, and the concentration of the salt in the reagent is from about 10 mM to about 50 mM, from about 15 mM to about 40 mM, or from about 20 mM to about 35 mM. In some embodiments, the salt is ammonium chloride in the reagent at a concentration from about 100 mM to about 500 mM, from about 200 mM to about 350 mM, or from about 250 mM to about 300 mM. Ranges include all whole and partial numbers therein.

**[00050]** In some embodiments, the detergent is one of lithium lauryl sulfate (LLS), nonyl phenoxypolyethoxylethanol (NP 40), sodium dodecyl sulfate (SDS), and Triton-X 100. In some embodiments, the detergent is an anionic detergent. In some embodiments, the detergent is LLS or SDS. In some embodiments, the detergent is present in the reagent at a concentration that is greater than about 1.5% (v/v). In some embodiments, the detergent is present in the reagent at a

concentration that is less than about 15.5% (v/v). In some embodiments, the detergent is present in the reagent at a concentration of from about 2% to about 15% (v/v). In some embodiments, the detergent is present in the reagent at a concentration from about 2% to about 15% (v/v). In some embodiments, the detergent is LLS and the concentration of LLS in the reagent is from about 2% to about 15% (v/v), from about 4% to about 10% (v/v), or from about 5% to about 8% (v/v). In some embodiments, the detergent is LLS and is present in the reagent at about 14 mM to about 50 mM. Ranges include all whole and partial numbers therein.

**[00051]** In some embodiments, the pH of the reagent is greater than a pH of 5.5. In some embodiments, the pH of the reagent is less than a pH of 10.5. In some embodiments, the pH of the reagent is from about 6.0 to about 10.0. In some embodiments, the pH of the reagent is from about 6.5 to about 8.0, or from about 7.0 to about 8.0, or from about 7.2 to about 7.6, or from about 6.7 to about 7.5, or about 6.7, or about 7.3 or about 7.5. Ranges include all whole and partial numbers therein.

**[00052]** In some embodiments of the reagent, the concentration of sodium bicarbonate is 14 mM, the concentration of ammonium chloride is 250 mM, the concentration of LLS is 8% (v/v), the concentration of EDTA is from about 0.1 mM to about 10 mM, and the pH is 7.2-7.6. In some embodiments of the reagent, the buffer is selected from the group consisting of sodium bicarbonate, sodium phosphate and TRIS, the detergent is from about 5% to about 10 % (v/v), the pH is from about 6.5 to about 8.0, and the salt is selected from the group consisting of magnesium chloride, ammonium chloride, and potassium chloride. In some embodiments of the reagent, the buffer is selected from the group consisting of sodium phosphate and TRIS and the salt is selected from the group consisting of magnesium chloride and ammonium chloride. In some aspects of this embodiment, the concentration of anti-coagulant is about 0 mM to about 1 mM. In some further aspects of this embodiment, the detergent is LLS at a concentration from about 6% to about 10% (v/v). In some further aspects of this embodiment, the buffer is TRIS at a concentration from about 90 mM to about 110 mM and the pH of the reagent is from about 7.2 to about 7.5. In some further aspects of this embodiment, the anti-coagulant is at a concentration of about 0.1 mM to about 5 mM and is EGTA, EDTA, EDTA-$Na_2$ or a combination thereof.

**[00053]** The lysis reagent serves to lyse blood cells, protect a released target from degradation in the lysate by nucleases or proteases, and is compatible with subsequent steps for use of the target, such as target capture, amplification, detection, and/or sequencing. The lysis reagent is particularly amenable for analysis of RNA from pathogens infecting red blood cells, including parasitic organisms such as *Babesia and Plasmodium species*. The lysis reagent is compatible with methods of nucleic acid detection without removal of the lysis reagent from samples

**III. Use of Lysis Reagents**

**[00054]** Blood cells can be obtained from any available source, such as whole blood or any fraction thereof including those containing red blood cells, such as pelleted red blood cells. Whole blood can be human whole blood, or non-human whole blood, or a combination thereof.

**[00055]** The lysis reagent can be admixed with blood cells for a time sufficient to induce cell lysis and cause release of the desired target(s) from cells. Exemplary times for maintaining blood cells admixed with lysis reagent include 1-30 minutes, 2-15 minutes, 3-10 minutes, 4-6 minutes, or 5 minutes. Preferably, the time is at least 5 minutes. Preferably the mixture lacks visible particles after lysis.

**[00056]** The temperature of incubation of the lysis reagent with blood cells can vary. The temperature is preferably chosen to maximize extent and rate of lysis to minimize degradation of target(s) or prevent inhibition of subsequent processing. Exemplary temperature ranges include 0-50°C, 5-45°C, 10-40°C, 15-37°C, 20-30°C, 22-27°C, or 25°C. Ambient temperature is suitable. Lysis of blood cells should release a sufficient amount of target molecules to be detectable by the methods described herein. Preferably lysis results in at least 50%, 60%, 70%, 80%, 90%, or 100% lysis of blood cells in a sample being lysed. Ranges include all whole and partial numbers therein.

**[00057]** The ratio at which a blood cell sample is combined with lysis reagent can affect the extent and rate of cell lysis and protection of target molecules from degradation after release from lysed cells. Exemplary ratios in which a blood cell sample is admixed with the lysis reagent include ratios of about 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, or in a range of ratios between 1:1 and 1:5 or 1:10 (v/v; sample:reagent). A preferred ratio is a sample aliquot admixed with the lysis reagent at a ratio of about 1:3 (v/v; sample:reagent). Another preferred ratio is a sample aliquot admixed with the lysis reagent at a ratio of about 1:4 (v/v; sample:reagent). When the sample comprises red blood cells isolated from whole blood, such as pelleted red blood cells, the red blood cells can be admixed with the lysis reagent at exemplary ratios of 1:1, 1:2, 1:3, 1:4, 1:5, 1:10, or in a range of ratios between 1:1 and 1:10 (v/v; red blood cells:reagent). Ranges include all whole and partial numbers therein.

**IV. Targets**

**[00058]** Targets released from blood cells by the present lysis reagent can include host-derived targets and pathogen derived targets. Targets include protein targets (e.g., peptide targets and antibody targets), nucleic acid targets (e.g., DNA target or RNA target), whole particles, lipids and carbohydrates. The RNA targets can be ribosomal RNA (rRNA) targets, messenger RNA (mRNA) targets, or heterogeneous nuclear RNA (hnRNA) targets. A preferred target for pathogen-derived targets is a ribosomal RNA target, particularly 18S rRNA targets, 5S rRNA targets, 5.8S rRNA

targets, or 28S rRNA targets. Targets can be released from white blood cells, red blood cells or be present in plasma. Targets include therapeutic targets. Therapeutic targets are preferably host derived targets (such as peptide targets (e.g., thrombin) and antibody targets).

**[00059]** Exemplary pathogenic organisms include hepatitis viruses, human immunodeficiency viruses, dengue viruses, west nile viruses, flaviviruses, zika virus, and parasitic organisms. Exemplary parasitic organisms include parasites from the genus *Babesia*, parasites from the genus *Plasmodium*, parasites from the genus *Trypanosoma*, parasites from the genus *Leishmania*, parasites from the genus *Anaplasma*, parasites from the genus *Toxoplasma*, *Babesia microti, Babesia divergens, Babesia duncani*, *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, and Plasmodium knowlesi*.

## V. Assays

**[00060]** Target molecules released from lysis of a sample aliquot containing blood cells are subject to analysis. Target molecules may or may not be separated from the lysis reagent (by centrifugation or otherwise) before analysis. Omission of a separation step can facilitate efficient work flow in performing the assay. The type of assay depends on the targets. The assay can be a real-time or end-point assay.

### A. Nucleic Acids

**[00061]** Analysis of nucleic acid targets often involves steps of target capture, amplification and detection. Alternatively, amplification and detection methods can be performed without prior target capture. Preferably amplification, and detection and target capture (if performed) occur without separation of target molecules from the lysis reagent. Thus, the entire process can be performed in a single vessel.

#### 1. Target Capture Assay

**[00062]** An exemplary target capture assay can be performed as follows using one or more capture probes, an immobilized probe, a sample, and a suitable medium to permit hybridization of the target capture oligomer to the target nucleic acid and of target capture oligomer to the immobilized probe. The target sample (preferably herein the target sample is the pooled lysate) can be heated (*e.g.*, from 65°C to 95°C) before performing the assay to denature any nucleic acids in double-stranded form. The components can be mixed in any order. For example the target capture oligomer can be added to the target sample and hybridized with the nucleic acid target in the target sample before adding the immobilized probe. However, for an automated assay, it is preferable to minimize the number of adding steps by supplying the target capture oligomer and immobilized probe at the same or

substantially the same time. In this case, the order of hybridization can be controlled by performing a first hybridization under conditions in which a duplex can form between the first segment of the target capture oligomer and the nucleic acid target but which exceeds the melting temperature of the duplex that would form between first and second segments of the capture probe and between the target capture oligomer and immobilized probe. Next is performed a second hybridization under conditions of reduced stringency, preferably below the melting temperature of the duplexes formed between the first segment of the target capture oligomer the nucleic acid target. Under the second hybridization conditions a duplex can form between the second segment of the capture probe and the immobilized probe. Stringency can be reduced by lowering the temperature of the assay mix. For example, the higher stringency hybridization can be performed at or around 60°C and the lower stringency hybridization by allowing cooling to room temperature or 25°C. Stringency can also be reduced by reducing salt concentration or adding or increasing concentration of a chaotropic solvent. In some methods, all steps (with the possible exception of an initial denaturation step at higher temperature to denature double stranded target) can be performed isothermally.

**[00063]** Following formation of the nucleic acid target:capture probe:immobilized probe hybrid (the capture complex), the capture complex is separated away from other sample components. One method for separating the capture complex from other sample components is by physically separating the solid support comprising the capture complex from other sample components by using any of a variety of known methods, *e.g.*, centrifugation, filtration, or magnetic attraction of a magnetic capture support. The separation is preferably performed at a temperature below the melting temperature of the duplexes formed between the nucleic acid target, the target capture oligomer, and the immobilized probe. In some methods, the separation is performed at a temperature less than but within 10°C of the melting temperature of the stem-loop structure (*e.g.*, at 60°C) to maintain stringency of hybridization conditions and consequent ability to distinguished matched and unmatched target nucleic acids. Another method includes allowing the capture complex to remain attached to a solid support placed in a column while the sample components elute out of the column and into one or more containers. The column comprising the capture complex is then subjected to eluting conditions to allow the captures nucleic acid target to elute into a separate container. ((See *e.g.*, US 6,110,678)

**[00064]** To further facilitate isolation of the target nucleic acid from other sample components that adhere non-specifically to any portion of the capture hybrid, the capture hybrid may be washed one or more times to dilute and remove other sample components. Washing may be accomplished by dissociating the capture hybrid into its individual components in an appropriate aqueous solution (*e.g.*, a solution containing Tris and EDTA. See *e.g.*, US 6,110,678) and appropriate conditions (*e.g.*, temperature above the $T_m$ of the components) and then readjusting the conditions to permit reformation of the capture hybrid. However, for ease of handling and minimization of steps, washing

preferably rinses the intact capture hybrid attached to the capture support in a solution by using conditions that maintain the capture hybrid. Preferably, capture of the target nucleic acid with washing, if performed, isolates at least 70%, preferably at least 90%, and more preferably about 95% of the target nucleic acids away from other sample components. Isolated nucleic acids can be used for a number of downstream processes, such as nucleic acid amplification.

**[00065]** A target capture assay may also be performed as part of a real-time, biphasic, target capture and amplification method. In such a method, 500 µL of sample and 400 µL of target capture reagent (TCR) are added to reaction tubes. The TCR contains magnetic particles, components to lyse organisms present in the sample, capture oligos, a T7 initiation promoter, and an internal calibrator. Fluid in the reaction tubes is mixed for a specific time and speed to ensure the mixture is homogeneous. Reaction tubes are then transferred to a transition incubator at 43.7°C to preheat the fluid in the reaction tubes. Reaction tubes are then transferred to an anneal incubator set at 64°C. During incubation at 64°C, any organisms present in the sample that were not previously disrupted by the lysis reagent are disrupted, causing release of the target. Reaction tubes are then moved to a transition incubator to start a cool down process, and are further cooled in a chiller ramp (17°C to 19°C), leading to binding of the T7 initiation promoter and capture of both the target and the internal calibrator to the magnetic particles via the capture oligos. The reaction tubes are moved to a magnetic parking station where they are subjected to magnets which pull the magnetic particles to the sides of the tubes prior to entering a wash station. In the wash station, potential interfering substances are removed from the reaction by washing the magnetic particles.

### 2. Amplification

**[00066]** A nucleic acid analyte can be amplified using methods such as isothermal amplification reactions (e.g., transcription mediated amplification (TMA), nucleic acid sequence based amplification (NASBA), loop mediated isothermal amplification, polymerase spiral reaction (PSR) (Liu, W. et al. Polymerase Spiral Reaction (PSR): A novel isothermal nucleic acid amplification method. Sci. Rep. 5, 12723; (2015)), ligase chain reaction, and other isothermal amplification methods), or temperature cycling amplification reactions (e.g., polymerase chain reaction (PCR), quantitative PCR (qPCT), real time PCR (rt-PCT), or other temperature cycling amplification methods), or other amplification methods. Detection of the amplified RNA analyte products can be performed during amplification (real-time) or following amplification (end-point).

#### i. Transcription Mediated Amplification

**[00067]** TMA has been previously described (*e.g.*, U.S. Pat. Nos. 5,399,491, 5,554,516, 5,824,518 and 7,833,716; and also *e.g.*, F. Gonzales and S. McDonough. Applications of Transcription-

Mediated Amplification to Quantification of Gene Sequences. Gene Amplification. 1998 Ed. François Ferre, Birkhauser, Boston. PP. 189-204). In TMA, a target nucleic acid that contains the sequence to be amplified is provided as single stranded nucleic acid (*e.g.*, ssRNA or ssDNA). Any conventional method of converting a double stranded nucleic acid (*e.g.*, dsDNA) to a single-stranded nucleic acid may be used. A promoter primer binds specifically to the target nucleic acid at its target sequence and a reverse transcriptase (RT) extends the 3' end of the promoter primer using the target strand as a template to create a cDNA copy, resulting in a RNA:cDNA duplex. RNase activity (*e.g.*, RNase H of RT enzyme) digests the RNA of the RNA:cDNA duplex and a second primer binds specifically to its target sequence in the cDNA, downstream from the promoter-primer end. Then RT synthesizes a new DNA strand by extending the 3' end of the second primer using the cDNA as a template to create a dsDNA that contains a functional promoter sequence. RNA polymerase specific for the functional promoter initiates transcription to produce about 100 to 1000 RNA transcripts (amplified copies or amplicons) complementary to the initial target strand. The second primer binds specifically to its target sequence in each amplicon and RT creates a cDNA from the amplicon RNA template to produce a RNA:cDNA duplex. RNase digests the amplicon RNA from the RNA:cDNA duplex and the target-specific sequence of the promoter primer binds to its complementary sequence in the newly synthesized DNA and RT extends the 3' end of the promoter primer as well as the 3' end of the cDNA to create a dsDNA that contains a functional promoter to which the RNA polymerase binds and transcribes additional amplicons that are complementary to the target strand. Autocatalytic cycles that use these steps repeatedly during the reaction produce about a billion-fold amplification of the initial target sequence. Optionally, amplicons may be detected during amplification (real-time detection) or at an end point of the reaction (end-point detection) by using a probe that binds specifically to a sequence contained in the amplicons. Detection of a signal resulting from the bound probes indicates the presence of the target nucleic acid in the sample.

**[00068]** TMA may also be performed as part of a real-time, biphasic, target capture and amplification method. In such a method, TMA can be performed by adding amplification reagent (50 μL /test) to reaction tubes containing captured target molecules and mixing in an amplification load station. The amplification reagent contains oligos and components necessary to build nucleic acids. The reaction tubes are moved to a transition incubator at 43.7°C to increase the temperature of the liquid in the reaction tubes, which are then moved back to the amplification load station where enzyme (25 μL /test) is added. Reaction tubes are moved to the amplification incubator set at 42.7°C and remain in the incubator for five minutes, during which the first rounds of amplification are initiated. Reaction tubes are moved back to the amplification load station where promoter reagent (25 μL /test) is added. Reaction tubes are moved back to the amplification incubator for further rounds of target amplification. The promoter reagent contains oligos and torches. The torches are complementary to the target or internal calibrator and fluoresce when bound, generating signal in

real-time. The signals for the target and internal calibrator preferably have different wavelengths and can be distinguished.

### ii. Polymerase Chain Reaction

**[00069]** Alternatively, PCR amplification (*e.g.*, reverse transcriptase or real-time PCR) can be used for amplification. PCR can be performed with or without prior release of the target nucleic acid from the capture complex. The PCR reaction can be performed in the same vessel (*e.g.*, a microfuge tube) as the capture step. The PCR reaction involves thermocycling between a high temperature of about 95°C (*e.g.*, 90-99°C) for dissociation and a low temperature of about 60°C *e.g.*, 40-75, or 50-70 or 55-64°C) for annealing. Typically, the number of complete thermocycles is at least 10, 20, 30 or 40. PCR amplification is performed using one or more primer pairs. A primer pair used for PCR amplification includes two primers complementary to opposite strands of a target nucleic acid flanking the region desired to be sequenced. For sequencing most of a viral genome (*e.g.*, more than 50, 75 or 99%), the primers are preferably located close to the ends of the viral genome. For amplification of related molecules (*e.g.*, mutant forms of the same virus present in a patient sample), the primers are preferably complementary to conserved regions of the target nucleic acid likely to be present in most members of the population. PCR amplification is described in PCR Technology: Principles and Applications for DNA Amplification (ed. H.A. Erlich, Freeman Press, NY, NY, 1992); PCR Protocols: A Guide to Methods and Applications (eds. Innis, et al., Academic Press, San Diego, CA, 1990); Mattila et al., Nucleic Acids Res. 19, 4967 (1991); Eckert et al., PCR Methods and Applications 1, 17 (1991); PCR (eds. McPherson et al., IRL Press, Oxford); and U.S. Patent 4,683,202.

### 3. Detection

**[00070]** Detection of a nucleic acid target can be performed following capture and either during (real-time) or following (end-point) amplification by using any known method. The amplification product of RNA is often in the form of DNA resulting from RT-PCR or RNA copies resulting from TMA. Amplified nucleic acids may be detected in solution phase or by concentrating them in or on a matrix and detecting labels associated with them (*e.g.*, an intercalating agent such as ethidium bromide). Some detection methods use probes complementary to a sequence in the amplified product and detect the presence of the probe:product complex, or use a complex of probes to amplify the signal detected from amplified products (*e.g.*, U.S. Pat. Nos. 5,424,413, 5,451,503 and 5,849,481). Other detection methods use a probe in which signal production is linked to the presence of the target sequence because a change in signal results only when the labeled probe binds to amplified product, such as in a molecular beacon, molecular torch, or hybridization switch probe (*e.g.*, U.S. Pat. Nos. 5,118,801, 5,210,015, 5,312,728, 5,538,848, 5,541,308, 5,656,207, 5,658,737,

5,925,517, 6,150,097, 6,361,945, 6,534,274, 6,835,542, and 6,849,412; and U.S. Pub. No. 2006/0194240 A1). Such probes typically use a label (*e.g.*, fluorophore) attached to one end of the probe and an interacting compound (*e.g.*, quencher) attached to another location of the probe to inhibit signal production from the label when the probe is in one conformation ("closed") that indicates it is not hybridized to amplified product, but a detectable signal is produced when the probe is hybridized to the amplified product which changes its conformation (to "open"). Detection of a signal from directly or indirectly labeled probes that specifically associate with the amplified product indicates the presence of the target nucleic acid that was amplified.

**4. Sequencing**

**[00071]** Following amplification, a target nucleic acid as well as or instead of undergoing qualitative or quantitative detection can be sequenced. Purification if desired can be performed on a silica column (*e.g.*, a Qiagen gravity flow column). The target nucleic acid binds to the column, where it can be washed and then eluted. Alternatively, purification can be performed using a nucleic acid probe-based purification system (*e.g.*, U.S. Pat. Nos. 6,110,678 or 8,034,554,. US 2013/0209992 or US 2009/0286249, or. WO 2012/037531 or WO 2013/116774). The amplified target DNA can also be adapted for some sequencing formats by attachment of an adapter. The amplified DNA can be tailed by Klenow-mediated addition of nucleotides (usually a homopolymer) followed by annealing to an oligonucleotide complementary to the added tail, and ligation. Depending on the sequencing platform used, special adaptors are ligated to the template before sequencing. For example, a SMRT bell adapter is ligated to the sample template for sequencing with a Pacific Biosciences' PacBio *RS* sequencer (*see, e.g.,* Travers *et al.* Nucl. Acids Res. (2010) 38 (15): e159).

**[00072]** The amplified target nucleic acid is suitable for sequence analysis by a variety of techniques. The capture of target nucleic acid can be coupled to several different formats of so-called next generation and third generation sequencing methods. Such methods can sequence millions of target templates in parallel. Such methods are particularly useful when the target nucleic acid is a heterogeneous mixture of variants. Among the many advantages, sequencing variants in parallel provides a profile of drug resistant mutations in the sample, even drug mutations present in relatively minor proportions within the sample.

**[00073]** Some next generation sequence methods amplify by emulsion PCR. A target nucleic acid immobilized to beads via a target capture oligomer provides a suitable starting material for emulsion PCR. The beads are mixed with PCR reagents and emulsion oil to create individual micro reactors containing single beads (Margulies et al., Nature 437, 376-80 (2005)). The emulsion is then broken and the individual beads with amplified DNA are sequenced. The sequencing can be pyrosequencing performed for example using a Roche 454 GS FLX sequencer (454 Life Sciences,

Branford, CT 06405). Alternatively, sequencing can be ligation/detection performed for example using an ABI SOLiD Sequencing System (Life Technologies, Carlsbad, CA 92008). In another variation, target nucleic acids are eluted from beads having target capture oligomers and are immobilized in different locations on an array (*e.g.*, the HiScanSQ (Illumina, San Diego, CA 92121)). The target nucleic acids are amplified by bridge amplification and sequenced by template directed incorporation of labeled nucleotides, in an array format (Illumina). In another approach, target nucleic acids are eluted from the target capture oligomer and single molecules are analyzed by detecting in real-time the incorporation nucleotides by a polymerase (single molecule real time sequencing or SMRT sequencing). The nucleotides can be labeled nucleotides that release a signal when incorporated (*e.g.*, Pacific Biosciences, Eid et al., Sciences 323 pp. 133 – 138 (2009) or unlabeled nucleotides, wherein the system measures a chemical change on incorporation (*e.g.*, Ion Torrent Personal Genome Machine (Life Technologies)).

**[00074]** Although captured target nucleic acids can be sequenced by any technique, third generation, next generation or massively parallel methods offer considerable advantages over Sanger and Maxam Gilbert sequencing. Several groups have described an ultrahigh-throughput DNA sequencing procedure (see. *e.g.*, Cheeseman, US Pat. No. 5,302,509, Metzker et al., Nucleic Acids Res. 22: 4259 (1994)). The pyrosequencing approach that employs four natural nucleotides (comprising a base of adenine (A), cytosine (C), guanine (G), or thymine (T)) and several other enzymes for sequencing DNA by synthesis is now widely used for mutation detection (Ronaghi, Science 281, 363 (1998); Binladin et al., PLoS ONE, issue 2, e197 (February 2007); Rehman et al., American Journal of Human Genetics, 86, 378 (March 2010); Lind et al., Next Generation Sequencing: The solution for high-resolution, unambiguous human leukocyte antigen typing, Hum. Immunol. (2010), doi 10.1016/jhumimm.2010.06.016 (in press); Shafer et al., J Infect Dis. 1;199(5):610 (2009)). In this approach, the detection is based on the pyrophosphate (PPi) released during the DNA polymerase reaction, the quantitative conversion of pyrophosphate to adenosine triphosphate (ATP) by sulfurylase, and the subsequent production of visible light by firefly luciferase. More recent work performs DNA sequencing by a synthesis method mostly focused on a photocleavable chemical moiety that is linked to a fluorescent dye to cap the 3'-OH group of deoxynucleoside triphosphates (dNTPs) (Welch et al. Nucleosides and Nucleotides 18, 197 (1999) & European Journal, 5:951-960 (1999); Xu et al., US Pat. No. 7,777,013; Williams et al., US Pat. No. 7,645,596; Kao et al, US Pat. No. 6,399,335; Nelson et al., US Pat. Nos. 7,052,839 & 7,033,762; Kumar et al., US Pat. No. 7,041,812; Sood et al, US Pat. App. No. 2004-0152119; Eid et al., Science 323, 133 (2009)). In sequencing-by-synthesis methodology, DNA sequences are being deduced by measuring pyrophosphate release on testing DNA/polymerase complexes with each deoxyribonucleotide triphosphate (dNTP) separately and sequentially. See Ronaghi et al., Science 281: 363 365 (1998); Hyman, Anal. Biochem. 174, 423 (1988); Harris, US Pat. No. 7,767,400.

### B. Other Targets

**[00075]** Antibodies, proteins, particles and other targets can be detected by formats such as immunoprecipitation, Western blotting, ELISA, radioimmunoassay, competitive and immunometric assays. See Harlow & Lane, Antibodies: A Laboratory Manual (CSHP NY, 1988); U.S. Patent Nos. 3,791,932; 3,839,153; 3,850,752; 3,879,262;4,034,074, 3,791,932; 3,817,837; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876. Sandwich assays are a preferred format (see US 4,376,110, 4,486,530, 5,914,241, and 5,965,375).

**[00076]** Competitive assays can also be used. In some methods, target antigen in a sample competes with exogenously supplied labeled target antigen for binding to an antibody detection reagent. The amount of labeled target antigen bound to the antibody is inversely proportional to the amount of target antigen in the sample. The antibody can be immobilized to facilitate separation of the bound complex from the sample prior to detection.

**[00077]** Lateral flow devices can also be used for detecting a target. Fluid is applied to a test strip that has been treated with a sample in which a target may be present. Labelled binding molecules pass through the strip and can be captured as they pass into a specific zone containing the sample with the target.

## VI. Sensitivity

**[00078]** The present methods can provide a high sensitivity of detection of an a target from a sample aliquot containing blood cells, such as whole blood, white blood cells, red blood cells, or other preparation of red blood cells. For pathogen-derived RNA targets, sensitivity can be expressed as a minimum number of pathogenic RNA copies present in a volume of whole blood. The volume of whole blood can be that contacted with lysis reagent directly, or can be that used to prepare a blood fraction, such as pelleted red cells, which are in turn contacted with the lysis reagent. Preferably the methods detect the presence of pathogenic RNA in whole blood with a sensitivity of about $2x10^3$ copies of ribosomal RNA/mL (equivalent to one parasite/1 mL) of whole blood or better, $2x10^3$ copies /5 mL of whole blood or better, $2x10^3$ copies /10 mL of whole blood or better, $2x10^3$ copies /50 mL of whole blood or better, or $2x10^3$ copies/100 mL of whole blood or better. Preferably the methods detect the presence of pathogenic RNA in whole blood with a sensitivity of about $8x10^3$ copies of ribosomal RNA/mL (equivalent to four parasites/1 mL) of whole blood or better, $8x10^3$ copies /5 mL of whole blood or better, $8x10^3$ copies /10 mL of whole blood or better, $8x10^3$ copies /50 mL of whole blood or better, or $8x10^3$ copies/100 mL of whole blood or better. Preferably the methods detect the presence of pathogenic RNA in whole blood with a sensitivity of about $24x10^3$ copies of ribosomal

RNA/mL (equivalent to 12 parasites/1 mL) of whole blood or better, $24x10^3$ copies /5 mL of whole blood or better, $24x10^3$ copies /10 mL of whole blood or better, $24x10^3$ copies /50 mL of whole blood or better, or $24x10^3$ copies/100 mL of whole blood or better.

## VII. Work Flow

**[00079]** Individually lysed aliquots from a plurality of blood samples are pooled. Different blood cell samples are typically from different subjects, often from different humans. An exemplary volume for individual blood samples is about 200-500 ml. Sample aliquots for pooling typically represent a small proportion of a blood cell sample such as 1 µL to 2 ml or 100 µL to 1 ml or less than 1% or less than 0.1% of the volume of the blood cell samples. Aliquots can be pooled after individual lysis from 2, 4, 8, 16, 24, or 200 samples (among other numbers). In some methods, lysed aliquots from at least four samples are pooled. In some methods, lysed aliquots from 2-200, 4-24, 14-16, or 4-20 samples are pooled. After lysis individual lysed sample aliquots can be pooled in whole or in part. Pooling in whole means that the complete volumes of each lysed sample aliquot are pooled. Pooling means that a specimen (i.e., fraction) of each lysed sample aliquot is pooled to form a pooled lysate. Analyses can then be performed to detect targets that may be present in blood cell samples. The analyses can look only for targets of pathogens that infect red blood cells, such as Babesia and Plasmodium species, or can also look for targets of any pathogens likely to be present in plasma or serum, such as HIV-1 and -2, hepatatis B, hepatitis C, West Nile, Dengue treponema pallidum, and Trypanosoma cruzi, Plasmodium species and Anaplasma, or can look for therapeutic targets. Such targets can be analyzed separately or together, such as by multiplex amplification and detection on a microarray.

**[00080]** The sensitivity of the method arises in part from performing lysis of blood cells before one or more samplings in which only a fraction of an original sample is passed on to a subsequent analyses. Such sampling may arise from using only a fraction of each lysed sample aliquot for pooling, or using only a fraction of the pooled lysate for analysis or other dilution. The fractionation performed by any or all samplings performed between lysis and detection can be less than or equal to 0.5 (i.e., 50%), 0. 25 (25%), 0.2 (20%), 0.125 (12.5%), 0.1 (10%), 0.0625 (6.25%), 0.05 (5%), 0.0416 (4.16%)or 0.01 (1%) of the initial volume of the aliquot that is ultimately analyzed. Because the number of blood cells containing a target may be the same order or less than the inverse of the sampling fraction, there is a high possiblity that for sampling without lysis, no target containing cells will be passed on to the subsequent analysis from a sample aliquot. For example, if a sample has 2 infected cells per ml, and 0.1 ml of the sample is used for analysis, on average only 1/5 samples used for analysis will contain a particle to allow a positive result. However, if lysis is performed before sampling to release target molecules from the blood cells, the situation is different. If each blood cell gives rise to 1000 different target molecules on lysis, the number of target molecules greatly exceeds the inverse of the fraction sampled, and there is a high probability of detecting the target. .

**[00081]** If the target is a pathogen-derived target that is not desired in the blood cell samples, and if the pooled lysates are negative for the tested target, the blood cell samples giving rise to the pool can be used (e.g., for blood transfusions and the like or banked for such use). Conversely, if pooled lysates are found to be positive for an undesired target, then individual aliquots from the samples previously combined can be tested to deconvolute which of the pooled samples contributed the pathogen. The blood cell sample(s) contributing the pathogen(s) can be removed from the collection of blood cell samples, and these other blood cell samples can be used (e.g., for blood transfusion and the like). If the target is one that is desired, then the testing is similar except that upon deconvolution the blood cell sample(s) containing the target are kept and can be used (e.g., harvesting a protein target for the blood cell sample(s) and/or from the donor(s)).

## EXAMPLES

### Example 1

**[00082]** This example compares the method of lysing infected hamster blood and then diluting the lysed sample in additional lysis buffer, to a method where the same hamster blood is first diluted with normal blood and then lysed. Condition A (Lyse-Dilute), 10 uL *Babesia*-infected whole hamster blood of which 59% of cells were infected was lysed in 20 uL PTM medium (250mM ammonium chloride, 14mM sodium bicarbonate, 10mM EDTA, and 8% lithium lauryl sulfate (LLS), with pH in the range of 7.2 to 7.6) and then serially diluted in the PTM. Condition B (Dilute-Lyse), 10 uL of the same infected hamster blood is first serially diluted with human whole blood and then lysed with PTM (1:3 (v/v)). Parasitemia of the infected hamster blood was determined by microscopy and by PCR wherein PCR results from the infected hamster blood were compared to PCR results of an *in vitro* transcript dilution curve. Parasitemia of the infected hamster blood was determined to be 43,130 parasites per mL (p/mL), and the dilutions for both Conditions A & B resulted in: 4,313 p/mL, 431 p/mL, 43 p/mL, 4.8 p/mL, 1.6 p/mL, 0.5 p/mL, 0.18 p/mL, 0.06 p/mL, 0.02 p/mL, and 0.01 p/mL. Lysis was done at ambient temperature for 5 minutes. Five 500 μL aliquots from each sample in the dilutions were tested for *Babesia* using a target capture and real-time transcription-mediated amplification (TMA) assay. Percent reactivity was determined as amplification/detection reactions having RLU signals greater than 100,000 being positive for detection of *Babesia* target.

**[00083]** Target capture was performed as generally described in U.S. Patent No. 6,110,678. *Babesia* 18S rRNA was amplified and detected in each sample as generally described in U.S. Pat. Nos. 5,399,491, 5,554,516, 5,824,518 and 7,833,716. Primers used to amplify *Babesia* 18S rRNA in the samples were as follows:

**Table 1**

| FUNCTION | Sequence (5'-3') |
|---|---|
| non T7 Primer | CTTGAATACTACAGCATGGAATAA |
| T7 Primer | AATTTAATACGACTCACTATAGGGAGATTCACCTCTGACAGTTAAATACGAA |
| Molecular Torch [1] | 5' CAGUCCAUAAUGAAGUAGGACUG 3' |
| TCO [2] | UAGGCCAAUACCCUACCGUCCTTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA |

[1] The molecular torch comprises Fam attached to its 5' end and Dabcyl attached to its 3' end. The molecular torch also comprises a linker between the underlined C and A residues (residues 7 & 8). The molecular torch also comprises 2' O-methyl residues.

[2] The target capture oligomer (TCO) comprises 2' O-methyl residues at the underlined residue positions.

**[00084]** The results are shown in Table 2, which compares % sample reactivity with number of particles/ml at the dilution tested. For samples diluted in whole blood, the reactivity drops to zero between a final concentration of 4.8 and 1.6 particles per ml. By contrast, when samples are diluted in PTM, 100% reactivity is obtained at least up to a final concentration of 0.18 particles per ml, which corresponds to about 200 18S ribosomal RNA copies per ml. Moreover, at 0.06 and 0.02 p/mL, the samples diluted in PTM provided 60% and 40% reactivity, respectively.

**Table 2**

| Sample Dilution (p/mL) | Condition A % Reactivity (N=5) | Condition B % Reactivity (N=5) |
|---|---|---|
| 4,313 p/mL | 100% | 100% |
| 431 p/mL | 100% | 100% |
| 43 p/mL | 100% | 100% |
| 4.8 p/mL | 100% | 100% |
| 1.6 p/mL | 100% | Not detectable |
| 0.5 p/mL | 100% | Not detectable |
| 0.18 p/mL | 100% | Not detectable |
| 0.06 p/mL | 60% | Not detectable |
| 0.02 p/mL | 40% | Not detectable |
| 0.01 p/mL | Not detectable | Not detectable |

**Example 2**

**[00085]** Twenty-four Babesia positive clinical RBC samples were obtained from the American Red Cross (ARC). These clinical samples in were each in Adsol solution (a preservation solution containing saline, adenine, dextrose and mannitol, Fenwal Laboratories, Deerfield, Illinois) and were stored at -20°C on receipt. The samples were thawed the day of testing and were mixed thoroughly by inversion before manipulation. Each sample was to be prepared for testing individually (individual donor testing or IDT), and testing in pools using the pre-lysed pooling method.

Individual Lysis of RBC Samples

**[00086]** To lyse the clinical samples, a 500µL aliquot of a sample was admixed with 3.5mL of parasite transport medium (PTM) and then mixed by gentle inversion at least 3 times at ambient temperature and allowed to stand for 5 minutes. This lysate is the IDT lysate sample.

**[00087]** The PTM that was used in this example contains 250mM ammonium chloride, 14mM sodium bicarbonate, 10mM EDTA, and 8% lithium lauryl sulfate (LLS), with pH in the range of 7.2 to 7.6.

**[00088]** Fifteen normal negative samples from whole blood donors were obtained from Bioreclamation Inc. (Long Island, NY) and were lysed by using a ratio of 1mL aliquot of whole blood to 3mL of PTM. An aliquot of lysed sample for each negative donor was set aside for negative controls.

Preparation of Pools

**[00089]** Donor pools of 1:4, 1:8 and 1:16 were prepared using the IDT lysate for each clinical sample. The total volume of each pooled sample was 4.8mL.

- A pool of 1:4 was prepared by combining 1200µL of the lysed clinical sample with 1200µL for each of 3 lysed negative donors.
- A pool of 1:8 was prepared by combining 600µL of the lysed clinical sample with 600µL for each of 7 lysed negative donors.
- A pool of 1:16 was prepared by combining 300µL of the lysed clinical sample with 300µL for each of 15 lysed negative donors.

**[00090]** Once the samples were prepared, they were mixed by inversion several times before testing.

**[00091]** Three replicates of 500µL for each IDT lysate, pooled, and negative control sample were tested on an automated Panther system using the Babesia TMA assay described above. A *B. microti* IVT panel at 100c/mL was included as a positive control.

**[00092]** Results are shown in Table 3 below. The data shows that the pooled method was capable of detecting Babesia infection in all clinical samples with a pooling factor of 1:4 and in the vast

majority of samples with a pooling factor up to 1:16. Sensitivity with the pooling method, followed by target capture and TMA was comparable to that of PCR of individual samples.

**Table 3**

| (RBC) | Research PCR | IDT | 1:4 (pool of 4) | 1:8 (pool of 8) | 1:16 (pool of 16) |
|---|---|---|---|---|---|
| 1 | Negative | Reactive | Reactive | NonReactive | NonReactive |
| 2 | Negative | Reactive | Reactive | Reactive | Reactive |
| 3 | Positive | Reactive | Reactive | Reactive | Reactive |
| 4 | Positive | Reactive | Reactive | Reactive | Reactive |
| 5 | Positive | Reactive | Reactive | Reactive | Reactive |
| 6 | Positive | Reactive | Reactive | Reactive | Reactive |
| 7 | Positive | Reactive | Reactive | Reactive | NonReactive |
| 8 | Positive | Reactive | Reactive | Reactive | Reactive |
| 9 | Positive | Reactive | Reactive | Reactive | Reactive |
| 10 | Positive | Reactive | Reactive | Reactive | Reactive |
| 11 | Positive | Reactive | Reactive | Reactive | Reactive |
| 12 | Positive | Reactive | Reactive | Reactive | Reactive |
| 13 | Positive | Reactive | Reactive | Reactive | Reactive |
| 14 | Positive | Reactive | Reactive | Reactive | Reactive |
| 15 | Positive | Reactive | Reactive | Reactive | Reactive |
| 16 | Positive | Reactive | Reactive | Reactive | Reactive |
| 17 | Positive | Reactive | not tested | Reactive | Reactive |
| 18 | Positive | Reactive | not tested | Reactive | Reactive |
| 19 | Positive | Reactive | not tested | Reactive | Reactive |
| 20 | Positive | Reactive | not tested | Reactive | Reactive |
| 21 | Positive | Reactive | not tested | Reactive | Reactive |
| 22 | Positive | Reactive | not tested | Reactive | Reactive |
| 23 | Positive | Reactive | not tested | Reactive | Reactive |
| 24 | Positive | Reactive | not tested | Reactive | Reactive |

## Example 3

**[00093]** In this example, *B. microti* infected hamster blood was diluted to 1 parasite per mL, pooled, and tested using nucleic acid amplification and detection for limit of detection in the pooled samples. An approximate parasitemia of *B. microti* infected hamster blood was calculated using microscopy. Based on the initial parasitemia estimate, a serial dilution of *B. microti* infected hamster blood was prepared in human whole blood. Nine separate 1 mL aliquots were made at the hamster blood dilution level estimated to have about 1p/mL of *B. microti*. Each of the separate aliquots were lysed by combining the 1 ml aliquot with 3 mL of a blood cell lysis solution (aqueous solution containing about 100 mM TRIS, 25-30 mM $MgCl_2$, 6% (v/v) lithium lauryl sulfate, and pH between about 7.3 and 7.6). These nine lysates were tested directly and in 1:20 & 1:200 pools.

**[00094]** Each 1:20 pool of the nine lysates were prepared by combining 250 uL of the lysates into an additional 4.75 mL of lysis reagent. The 1:200 pool was prepared by combining 500 uL of the 1:20

pool with 4.5 mL of the lysis reagent. The nine aliquots of lysate were tested at 5 replicates using an isothermal amplification and detection assay on the automated Panther system (Hologic, Inc. San Diego, CA). The 1:20 pools and the 1:200 pools made from each of the nine lysates were tested at 7 replicates using isothermal amplification and detection assay on the automated Panther system. Results are shown in Table 4.

**Table 4**

| Lysate Aliquot Number | ~1 p/mL Aliquot Reactivity | 1:20 Pool Reactivity | 1:200 Pool Reactivity |
|---|---|---|---|
| 1 | 0/5 | 0/7 | 0/7 |
| 2 | 5/5 | 7/7 | 6/7 |
| 3 | 5/5 | 7/7 | 7/7 |
| 4 | 0/5 | 0/7 | 0/7 |
| 5 | 0/5 | 0/7 | 0/7 |
| 6 | 5/5 | 7/7 | 4/7 |
| 7 | 0/5 | 0/7 | 0/7 |
| 8 | 0/5 | 0/7 | 0/7 |
| 9 | 5/5 | 7/7 | 7/7 |

**[00095]** These data show that in samples containing as little as 1 p/mL, a 1:20 pool is 100% reactive. Furthermore, a 1:200 pool showed good sensitivity, though not 100% reactive. Thus, for an assay having limit of detection sensitivity requirements at 100%, the lysate pooling results shown in this examples indicate that at least a 1:20 pool meets the requirements. For an assay with less than 100% sensitivity requirements, this example indicates that a pool as dilute as 1:200 provides remarkably good results.

**[00096]** Although the invention has been described in detail for purposes of clarity of understanding, certain modifications may be practiced within the scope of the appended claims. All publications including accession numbers, websites and the like, and patent documents cited in this application are hereby incorporated by reference in their entirety for all purposes to the same extent as if each were so individually denoted. To the extent difference version of a sequence, website or other reference may be present at different times, the version associated with the reference at the effective filing date is meant. The effective filing date means the earliest priority date at which the accession number at issue is disclosed. Unless otherwise apparent from the context any element, embodiment, step, feature or aspect of the invention can be performed in combination with any other.

WHAT IS CLAIMED IS:

1. A method of testing a sample aliquot containing blood cells for presence of a pathogen, comprising

(a) providing a sample aliquot from each of a plurality of samples, thereby providing a plurality of sample aliquots;

(b) separately contacting each of the sample aliquots of the a plurality of sample aliquots with a lysis reagent, whereby at least a portion of the blood cells in each of the first sand second sample aliquots lyse;

(c) pooling the lysed sample aliquots of step (b) to form a pooled lysate;

(d) testing the pooled lysate for presence of a pathogen, whereby identification of the presence of the pathogen in the pooled lysate indicates the presence of the pathogen in at least one of the sample aliquots.

2. The method of claim 1, wherein the lysis reagent comprises: (i) a buffer; (ii) lithium lauryl sulfate (LLS); and (iii) one or both of a chloride containing salt and an anti-coagulant selected from the group consisting of EDTA, EDTA-$Na_2$, EGTA, and combinations thereof, and wherein the reagent has a pH that is greater than 5.5.

3. The method of claim 2, wherein the buffer is sodium bicarbonate, and the reagent comprises a chloride containing salt that is ammonium chloride, and wherein the pH of the reagent is from about 7.0 to about 8.0.

4. The method of claim 3, wherein the ammonium chloride is present in the reagent at a concentration from about 100 mM to about 500 mM, or at a concentration of about 250 mM.

5. The method of claim 3 or claim 4, wherein the sodium bicarbonate is present in the reagent at a concentration of about 5 mM to about 30 mM, or at a concentration of about 14 mM.

6. The method of any one of claims 2 to 5, wherein the reagent comprises an anti-coagulant that is EDTA-$Na_2$ present in the reagent at a concentration of about 1 mM, EGTA present in the reagent at a concentration of about 1 mM, or is a combination of EDTA-$Na_2$ and EGTA, wherein the EDTA-$NA_2$ is present in the reagent at a concentration of about 1 mM and the EGTA is present in the reagent at a concentration of about 1 mM.

7. The method of claim 2, wherein the lysis reagent comprises (i) about 14 mM sodium bicarbonate, (ii) about 5% (v/v) LLS (iii) a chloride containing salt and an anticoagulant, wherein the chloride containing salt is ammonium chloride and the ammonium chloride is present in the reagent at a concentration of about 250 mM, and wherein the anti-coagulant is EDTA and the EDTA is present in the reagent at a concentration from about 0.1 mM to about 10 mM, and wherein the pH of the reagent is from about 7.2 to about 7.5.

8. The method of claim 2, wherein the buffer is a TRIS buffer present in the reagent at a concentration from about 75 mM to about 150 mM, or at a concentration of about 100 mM.

9. The method of claim 2 or claim 8, wherein the reagent comprises a chloride salt that is magnesium chloride, and the magnesium chloride is present in the reagent at a concentration from about 20 mM to about 35 mM, or at a concentration of about 30 mM

10. The method of claim 9, wherein the lysis reagent comprises about 100 mM TRIS buffer and about 6% (v/v) LLS, and wherein the pH of the reagents is about 7.5.

11. The method of any one of claims 2 to 6 and 8 to 9, wherein the LLS is present in the reagent at a concentration from about 4% (v/v) to about 15% (v/v), or at a concentration of about 10% (v/v), or at a concentration of about 8% (v/v), or at a concentration of about 6% (v/v), or at a concentration of about 5% (v/v).

12. The method of 2, wherein the reagent comprises (i) a buffer comprising sodium phosphate, about 10% (v/v) LLS, and an anti-coagulant, wherein the anti-coagulant is EDTA-$Na_2$ present in the reagent at a concentration of about 1 mM, EGTA present in the reagent at a concentration of about 1 mM, or is a combination of EDTA-$Na_2$ and EGTA, wherein the EDTA-$NA_2$ is present in the reagent at a concentration of about 1 mM and the EGTA is present in the reagent at a combination of about 1 mM.

13. The method of claim 1, wherein the entire volumes of each of the lysed sample aliquots are pooled.

14. The method of claim 1, wherein up to 25% of the volumes of each of the lysed sample aliquots are pooled.

15. The method of any preceding claim, wherein the plurality of sample aliquots is at least two sample aliquots.

16. The method of any preceding claim, wherein the plurality of sample aliquots are from 4 sample aliquots to 200 sample aliquots.

17. The method of claim 16, wherein the plurality of sample aliquots is up to 20 sample aliquots.

18. The method of claim 16, wherein the plurality of sample aliquots is up to 16 sample aliquots.

19. The method of claim 1, wherein if at the testing step (d) it is determined that a pathogen is present in the pooled lysate, the method further comprises the step of individually testing the sample aliquots to identify which of the samples is infected with the pathogen.

20. The method of any of the preceding claims, wherein each of the sample aliquots are whole blood.

21. The method of claim 20, wherein the whole blood is human whole blood, non-human whole blood, or a mixture thereof.

22. The method of any of the preceding claims, wherein the sample aliquots comprise red blood cells.

23. The method of any of the preceding claims, wherein the sample aliquots comprises white blood cells.

24. The method of any of the preceding claims, wherein each sample aliquot is contacted with the lysis reagent at a volume ratio of sample aliquot to lysis reagent that is from about 1:2 to about 1:10 (v/v).

25. The method of claim 24, wherein the volume ratio of sample aliquot to lysis reagent is selected from the group consisting of: 1:2 (v/v), 1:3 (v/v), 1:4 (v/v), 1:5 (v/v), 1:6 (v/v), 1:7 (v/v), 1:8 (v/v), 1:9 (v/v), and 1:10 (v/v).

26. The method of claim 24, wherein the volume ratio of sample aliquot to lysis reagent is about 1:3 (v/v).

27. The method of claim 24, wherein the volume ratio of sample aliquot to lysis reagent is about 1:4 (v/v).

28. The method of any preceding claim, wherein the testing step (d) tests for presence of a pathogen-derived target released from the blood cells by the reagent.

29. The method of claim 28, wherein the pathogen-derived target is a nucleic acid target.

30. The method of claim 28 or claim 29, wherein the pathogen-derived target is derived from a pathogen selected from the group consisting of: hepatitis viruses, human immunodeficiency viruses, dengue viruses, west nile viruses, flaviviruses, zika virus, and parasitic organisms.

31. The method of claim 30, wherein the pathogen is a parasitic organism selected from the group consisting of: parasites from the genus *Babesia*, parasites from the genus *Plasmodium*, parasites from the genus *Trypanosoma*, parasites from the genus *Leishmania*, parasites from the genus *Anaplasma*, parasites from the genus *Toxoplasma*, *Babesia microti, Babesia divergens, Babesia duncani*, *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, and Plasmodium knowlesi*.

32. The method of any one of claims 29-31, wherein the pathogen-derived target is an RNA target.

33. The method of claim 32, wherein the pathogen is *Babesia microti*.

34. The method of claim 32 or 33, wherein the RNA target is a ribosomal RNA target.

35. The method of claim 32 or 33, wherein the RNA target is an 18S ribosomal RNA target.

36. The method of claim 32, wherein the RNA target is from a pathogen of the genus Plasmodium.

37. The method of claim 32, wherein the RNA target is from the Anaplasma genus of bacterium

38. The method of any one of claims 29 to 37, wherein the testing step (d) comprises contacting the pooled lysate with a capture probe and an immobilized probe, the capture probe having a first segment complementary to the nucleic acid target, and a second segment complementary to the immobilized probe, wherein the nucleic acid target binds to the capture probe, and wherein the bound capture probe binds to the immobilized probe.

39. The method of claim 38, wherein the immobilized probe is attached to a solid support.

40. The method of any one of claims 29 to 39, further comprising performing a transcription mediated amplification of the nucleic acid target and detecting the resulting amplification product with a detection probe.

41. The method of claim 1, wherein the testing step (d) comprises performing a nucleic acid amplification and detection reaction to detect a pathogen-derived target in the pooled lysate.

42. The method of any of the preceding claims, wherein the method is performed without a centrifugation step.

43. A method for detecting a target in a sample aliquot comprising the steps of:

(a) providing a sample aliquot from each of a plurality of whole blood samples, thereby providing a plurality of sample aliquots;

(b) separately performing a lysing reaction on each of the sample aliquots, wherein the lysing step comprises contacting each of the sample aliquots with a lysis reagent comprising

(i) a buffer,

(ii) lithium lauryl sulfate (LLS), and

(iii) at least one of a chloride containing salt and an anticoagulant selected from the group consisting of: EDTA, EDTA-$Na_2$, EGTA, and combinations thereof;

wherein the reagent has a pH that is greater than 5.5, and whereby at least a portion of the blood cells in each of the sample aliquots lyse;

(c) combining the lysed sample aliquots into a single pooled sample to form a pooled lysate;

(d) separating a target from the pooled lysate; and

(e) performing a reaction to detect the separated target from step (d), wherein detection of the presence of the target in the pooled lysate indicates the presence of the target in at least one of the sample aliquots provided in step (a).

44. The method of claim 43, further comprising when the target is detected in step (e), individually testing the separate whole blood samples to identify which of the whole blood samples contain(s) the target.

45. The method of claim 44, wherein the separate whole blood samples that are tested and determined to not contain the target are stored as blood bank samples.

46. The method of claim 43, wherein the target is a nucleic acid target, and wherein the pooled lysate is tested for the presence or absence of the nucleic acid target using a nucleic acid amplification reaction and a detection reaction.

47. The method of claim 46, wherein the amplification reaction is an isothermal amplification reaction.

48. The method of claim 46, wherein the amplification reaction comprises performing a transcription mediated amplification reaction to generate an amplification product and wherein the detection reaction comprises detecting the amplification product with a detection probe.

49. The method of any one of claims 46 to 48, wherein the amplification reaction and the detection reaction are performed simultaneously.

50. The method of any one of claims 46 to 49, wherein the amplification reaction and the detection reaction are performed on an automated device.

51. The method of claim 50, wherein step (d) is performed on the automated device.

52. The method of any one of claims 43 to 49, wherein steps (b) through (e) are performed on an automated device.

53. The method of claim 43, wherein at step (b) the lysing reaction is performed on at least 16 sample aliquots.

54. The method of claim 43, wherein at step (b) the lysing reaction is performed on at least 20 sample aliquots.

55. The method of any one of claims 43 to 54, wherein at step (b) the lysing reaction is performed on up to 200 sample aliquots.

56. The method of claim 43, wherein the buffer is sodium bicarbonate, and the reagent comprises a chloride containing salt that is ammonium chloride, and wherein the pH of the reagent is from about 7.0 to about 8.0.

57. The method of claim 56, wherein the ammonium chloride is present in the reagent at a concentration from about 100 mM to about 500 mM, or at a concentration of about 250 mM.

58. The method of claim 56 or claim 57, wherein the sodium bicarbonate is present in the reagent at a concentration of about 5 mM to about 30 mM, or at a concentration of about 14 mM.

59. The method of any one of claims 43 to 58, wherein the reagent comprises an anti-coagulant that is EDTA-$Na_2$ present in the reagent at a concentration of about 1 mM, EGTA present in the reagent at a concentration of about 1 mM, or is a combination of EDTA-$Na_2$ and EGTA, wherein the EDTA-$NA_2$ is present in the reagent at a concentration of about 1 mM and the EGTA is present in the reagent at a concentration of about 1 mM.

60. The method of claim 43, wherein the lysis reagent comprises (i) about 14 mM sodium bicarbonate, (ii) about 5% (v/v) LLS (iii) a chloride containing salt and an anticoagulant, wherein the chloride containing salt is ammonium chloride and the ammonium chloride is present in the reagent at a concentration of about 250 mM, and wherein the anti-coagulant is EDTA and the EDTA is present in the reagent at a concentration from about 0.1 mM to about 10 mM, and wherein the pH of the reagent is from about 7.2 to about 7.5.

61. The method of claim 43, wherein the buffer is a TRIS buffer present in the reagent at a concentration from about 75 mM to about 150 mM, or at a concentration of about 100 mM.

62. The method of claim 43 or claim 61, wherein the reagent comprises a chloride salt that is magnesium chloride, and the magnesium chloride is present in the reagent at a concentration from about 20 mM to about 35 mM, or at a concentration of about 30 mM

63. The method of claim 62, wherein the lysis reagent comprises about 100 mM TRIS buffer and about 6% (v/v) LLS, and wherein the pH of the reagents is about 7.5.

64. The method of any one of claims 43 to 59 and 61 to 62, wherein the LLS is present in the reagent at a concentration from about 4% (v/v) to about 15% (v/v), or at a concentration of about 10% (v/v), or at a concentration of about 8% (v/v), or at a concentration of about 6% (v/v), or at a concentration of about 5% (v/v).

65. The method of 43, wherein the reagent comprises (i) a buffer comprising sodium phosphate, about 10% (v/v) LLS, and an anti-coagulant, wherein the anti-coagulant is EDTA-$Na_2$ present in the reagent at a concentration of about 1 mM, EGTA present in the reagent at a concentration of about 1 mM, or is a combination of EDTA-$Na_2$ and EGTA, wherein the EDTA-$NA_2$ is present in the reagent at a concentration of about 1 mM and the EGTA is present in the reagent at a concentration of about 1 mM.

66. The method of any of claims 43 to 65, wherein the whole blood samples are each human whole blood samples, each non-human whole blood samples, or a combination of human whole blood samples and non-human whole blood samples.

67. The method of any of 43 to 66, wherein each sample aliquot is contacted with the lysis reagent at a volume ratio of sample aliquot to lysis reagent that is from about 1:2 to about 1:10 (v/v).

68. The method of claim 67, wherein the volume ratio of sample aliquot to lysis reagent is selected from the group consisting of: 1:2 (v/v), 1:3 (v/v), 1:4 (v/v), 1:5 (v/v), 1:6 (v/v), 1:7 (v/v), 1:8 (v/v), 1:9 (v/v), and 1:10 (v/v).

69. The method of claim 67, wherein the volume ratio of sample aliquot to lysis reagent is about 1:3 (v/v).

70. The method of claim 67, wherein the volume ratio of sample aliquot to lysis reagent is about 1:4 (v/v).

71. The method of any of claims 43 to 70, wherein the target is released from a red blood cell.

72. The method of any one of claims 43 to 71, wherein the target is a pathogen-derived target.

73. The method of any one of claims 43 to 72, wherein the target is an RNA target.

74. The method of claim 72, wherein the pathogen-derived target is an RNA target from a pathogen of the genus *Babesia*.

75. The method of claim 73 or 74, wherein the RNA target is a ribosomal RNA target.

76. The method of claim 75, wherein the RNA target is an 18S ribosomal RNA target.

77. The method of one of claims 74 to 76, wherein the RNA target is from a pathogen of the species *Babesia microti*.

78. The method of claim 72, wherein the pathogen-derived target is an RNA target from a pathogen of the genus Plasmodium.

79. The method of claim 72, wherein the pathogen-derived target is a nucleic acid target from the Anaplasma genus of bacterium.

80. The method of claim 72, wherein the pathogen-derived target is derived from a pathogen selected from the group consisting of: hepatitis viruses, human immunodeficiency viruses, dengue viruses, west nile viruses, flaviviruses, zika virus, and parasitic organisms.

81. The method of claim 80, wherein the pathogen is a parasitic organism selected from the group consisting of: parasites from the genus *Babesia*, parasites from the genus *Plasmodium*, parasites from the genus *Trypanosoma*, parasites from the genus *Leishmania*, parasites from the genus *Anaplasma*, parasites from the genus *Toxoplasma*, *Babesia microti, Babesia divergens, Babesia duncani*, *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, and Plasmodium knowlesi*.

82. The method of claim 43, wherein at least 50% of the blood cells are lysed in five minutes or less after contacting the sample aliquot with the lysis reagent.

83. The method of any of claims 43 to 82, wherein the target is a nucleic acid target, wherein the separating step (d) comprises contacting the pooled lysate with a capture probe and an immobilized probe, the capture probe having a first segment complementary to the nucleic acid target, and a second segment complementary to the immobilized probe, wherein the nucleic acid target binds to the capture probe, and wherein the bound capture probe binds to the immobilized probe.

84. The method of claim 83, wherein the immobilized probe is attached to a solid support.

85. The method of any of claim 43 to 84, wherein the method is performed without a centrifugation step.

86. The method of any of claims 43 to 85, wherein the entire volumes of the lysed sample aliquots are combined.

87. The method of any of claims 43 to 85, wherein 25% or less of volumes of the lysed sample aliquots are combined.

88. A method of separating a target from a plurality of sample aliquots, comprising the steps of:

(a) providing a sample aliquot from each of a plurality of whole blood samples, thereby providing a plurality of sample aliquots;

(b) separately performing a lysing reaction on each of the sample aliquots, wherein the lysing step comprises contacting each of the sample aliquots with a lysis reagent comprising

(i) a buffer,

(ii) lithium lauryl sulfate (LLS), and

(iii) at least one of a chloride containing salt and an anticoagulant selected from the group consisting of: EDTA, EDTA-$Na_2$, EGTA, and combinations thereof;

wherein the reagent has a pH that is greater than 5.5, and whereby at least a portion of the blood cells in each of the sample aliquots lyse;

(c) combining the lysed sample aliquots into a single pooled sample to form a pooled lysate; and

(d) separating a target from the pooled lysate.

89. The method of claim 88, further comprising contacting the pooled lysate with a solid support configured to immobilize the target; and separating the immobilized target from the pooled lysate.

90. The method of claim 89, wherein the target is a nucleic acid target and wherein the solid support comprises an attached immobilized probe.

91. The method of claim 90, further comprising contacting the pooled lysate with a capture probe comprising a first segment complementary to the nucleic acid target, and a second segment complementary to the immobilized probe.

92. The method of claim 91, further comprising providing hybridization conditions that favor formation of a hybridization complex between the first segment of the capture probe and the nucleic acid target.

93. The method of claim 92, further comprising providing hybridization conditions that favor formation of a hybridization complex between the second segment of the capture probe and the immobilized probe attached to the solid support.

94. The method of any one of claims 89-93, wherein the solid support is a magnetic bead solid support.

95. The method of any one of claims 89-93, wherein the solid support is a silica solid support.

96. The method of claim 95, wherein the silica solid support is glass wool.

97. The method of claim 95, wherein the silica solid support is a bead.

98. The method of any one of claims 89-97, wherein the solid support is contained within a column.

99. The method of any one of claims 88-98, wherein the target is a pathogen-derived target.

100. The method of claim 99, wherein the pathogen derived target is from a pathogenic organism selected from the group consisting of: hepatitis viruses, human immunodeficiency viruses, dengue viruses, west nile viruses, flaviviruses, zika virus, and parasitic organisms.

101. The method of claim 100, wherein the pathogenic organism is a parasitic organism selected from the group consisting of: parasites from the genus *Babesia*, parasites from the genus *Plasmodium*, parasites from the genus *Trypanosoma*, parasites from the genus *Leishmania*, parasites from the genus *Anaplasma*, parasites from the genus *Toxoplasma*, *Babesia microti, Babesia divergens, Babesia duncani*, *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, and Plasmodium knowlesi*.

102. The method of any one of claims 88-101, wherein the target is an RNA target.

103. The method of claim 101, wherein the parasitic organism is from the genus *Babesia,* and wherein the target is a ribosomal RNA target.

104. The method of claim 103, wherein the parasitic organism is *Babesia microti*.

105. The method of claim 104, wherein the RNA target is a ribosomal RNA target.

106. The method of any one of claims 88 to 105, wherein the buffer is sodium bicarbonate, and the reagent comprises a chloride containing salt that is ammonium chloride, and wherein the pH of the reagent is from about 7.0 to about 8.0.

107. The method of claim 106, wherein the ammonium chloride is present in the reagent at a concentration from about 100 mM to about 500 mM, or at a concentration of about 250 mM.

108. The method of claim 106 or claim 107, wherein the sodium bicarbonate is present in the reagent at a concentration of about 5 mM to about 30 mM, or at a concentration of about 14 mM.

109. The method of any one of claims 106 to 108, wherein the reagent comprises an anti-coagulant that is EDTA-$Na_2$ present in the reagent at a concentration of about 1 mM, EGTA present in the reagent at a concentration of about 1 mM, or is a combination of EDTA-$Na_2$ and EGTA, wherein the EDTA-$NA_2$ is present in the reagent at a concentration of about 1 mM and the EGTA is present in the reagent at a concentration of about 1 mM.

110. The method of any one of claims 88 to 106, wherein the lysis reagent comprises (i) about 14 mM sodium bicarbonate, (ii) about 5% (v/v) LLS, (iii) a chloride containing salt and an anticoagulant, wherein the chloride containing salt is ammonium chloride and the ammonium chloride is present in the reagent at a concentration of about 250 mM , and wherein the anti-coagulant is EDTA and the EDTA is present in the reagent at a concentration from about 0.1 mM to about 10 mM, and wherein the pH of the reagent is from about 7.2 to about 7.5.

111. The method of any one of claims 88 to 105, wherein the buffer is a TRIS buffer present in the reagent at a concentration from about 75 mM to about 150 mM, or at a concentration of about 100 mM.

112. The method of any one of claims 88 to 106 and 111, wherein the reagent comprises a chloride salt that is magnesium chloride, and the magnesium chloride is present in the reagent at a concentration from about 20 mM to about 35 mM, or at a concentration of about 30 mM.

113. The method of claim 112, wherein the lysis reagent comprises about 100 mM TRIS buffer and about 6% (v/v) LLS, and wherein the pH of the reagents is about 7.5.

114. The method of any one of claims 88 to 109 and 111 to 112, wherein the LLS is present in the reagent at a concentration from about 4% (v/v) to about 15% (v/v), or at a concentration of about 10% (v/v), or at a concentration of about 8% (v/v), or at a concentration of about 6% (v/v), or at a concentration of about 5% (v/v).

115. The method any one of claims 88-105, wherein the reagent comprises (i) a buffer comprising sodium phosphate, about 10% (v/v) LLS, and an anti-coagulant, wherein the anti-coagulant is EDTA-$Na_2$ present in the reagent at a concentration of about 1 mM, EGTA present in the reagent at a concentration of about 1 mM, or is a combination of EDTA-$Na_2$ and EGTA, wherein the EDTA-$NA_2$ is present in the reagent at a concentration of about 1 mM and the EGTA is present in the reagent at a concentration of about 1 mM.

116. The method of any one of claims 88 to 115, wherein the entire volumes of each of the lysed sample aliquots are pooled.

117. The method of any one of claims 88 to 115, wherein up to 25% of the volumes of each of the lysed sample aliquots are pooled.

118. The method of any one of claims 88 to 117, wherein the plurality of sample aliquots is at least two sample aliquots.

119. The method of any one of claims 88 to 118, wherein the plurality of sample aliquots are from 4 sample aliquots to 200 sample aliquots.

120. The method of claim 119, wherein the plurality of sample aliquots is up to 20 sample aliquots.

121. The method of claim 119, wherein the plurality of sample aliquots is up to 16 sample aliquots.

122. The method of any one of claims 88 to 121, wherein the whole blood samples are each human whole blood samples, each non-human whole blood samples, or a combination of human whole blood samples and non-human whole blood samples.

123. The method of any one of claims 88 to 122, wherein the sample aliquots comprise red blood cells.

124. The method of any one of claims 88 to 123, wherein the sample aliquots comprises white blood cells.

125. The method of any one of claims 88 to 124, wherein each sample aliquot is contacted with the lysis reagent at a volume ratio of sample aliquot to lysis reagent that is from about 1:2 to about 1:10 (v/v).

126. The method of claim 125, wherein the volume ratio of sample aliquot to lysis reagent is selected from the group consisting of: 1:2 (v/v), 1:3 (v/v), 1:4 (v/v), 1:5 (v/v), 1:6 (v/v), 1:7 (v/v), 1:8 (v/v), 1:9 (v/v), and 1:10 (v/v).

127. The method of claim 125, wherein the volume ratio of sample aliquot to lysis reagent is about 1:3 (v/v).

128. The method of claim 125, wherein the volume ratio of sample aliquot to lysis reagent is about 1:4 (v/v).

129. A method for performing a target analysis reaction, comprising the steps of:

(a) separating a target from a whole blood sample according to the methods of any one of claims 83 to 128; and

(b) performing an analysis reaction of the separated target to determine a characteristic of target.

130. The method of claim 129, wherein the characteristic of the target is determining that the target is a pathogen-derived target.

131. The method of claim 129 or claim 130, wherein the target is a nucleic acid target.

132. The method of any one of claims 129 to 131, wherein the analysis reaction si a nucleic acid analysis reaction.

133. The method of claim 132, wherein the nucleic acid analysis reaction is selected from the group consisting of: nucleic acid amplification, isothermal amplification, polymerase chain reaction, sequencing, real-time nucleic acid amplification, nucleic acid hybridization, and combinations thereof.

**INTERNATIONAL SEARCH REPORT**

International application No.
**PCT/US2016/032011**

**A. CLASSIFICATION OF SUBJECT MATTER**

**C12Q 1/68(2006.01)I**

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)
C12Q 1/68; C07H 21/04; C07H 21/00; C40B 30/04

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: sample aliquot, blood cells, lysis reagent, pooling

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 5973137 A (HEATH, ELLEN M.) 26 October 1999<br>See claims 1 and 5; example 1; column 4, lines 12-15; column 5, lines 62-66; column 6, lines 11-17 and column 9, lines 2-10. | 1-5,7-10,12-14,19,41,43-49,53-54,56-58,60-63,65,82,88-97 |
| Y | US 6063563 A (PEDDADA, LORRAINE B. et al.) 16 May 2000<br>See column 2, lines 29-33. | 1-5,7-10,12-14,19,41,43-49,53-54,56-58,60-63,65,82,88-97 |
| Y | US 2007-0042384 A1 (LI, WEIWEI et al.) 22 February 2007<br>See claims 1-2. | 9-10,12,62-63,65 |
| Y | EP 0133288 A2 (MILES LABORATORIES, INC.) 20 February 1985<br>See claims 1 and 3. | 89-97 |
| A | US 2011-0086769 A1 (OLIPHANT, ARNOLD R. et al.) 14 April 2011<br>See claims 1-4. | 1-5,7-10,12-14,19,41,43-49,53-54,56-58,60-63,65,82,88-97 |

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

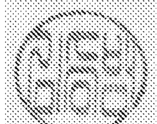
☒ See patent family annex.

* 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 | Date of mailing of the international search report |
|---|---|
| 22 August 2016 (22.08.2016) | **22 August 2016 (22.08.2016)** |


Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea
Facsimile No. +82-42-481-8578

Authorized officer
KIM, Seung Beom
Telephone No. +82-42-481-3371

## INTERNATIONAL SEARCH REPORT

International application No.
**PCT/US2016/032011**

**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.: See below .
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:
Each of claims 17-18, 21, 25-27, 29, 31, 33, 36-37, 39, 51, 68-70, 74, 76, 78-81, 84, 100-101, 103-105, 107, 113, 120-121, 126-128, 130 and 133 directly or indirectly refers to a claim which is not drafted in accordance with the third sentence of Rule 6.4(a).

3. ☒ Claims Nos.: See Extra sheet.
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:

1. ☐ As all required addtional 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 an additional fees, this Authority did not invite payment of any 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.:

**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.

(Continuation of Box No.II)

3. Claims Nos.
6, 11, 15-16, 20, 22-24, 28, 30, 32, 34-35, 38, 40, 42, 50, 52, 55, 59, 64, 66-67, 71-73, 75, 77, 83, 85-87, 98-99, 102, 106, 108-112, 114-119, 122-125, 129 and 131-132

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

**PCT/US2016/032011**

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| US 5973137 A | 26/10/1999 | None | |
| US 6063563 A | 16/05/2000 | AT 417940 T | 15/01/2009 |
| | | AU 717157 B2 | 16/03/2000 |
| | | BR 9807466 A | 02/05/2000 |
| | | CA 2276570 A1 | 16/07/1998 |
| | | CA 2276570 C | 29/06/2004 |
| | | CN 100354431 C | 12/12/2007 |
| | | CN 1246158 A | 01/03/2000 |
| | | DK 0975810 T3 | 02/03/2009 |
| | | EP 0975810 A1 | 02/02/2000 |
| | | EP 0975810 A4 | 15/09/2004 |
| | | EP 0975810 B1 | 17/12/2008 |
| | | ES 2319937 T3 | 14/05/2009 |
| | | HK 1025361 A1 | 01/08/2008 |
| | | ID 22743 A | 09/12/1999 |
| | | JP 2000-508181 A | 04/07/2000 |
| | | JP 2004-226411 A | 12/08/2004 |
| | | JP 2006-149391 A | 15/06/2006 |
| | | JP 2006-174843 A | 06/07/2006 |
| | | JP 2008-220369 A | 25/09/2008 |
| | | KR 10-0351472 B1 | 05/09/2002 |
| | | KR 10-2000-0069936 A | 25/11/2000 |
| | | NZ 336514 A | 22/12/2000 |
| | | PL 189041 B1 | 30/06/2005 |
| | | PT 975810 E | 07/01/2009 |
| | | RU 2198405 C2 | 10/02/2003 |
| | | US 2003-0204321 A1 | 30/10/2003 |
| | | US 2006-0136148 A1 | 22/06/2006 |
| | | US 2006-0160073 A1 | 20/07/2006 |
| | | US 2009-0263788 A1 | 22/10/2009 |
| | | US 5780222 A | 14/07/1998 |
| | | US 6566052 B1 | 20/05/2003 |
| | | US 8158341 B2 | 17/04/2012 |
| | | WO 98-30723 A1 | 16/07/1998 |
| | | YU 31899 A | 19/06/2002 |
| US 2007-0042384 A1 | 22/02/2007 | US 2009-0326209 A1 | 31/12/2009 |
| EP 0133288 A2 | 20/02/1985 | AU 3138884 A | 07/02/1985 |
| | | ES 8507178 A1 | 01/12/1985 |
| | | JP 60-110299 A | 15/06/1985 |
| US 2011-0086769 A1 | 14/04/2011 | CA 2748030 A1 | 01/07/2010 |
| | | CN 102325901 A | 18/01/2012 |
| | | EP 2379746 A1 | 26/10/2011 |
| | | JP 2012-513217 A | 14/06/2012 |
| | | KR 10-2011-0137286 A | 22/12/2011 |
| | | US 2015-0232937 A1 | 20/08/2015 |

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

**PCT/US2016/032011**

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| | | US 9051602 B2 | 09/06/2015 |
| | | WO 2010-075459 A1 | 01/07/2010 |