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Canadian Intellectual Property Office

CA 3153071 A1 2021/03/11

(21) 3 153 071

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2020/09/03
(87) Date publication PCT/PCT Publication Date: 2021/03/11
(85) Entrée phase nationale/National Entry: 2022/03/01
(86) N° demande PCT/PCT Application No.: US 2020/049290
(87) N° publication PCT/PCT Publication No.: 2021/046278
(30) Priorités/Priorities: 2019/09/03 (US62/895,387); 2019/11/13 (US62/934,754); 2020/04/07 (US63/006,570)

(51) Cl.Int./Int.Cl. *B01L 3/00* (2006.01), *C12Q 1/6813* (2018.01), *C12Q 1/6844* (2018.01), *C12Q 1/6876* (2018.01)

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(54) Titre : SYSTEMES, METHODES ET COMPOSITIONS POUR LA DETECTION PRECOCE RAPIDE DE BIOMARQUEURS D'ARN HOTE D'INFECTION ET L'IDENTIFICATION PRECOCE D'UNE INFECTION A CORONAVIRUS COVID-19 CHEZLES ETRES HUMAINS

(54) Title: SYSTEMS, METHODS, AND COMPOSITIONS FOR THE RAPID EARLY-DETECTION OF HOST RNA BIOMARKERS OF INFECTION AND EARLY IDENTIFICATION OF COVID-19 CORONAVIRUS INFECTION IN HUMANS

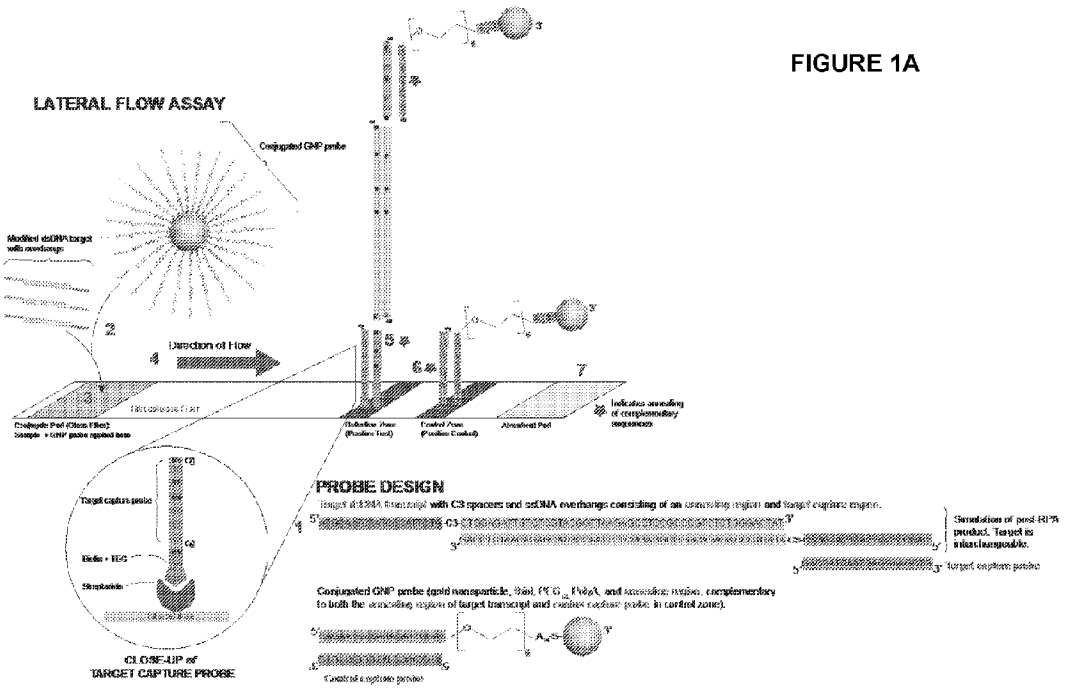

FIGURE 1A

(57) Abrégé/Abstract:
The current inventive technology is directed to systems, methods, and compositions detection of host signatures of pathogenic infection, and in particular a rapid detection assay configured to detect target RNA transcripts that may be biomarkers of infection. In one embodiment, the invention includes systems, methods and compositions for the early detection of pathogens or infection in an asymptomatic subject through a novel lateral flow assay, which in a preferred embodiment may include a rapid self-administered test strip configured to detect one or more RNA transcript biomarkers produced by a subject's innate immune system in response to a pathogen or infection and present in saliva.

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 March 2021 (11.03.2021)

WIPO | PCT

(10) International Publication Number
WO 2021/046278 A1

(51) International Patent Classification:
B01L 3/00 (2006.01) *C12Q 1/6844* (2018.01)
C12Q 1/6813 (2018.01) *C12Q 1/6876* (2018.01)

(21) International Application Number:
PCT/US2020/049290

(22) International Filing Date:
03 September 2020 (03.09.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/895,387 03 September 2019 (03.09.2019) US
62/934,754 13 November 2019 (13.11.2019) US
63/006,570 07 April 2020 (07.04.2020) US

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(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,

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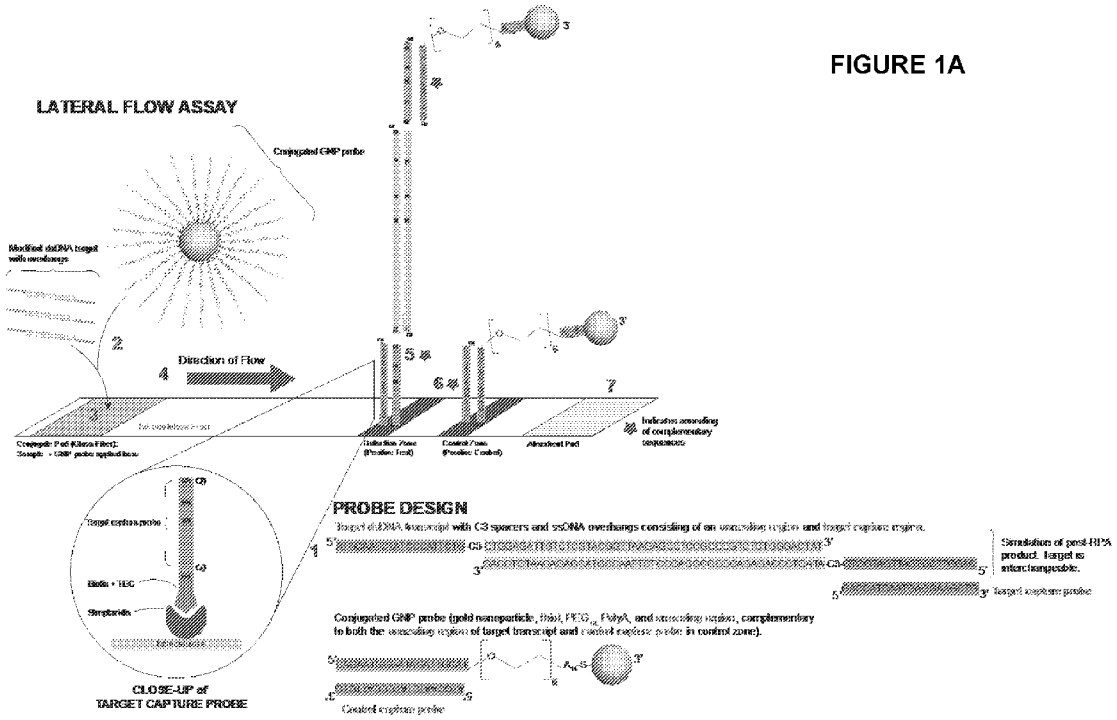
FIGURE 1A



(57) Abstract: The current inventive technology is directed to systems, methods, and compositions detection of host signatures of pathogenic infection, and in particular a rapid detection assay configured to detect target RNA transcripts that may be biomarkers of infection. In one embodiment, the invention includes systems, methods and compositions for the early detection of pathogens or infection in an asymptomatic subject through a novel lateral flow assay, which in a preferred embodiment may include a rapid self-administered test strip configured to detect one or more RNA transcript biomarkers produced by a subject's innate immune system in response to a pathogen or infection and present in saliva.

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

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

Published:

— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

SYSTEMS, METHODS, AND COMPOSITIONS FOR THE RAPID EARLY-DETECTION OF HOST RNA BIOMARKERS OF INFECTION AND EARLY IDENTIFICATION OF COVID-19 CORONAVIRUS INFECTION IN HUMANS

This application claims the benefit of and priority to U.S. Provisional Application No. 62/895,387, filed September 3, 2019, and U.S. Provisional Application No. 62/934,754, filed November 13, 2019, and U.S. Provisional Application No. 63/006,570, filed April 07, 2020. The entire specification and figures of the above-referenced applications are hereby incorporated, in their entirety by reference.

STATEMENT OF FEDERALLY SPONSORED RESEARCH

This invention was made with government support under grant number HDTRA1-18-1-0032 awarded by Defense Threat Reduction Agency (DTRA). The government has certain rights in the invention.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on August 30, 2020, is named “90245.00432-Sequence-Listing.txt” and is 2476 Kbytes in size.

TECHNICAL FIELD

The current inventive technology is directed to systems, methods, and compositions detection of host signatures of pathogenic infection, and in particular a rapid detection assay configured to detect target RNA transcripts that may be biomarkers of infection.

BACKGROUND

Early detection of infection by pathogenic microorganisms is vital for proper treatment and positive clinical outcomes. However, infected individuals may remain asymptomatic for several days post-infection while actively transmitting the pathogen to others. Traditional pathogen detection systems are often not effective at detecting the infection until after the onset of symptoms. Traditional pathogen testing includes serology or antibody-based tests, bacterial/viral/fungal growth cultures, and nucleic acid-based detection such as PCR (polymerase chain reaction). Such traditional tests are often time and labor intensive and are only effective after a patient has begun to show symptoms of the infection. Additionally, traditional diagnostic tests

require clinical suspicion for a specific pathogen, expensive laboratory equipment, trained personnel, and have increased upstream and end-user costs.

For example, as highlighted in Figure 2, in a typical infection course exposure to an unknown pathogen occurs at day zero and then progresses through subsequent clinical stages of infection as indicated by the timeline running vertically along the left side of the figure. As the pathogen replicates within the infected person, standard diagnostic tests are typically designed to work after the onset of symptoms, when people know there is something wrong and seek healthcare and diagnosis. However, at that point the person may have been contagious to others for several days or weeks. The opportunity to implement early quarantine and limit destructive downstream effects of unimpeded pathogen transmission has passed. This time delay to diagnosis can result in poorer patient outcomes and ongoing disease transmission before patients know they are contagious.

As opposed to the specialized, and later developing adaptive immune response, a host's first line of defense against pathogenic microorganisms is the "innate immune" response. The body's innate immunity is a self-amplifying and non-specific physiological response that occurs within hours of infection. As such, the ability to detect the presence of molecules produced by a host's innate immune response may provide the ability to rapidly detect infection at the earliest stages while a patient is still asymptomatic. Such advancement would allow for more effective quarantine protocols, as well as improved treatment and clinical outcomes.

The need for improved methods of detecting pathogens, especially early in the infection cycle, has been magnified by the worldwide coronavirus pandemic. Specifically, in 2019, a novel coronavirus identified as COVID-19, having a high infection and mortality rate, emerged in the Wuhan region of China and later spread throughout the world resulting in sever public health crisis. Coronaviruses, members of the Coronaviridae family and the Coronavirinae subfamily, are found in mammals and birds. A prominent member is severe acute respiratory syndrome coronavirus (SARS-CoV), which killed almost 10% of the affected individuals during an outbreak in China between 2002 and 2003. Another prominent coronaviruses called Middle East Respiratory Syndrome Coronavirus (MERS coronavirus or MERS-CoV) MERS-CoV shares some similarities with the SARS-CoV outbreak. Typical symptoms of a SARS. MERS and COVID-19 coronavirus infection include fever, cough, shortness of breath, pneumonia and gastrointestinal symptoms. Severe illness can lead to respiratory failure that requires mechanical ventilation and support in an

intensive care unit. Both coronavirus appears to cause more severe disease in older people, people with weakened immune systems and those with chronic diseases, such as cancer, chronic lung disease and diabetes. At present no vaccine or specific treatment is available for COVID-19. Patients diagnosed with a COVID-19 coronavirus infection merely receive supportive treatment based on the individual 's symptoms and clinical condition.

As outlined below, the present inventors have overcome the limitations of traditional pathogen detection systems while leveraging the host's early innate immune response (including but not exclusive to the interferon response) to rapidly detect RNA biomarkers indicative of infection, and particular infection with COVID-19 coronavirus. This rapid point-of-care diagnostic application allows detection of infection at the earlies stages when patients are typically asymptomatic. Such early detection is directly correlated with more targeted and effective therapeutic interventions as well as overall improved clinical outcomes.

SUMMARY OF THE INVENTION

The inventive technology may include systems, methods and compositions for the early detection of pathogens and/or infection in an asymptomatic subject through a novel lateral flow assay, which in a preferred embodiment may include a rapid test strip configured to detect one or more RNA transcript biomarkers produced by a subject's innate immune system in response to a pathogen or infection and present in saliva.

In another aspect the inventive technology may include systems, methods and compositions for the early detection of pathogens and/or infection in an asymptomatic subject through a novel lateral flow assay, which in a preferred embodiment may include a rapid test strip configured to detect one or more RNA transcript biomarkers encoded by one or more of the nucleotide sequences according to SEQ ID NOs. 1-444, and 657-815 produced by a subject's innate immune system in response to a pathogen or infection, and which may be present in saliva.

Additional aspects of the invention include the use of one or more biomarkers for infection, and preferably pathogen infection in humans according to the nucleotide sequences identified in SEQ ID NOs. 1-444, and 657-815.

In another aspect, the inventive technology may include systems, methods and compositions for the detection of these target RNA transcripts, which may act as biomarkers for early-infection in a subject.

In another aspect, the inventive technology may include systems, methods and compositions for the detection of early-infection in a subject which may include at least: a lateral flow assay test strip device which may preferably include a fibrous or paper-based lateral flow strip configured to allow liquid flow *via* capillary action; 2) a RT-RPA (reverse transcription recombinase polymerase amplification) reaction which may occur in a pre-prepared reaction cylinder, which may include a collective container configured to receive a fluid sample from a subject and pre-prepared to perform a RT-RPA reaction; and 3) one or more RNA biomarkers transcripts, for example one or more biomarkers encoded by the nucleotide sequences identified as SEQ ID NOs. 1-444, and 657-815, also generally referred to as biomarkers, supplied in a fluid sample, which in a preferred embodiment may include a saliva sample provided by a subject. In a preferred embodiment, an RNA biomarkers transcript may be amplified in a reaction cylinder in an isothermal amplification RT-RPA reaction to form either a hybrid dsDNA probe having single-stranded adapter sequences or a dsDNA product containing 5' modifications for downstream hybridization.

Additional aspects may include novel conjugated reporter probes that may be coupled with a hybrid dsDNA probe. In certain aspects, a novel conjugated probe may include a GNP, or other single reporter conjugated with a ssDNA sequence or antibody or antibody fragment that may bind to the dsDNA probe. While still, further aspects of the invention may include novel target capture probes that may bind to and form an immobilized "sandwiched" complex aggregate comprising an embedded capture probe coupled with the hybrid dsDNA probe which is further coupled to a conjugated reporter probe, and preferably a GNP reporter probe. In this aspect, the localized immobilization may facilitate the generation of a visual signal, for example on a test strip, or even solution.

Additional aspects of the invention include systems, methods, and compositions for the quantification of early host-derived biomarkers of infection that may or may not be combined with quantified data directed to pathogen specific biomarkers, preferably generated by PCR, RT-PCR, or qRT-PCR.. In one preferred aspect, RNA may be extracted from a biological sample provided by a potentially exposed or infected subject. The RNA may undergo qRT-PCT reaction to determine the levels of pathogen biomarkers, as well as host-derived biomarkers of infection, and preferably host-derived RNA biomarkers present in the subject's saliva. A plurality of biological samples may be taken from one or more subjects to generate a time-course of infection showing

the relative levels of pathogen, and host-derived biomarkers over time. This data may be used to generate biomarker candidates for a lateral flow assay to detect pathogen specific host-derived biomarkers. This lateral flow assay may be administered to a subject in need thereof and provide an indication of infection, as well as the stage of infection by one or more specific pathogens. In one preferred aspect, the specific pathogen may include the SARS-CoV-2, commonly referred to as the COVID-19 coronavirus.

Additional aspects of the invention may include one or more of the preferred embodiments set forth in the claims.

Additional aspects of the invention may be evidenced from the specification, claims and figures provided below.

BRIEF DESCRIPTION OF DRAWINGS

The novel aspects, features, and advantages of the present disclosure will be better understood from the following detailed descriptions taken in conjunction with the accompanying figures, all of which are given by way of illustration only, and are not limiting the presently disclosed embodiments, in which:

Figure 1 (A) show a general schematic diagram of a lateral flow assay in one embodiment of the invention thereof; (B) show another general overview of a lateral flow assay test strip in one embodiment of the invention thereof.

Figure 2 shows a representative example of an infection course.

Figure 3 (A) shows an exemplary *in vivo* mouse experiment demonstrating the current state of the art for detection of pathogen infection. In this case, a group of mice may be infected with a pathogen and blood samples will be collected at the indicated days post infection. These samples will be used to carry out high throughput sequencing in order to characterize the presence of biomarkers and may also be used to carry out tests to compare the current invention with current state-of-the art detection methods. Below shows exemplary data showing the invention's ability to detect pathogen infection several days before other methods. All of the illustrated assays will be carried out during prior *in vivo* experiments. (B) Shows a timeline of a hypothetical viral infection and various tests designed to detect that infection.

Figure 4 shows an exemplary pathogen detection device in one embodiment thereof and in particular highlights the device's capability for multiplexing. The technology of the invention, and in particular a lateral flow assay test strip or test strip, is adaptable to multiple configurations

depending on the aims of the end user. (A) As an initial screening test, the most important parameter is sensitivity to ensure no infected individuals are inadvertently labeled as "not sick" when they are in fact "sick." A highly sensitive test identifies near 100% of the true positive cases of illness and has a near 0% false negative rate. Sensitivity of RNA transcript biomarker assay is tunable by addition of multiple test lines for different biomarkers, which if detected in combination increases the probability of identifying all true positives. (B) For clinicians assessing already symptomatic patients among diverse medical settings (e.g. emergency departments, primary care offices, assisted care facilities, field hospitals, etc.), it is important to distinguish between the general category of pathogen (i.e. viral vs. bacterial vs fungal) to begin the best early treatment prior to full identification of the causative agent. The inventive assay could inform treatment plans and dramatically reduce the use of antibiotics in cases of non-bacterial infections to help limit the spread of antibiotic resistant bacteria. (C) Early investigation of host signals in response to specific organisms may allow for an assay configuration in which infection by a specific pathogenic organism may be identified. The panel of microbes tested for could be specified by the end users' needs. For example, the military may be most interested in varieties of airborne and weaponizable pathogens while a domestic clinic needs to evaluate patients for seasonal flu, RSV, rhinovirus, and norovirus.

Figure 5 shows the use of an exemplary pathogen detection device in one embodiment thereof. In this embodiment, the patient provides a saliva sample into a reaction cylinder, which may be represented here as a tube container preloaded with reaction reagents that may allow amplification reaction to proceed at room temperature to increase the biomarker concentration. Following this, the solution containing the amplified biomarkers may be applied to the lateral flow test strip. As fluid flows down the strip, a visible pink signal appears. In the simplest iteration of the strip, one band means a negative result and two bands equal a positive result indicating infection. In a consumer product embodiment, the strip will be contained in housing for ease of results interpretation.

Figure 6 (A) shows a Venn diagram indicating significant overlap in the identities of RNA transcripts expressed in saliva and PBMCs (peripheral blood mononucleated cells) according to sequencing data of healthy human samples. This overlap implies that transcripts present in the blood are also likely to appear in the saliva. Note, this transcript sequencing data was normalized to an average of 10 million reads coverage and does not describe abundance of these transcripts.

(B) Representative PCC (Pattern Correlation Coefficient) Plot showing relative expression levels of RNA transcripts present in both saliva and PBMCs (two samples from the same individual). Every dot in this graph symbolizes a different transcript in the overlapping section of the Venn diagram in A. The average r value = 0.64 (> 0.5 is considered significant correlation). Overall, there are higher levels of expression of most transcripts in PBMCs vs. saliva, but also a subset of transcripts that are upregulated in saliva relative to PBMCs. Due to this data, the present inventors can pursue saliva as our sample type of choice from which to identify key signals of early infection.

Figure 7 shows a general approach for identifying biomarkers of infection in one embodiment thereof.

Figure 8 shows an example of a host RNA biomarker for infection, IFIT2 that was identified using *in vitro* transcriptomic datasets. Horizontally, the gene structure is shown with dark blue bar indicating the coding region of the gene. Vertically, the height of the peaks represents the relative abundance of the indicated RNA. For each study, the "-" lane indicates non-infected sample, while "+" lane indicates various types of viral infection. The changes in abundance for different studies were highlighted in different colors. Together, the identified RNA biomarker is upregulated across 9 different cell types and 10 different viral infections. The upregulation of this biomarker can be detected *in vitro* as early as 4 hours post infection which is well prior to any observable symptoms. Additional biomarkers may be identified and selected for use in the invention in a similar procedure as described generally above.

Figure 9 shows qPCR of biomarker candidates in infected cells. Human lung cells (A549) were mock infected or infected with either influenza virus (left) or vesicular stomatitis virus (VSV, right) for 24 hours. RNA was collected and quantified using qPCR. Results are shown as 'fold change over mock,' and a dotted line indicates no change during infection. IFIT2 is an example of an RNA that is global marker of infection, as illustrated in Figure 8. In this example, NEAT1 would distinguish VSV from influenza, and OAS1 would distinguish influenza from VSV.

Figure 10 shows a schematic representation of optimization steps used to amplify and detect biomarkers from human saliva. Step 3.1, the RNA from 2 μL human saliva was successfully reverse transcribed into DNA and amplified using a customized RT-RPA kit. The reaction was achieved at constant 37°C within 20 minutes. Step 3.2, upon successful detection of the potential biomarker for infection, multiple primer sets with different lengths and sequences were designed to optimize the biomarker amplification. The primer set that resulted in the highest amplification

efficiency (reflected by the intensity of the band on the gel image) was chosen to be used in actual diagnosis. Step 3.3, the selected primers from previous step is modified to carry adapter sequences to allow downstream hybridization to lateral flow assay test strip and gold nanoparticle reporter probe. After RT-RPA amplification at 37°C for 20 minutes, the resulting amplicon contains both adapter sequences and the sequences from the target biomarker. The final reaction product can then be directly applied to test strip for visualization.

Figure 11 demonstrates complementary DNA binding forms nucleic acid "sandwiches" that aggregate for visual readout. The amplified biomarker has a double-stranded DNA (dsDNA) region flanked by specific single-stranded overhanging adapters. The solution with this biomarker is mixed with a gold nanoparticle reporter, which itself is conjugated to a single stranded DNA adapter complementary to adapters of the amplified biomarker and the control capture probe on the nitrocellulose. Due to the mechanism of complementary DNA base pairing, as these overhanging DNA adapter strands interact in solution flowing through the membrane they will bind and form dsDNA structures with the ssDNA conjugated gold nanoparticles and stationary oligo capture probes forming nucleic acid "sandwiches" (Fig. 4A). As more and more of these reporter-amplified biomarker-capture probe sandwich structures form and aggregate, a visible pink signal appears on the nitrocellulose in the target detection zone (B), indicating the presence of that biomarker in the original sample. Here, the leftmost pink dot is representative of the complex illustrated in panel A, and the second pink dot is a control where the gold reporter alone is binding to its complimentary probe. This control verifies that the sample flowed correctly over the strip.

Figure 12 shows colorimetric image of a series of test strips run with 10-fold dilutions of a synthetic RT-RPA product.

Figure 13A-D shows a lateral flow assay test strip having an external cover for ease of use in one embodiment thereof.

Figure 14 shows a general schematic diagram of a lateral flow assay incorporating an antibody-based capture mechanism in one embodiment of the invention thereof.

Figure 15 shows a general flow diagram of an exemplary laboratory-based test and lateral flow test for detection of biomarkers.

Figure 16 shows a flow-chart diagram for a designing and validating primers for biomarker candidates. The system being described in US Provisional Application Nos. 62/934,873, and 63/006561, incorporated herein by references with respect to the disclosure of Figure 16.

Figure 17A-B: show host RNA biomarkers are gene transcripts deriving from the earliest immune responses of infected cells. The heatmap was generated from published RNA sequencing datasets and shows the level of expression change (color code at left) of certain RNA species upon infection of cultured human cells with different pathogens (top). In all cases, mock infected (-) and infected (+) cells are compared. Some of the SARS-CoV-2- and Influenza A- specific biomarkers are shown in the orange and green highlighted boxes.

Figure 18 shows various RNA biomarkers upregulated in response to diverse types of infections and are detectable in human saliva. (A) The heatmap was generated from published RNA sequencing datasets and shows the level of expression change (color code below) of certain RNA species upon infection of cultured human cells with different pathogens (top). (B) In all cases, mock infected (-) and infected (+) cells are compared. Here, we have saliva samples from 3 patients in the infectious disease unit. These represent acute infections with either a fungus (patient 1; *Coccidioides*), a virus (patient 2; Varicella-zoster virus), and a bacteria (patient 3; *E. coli*). Quantitative RT-PCR was carried out to measure the fold change of eight of our biomarker RNAs, relative to a healthy saliva control. Note the log scale on the Y-axis, indicating that these biomarkers are found at levels 10-10,000 times higher in the saliva of infected individuals compared to the saliva of healthy individuals. There are also saliva biomarkers that may be able to differentiate one type of infection from others, such as EGR1 which does not respond to fungal infection but is upregulated 100,000-fold in viral infection.

Figure 19 shows host biomarker upregulation can be detected in a multiplexed RT-qPCR reaction. Human lung cells (A549) were either mock infected or infected with influenza virus and RNA was purified from cell lysates 24 hours after infection. RNA was then subjected to an RT-qPCR reaction using Taqman probes and chemistry. The biomarkers indicated on the X-axis were either measured in singleplex (black bars) or multiplex (orange bars) reactions using the primers and probes listed in Table 4. Relative mRNA expression (Y-axis) was calculated by first using a host control gene to internally normalize samples, and then compared to the mock infected samples.

Figure 20 shows some host biomarker upregulation precedes viral RNA detection. A human liver cell line (Huh7) was either mock infected or infected with the SARS-CoV-2 coronavirus. RNA was purified from cell lysates at 0, 2, 4, 8, 12, 24, and 48 hours post infection (X-axis). RNA was then subjected to RT-qPCR using the primers and probes listed in **Table 4**.

Relative mRNA expression (Y-axis) was calculated by first using a host control gene to internally normalize samples, and then compared to the mock infected samples. A full panel of biomarkers is shown on the left, whereas a subset of biomarkers are shown on the right that highlights biomarkers that are upregulated in the early-stage of infection (blue), late-stage of infection (green), and host control biomarkers that are no upregulated (gray). Detection of the SARS-CoV-2 nucleoprotein gene (N2) is also shown in red.

Figure 21 show an exemplary lateral flow strip with antibody capturing scheme. Lateral flow strips were striped according to the schematic of Figure 4 sMimic amplicons were generated in order to test the sensitivity of the lateral flow strip. The 'excess' line is capturing excess anti-FITC conjugated gold nanoparticles. The 'control' line is capturing mimic amplicons conjugated with FITC and Biotin. The 'test' line is capturing mimic amplicons conjugated with FITC and DIG.

Figure 22 shows Table 3 which includes primers for detecting host biomarkers of infection. A subset of candidate biomarkers was chosen for primer optimization. Listed primer sets were used to carry out RT-qPCR to optimize primer efficiency, Ct values, melting curves, and log fold-change with respect to two host control biomarkers (RACK1 or CALR). Expression in untreated human lung cells (A549) was compared to either interferon treated A549 cells (A549+IFN) or influenza virus infected A549 cells (A549+flu).

Figure 23 shows Table 4 which includes primers and probes for multiplexed detection of host biomarkers. A subset of candidate biomarkers from Table 3 was chosen based on their large fold-changes. Taqman probes were designed for each primer set to be compatible with Taqman fluorescent chemistry in an RT-qPCR reaction. Biomarkers were grouped into triplets based on Ct values in order to be compatible for multiplexing.

Figure 24 shows Table 5 which includes primers for amplifying host biomarkers using isothermal RT-RPA. A subset of candidate biomarkers was chosen for optimization of RT-RPA reactions (A). Those primer sets that satisfied conditions presented in Figure 16 were then modified to contain 5' modifications (FITC, Biotin, or DIG) for compatibility with the lateral flow assay of the invention (B).

Figure 25 shows amplified products from RT-RPA reactions can be detected on a lateral flow strip. (A) Lateral flow strips striped with secondary anti-rabbit antibody (gold nanoparticle excess line), streptavidin (control line) or anti-DIG antibody (biomarker line) were used to resolve

the indicated RT-RPA reactions. Sample #1 only contains PBS and no RT-RPA reaction products, whereas all the other samples contain RT-RPA reaction (20-minute reaction) products. RT-RPA was carried out using purified RNA from influenza infected human lung cells (A549) as a template. (B) Lateral flow strips as described in panel A were used to confirm that primer sets on their own do not produce a false positive signal. Indicated primer sets were mixed with PBS at the same concentration of an RT-RPA reaction and run out on the strips.

DETAILED DESCRIPTION OF INVENTION

The inventive technology may include systems, methods and compositions for the early detection of pathogens and/or infection in an asymptomatic subject through a novel lateral flow assay, which in a preferred embodiment may include a rapid self-administered test strip configured to detect one or more host RNA transcript biomarkers (coding or non-coding) produced by a subject's innate immune system in response to a pathogen or infection and present in saliva.

As generally shown in Figure 1B, one embodiment the inventive technology may include systems, methods and compositions for the detection of early-infection in a subject which may include at least: a lateral flow assay test strip device (also refer to as a test strip, or lateral flow strip), which may preferably include a fibrous or paper-based lateral flow strip configured to allow liquid flow *via* capillary action; 2) a RT-RPA (reverse transcription recombinase polymerase amplification) reaction which may occur in a pre-prepared reaction cylinder , which may include a collective container configured to receive a fluid sample from a subject and pre-prepared to perform a RT-RPA reaction; and 3) one or more RNA biomarkers transcripts, also generally referred to as biomarkers, supplied in a fluid sample, which in a preferred embodiment may include a saliva sample provided by a subject.

Specific target RNA transcripts or biomarkers produced by a patient's immune response (generally innate immune response or any other cellular pathway upregulated upon infection) and found in saliva may be indicative of early infection. As a result, in one embodiment of the inventive technology may include systems, methods and compositions for the detection of these target RNA transcripts, which may act as biomarkers for early-infection in a subject. However, as noted above, target RNA transcript biomarkers present in a typical fluid sample provided by, in this embodiment a human subject, are generally present at low concentrations and require amplification to be detected. To overcome this physical limitation, as further shown in Figure 1B, in one embodiment of the invention, a subject may deposit a fluid sample, which in this case may comprise a saliva

sample, into a reaction cylinder where it may undergo an amplification step. Specifically, a reaction cylinder may receive a fluid sample where it may undergo a RT-RPA reaction to amplify the RNA biomarker transcripts present in a fluid sample. In this preferred embodiment, a reaction cylinder may be pre-loaded with a quantity of pre-prepared proteins, enzymes, salts, and other reagents that may allow for a RT-RPA reaction to proceed within the reaction cylinder. As shown in Figure 1A, the reaction cylinder may be pre-loaded with primers directed to target RNA biomarker transcripts that may further include C3 spacer elements. In another preferred embodiment, a reaction cylinder may further be pre-loaded with one or more conjugated reporter probes, such as a conjugated gold nanoparticle (GNP) reporter probe.

In other embodiments, conjugated reporter probes, such as a conjugated gold nanoparticle (GNP) reporter probe may be pre-embedded, dried, lyophilized, or otherwise attached to the conjugate pad instead of being pre-loaded into the reaction cylinder. This specific embodiment may allow for the generation of a lateral flow assay test strip having multiple pre-embedded conjugate pads with different conjugated reporter probes.

Again, as shown in Figure 1B, a fluid sample may be introduced into a reaction cylinder manually by a subject, or through another automated, or semi-automated process, such that one or more RNA biomarker transcripts present in a fluid sample interact with the RT-RPA components, including the modified primers pre-loaded into the reaction cylinder to facilitate a RT-RPA amplifying reaction. Importantly, in this preferred embodiment, the reaction cylinder may be configured to generate the RT-RPA reaction isothermally.

In one embodiment, a reaction cylinder may contain the necessary pre-prepared proteins, enzymes, salts, and other reagents necessary for a RT-RPA reaction to proceed isothermally at approximately room temperature (~25°C) or body temperature (~37°C) by holding in one's hand, eliminating the need for the laboratory equipment generally required to amplify nucleic acids. In one preferred embodiment, the RT-RPA reaction may proceed in the reaction cylinder for a period of approximately 30 minutes or less.

As highlighted in Figure 1A, the result of this isothermal RT-RPA reaction may include an engineered probe having a hybrid double stranded DNA (dsDNA) probe of a target biomarker sequence (GREEN) coupled, in this case through a C-3 spacer, with overhanging single-stranded DNA (ssDNA) regions at its 3' and 5' ends. A first overhanging ssDNA region, in Figure 1a at the 5' end of the dsDNA probe, may include an annealing region (ORANGE), while a second

overhanging ssDNA region, shown here at the 5' end of the dsDNA probe may include a target capture region (BLUE).

Once the RT-RPA reaction is completed, the contents of the reaction cylinder may be introduced to one or more conjugated reporter probes, which in a preferred embodiment may act as visual reporters by producing an observable indication of, for example the presence of a target RNA biomarker transcript in a sample. As shown above, a conjugated reporter probe may include a conjugated gold nanoparticle (GNP) conjugated to single stranded DNA (ssDNA) molecule complementary to both the annealing regions of the hybrid double stranded DNA molecules and a control capture probe as discussed below. Naturally, the use of a GNP is exemplary only, as a variety of metalloid nanoparticle reporters of various geometries and sizes may be incorporated into the inventive technology. Additional embodiments may also include one or more non-metalloid reporter probes, such as fluorescence, enzymatic, or antibody reporters.

Again, referring to Figure 1A, in the preferred embodiment highlighted above, this annealing region may be coupled with a GNP through a thiol, PEG_{18} and PolyA construct. Notably, in this configuration, when a conjugated GNP reporter probes are concentrated in solution or in a small surface area, such as one or more discrete bands on the lateral flow test strip shown in Figure 13, they may provide a visual signal, which in this embodiment may include a colored band, shown as a red band in Figures 1B and 13.

As further shown in Figure 1B, the hybrid dsDNA probe containing the target dsDNA transcript sequence with an annealing region and target capture region generated in the amplifying reaction in reaction cylinder may be combined with a DNA-conjugated GNP reporter probe. In this embodiment, in the presence of an optimal running buffer, the complementary regions of the hybrid DNA molecule and DNA-conjugated GNP reporter probe may anneal forming an aggregated complex. As should be understood from the disclosure, such aggregate complexes may only form if the expected target sequence, in this case a biomarker indicative of early-infection, is both present in the sample and amplified via the RT-RPA reaction localized in the reaction cylinder.

Referring now to Figures 1A-B, in a preferred embodiment, the combined solution containing the aggregate complexes formed by the hybrid dsDNA probe coupled with the DNA-conjugated GNP reporter probe may be introduced to the lateral flow strip. In a preferred embodiment, this combined solution may be introduced into a conjugate pad region made

preferably of glass fiber. The combined solution may flow *via* capillary action through a membrane, such as a nitrocellulose fiber membrane, towards an absorbent pad region on the lateral flow strip that may include a detection zone having one or more capture probes embedded to the surface of the lateral flow strip, and preferably the surface of a nitrocellulose membrane of a test strip. The position and orientation of the capture probes embedded in nitrocellulose membrane of a test strip may be adjusted to optimize signal generation or sample-probe interactions. Notably, the absorbent pad region may be positioned at the distal end of the lateral flow strip to facilitate sample flow *via* capillary action through the detection zone.

As highlighted in Figure 1A, a capture probe may include an immobilized streptavidin base tetramer embedded in the nitrocellulose surface of a lateral flow strip. This immobilized streptavidin base may be coupled with a biotin-TEG linker that may further be coupled with a ssDNA target capture probe sequence that may be complementary to a target capture region on a hybrid dsDNA probe.

Again, in the preferred embodiment shown in Figure 1A, the target capture region of a hybrid dsDNA probe may anneal to a complementary capture probe ssDNA sequence forming an immobilized "sandwiched" complex aggregate comprising an embedded capture probe coupled with the hybrid dsDNA probe which is further coupled to the DNA-conjugated GNP reporter probe. As can be seen in Figures 1A-1B, where a biomarker of interest is present (i.e. a biomarker indicative of pathogen infection in a subject), the "sandwich" complex may be immobilized at a discrete position along the lateral flow strip. As noted above, the GNP reporter probes of the invention produce a red color signal in solution or when immobilized on the lateral flow strip. As such, when a certain concentration of complex aggregates is captured in close proximity to one another a visible signal within the detection zone may be generated, which in this exemplary embodiment is shown as a red-pink band on the lateral flow strip. This visible signal within the detection zone may indicate a positive result indicating the presence of a target pathogen, or an early-indication of infection in a subject. Notably, this process as generally described above may take less than 10 minutes and, in some instances, less than 3 minutes to run to completion and provide a discernable signal.

As further shown in Figure 1A, any unbound GNP reporter probes not immobilized within the detection zone may continue to flow through the lateral flow strip towards a distal absorbent pad and anneal to a control capture probe immobilized to a control region on the surface of the

lateral flow strip. In this manner, the unbound GNP reporter probes immobilized in the control region will also produce a visible signal providing a positive control for the system.

In an alternative embodiment, the invention may include a lateral flow assay strip having an antibody-based capture mechanism. Similar to the lateral flow assay described in Figure 1A, the result of this isothermal RT-RPA reaction may include an amplified RPA product that may act as a control biomarker, and another amplified RPA product that may act as an infection biomarker. Once the RT-RPA reaction is completed, the contents of the reaction cylinder may be introduced to one or more conjugated antibody reporter probes, which in a preferred embodiment may act as visual reporters by producing an observable indication of, for example the presence of a target RNA biomarker transcript in a sample. More specifically, as shown in Figure 14, the isothermal RT-RPA reaction may generate at least two amplified RPA products, or amplicons, namely a control biomarker and infection biomarker respectively having modified 5' ssDNA overhang regions forming a probe capture region and a target capture region respectively. In this embodiment, a control biomarker may include a dsDNA transcript region coupled with a 5' FITC forward ssDNA oligo (GREEN) and 5' biotin reverse ssDNA oligo (ORANGE). The infection biomarker of this embodiment may include a dsDNA transcript region coupled with a 5' FITC forward ssDNA oligo (GREEN and PINK) and a 5' DIG ssDNA reverse oligo (BLUE).

As further shown in Figure 14, GNP may be conjugated with an anti-FITC (fluorescein isothiocyanate) antibody, and preferably an anti-FITC antibody produced in a rabbit. As also shown in Figure 14, streptavidin may also be stripped onto the membrane as generally described above to capture control biomarker amplicons present in the amplified RPA product. In this embodiment, an anti-DIG (Digoxigenin) antibody, and preferably an anti-DIG antibody raised in mouse, may also be stripped onto the lateral flow membrane to capture infection biomarker amplicons present in the amplified RPA product.

As further shown in Figure 14, the hybrid dsDNA control and infection amplicon probes generated in the amplifying reaction may be combined with an anti-FITC antibody-conjugated GNP reporter probe. In this embodiment, the anti-FITC antibody may bind to the 5' FITC-forward oligo of the control and infection biomarker forming an aggregated complex. In this embodiment, the aggregated complexes may further be introduced to the lateral flow strip of the invention. In a preferred embodiment, this combined solution may be introduced into a conjugate pad region made preferably of glass fiber. The combined solution may flow *via* capillary action through a

membrane, such as a nitrocellulose fiber membrane, towards an absorbent pad region on the lateral flow strip that may include a detection zone having one or more capture probes embedded to the surface of the lateral flow strip, and preferably the surface of a nitrocellulose membrane of a test strip. The position and orientation of the capture probes embedded in nitrocellulose membrane of a test strip may be adjusted to optimize signal generation or sample-probe interactions. Notably, the absorbent pad region may be positioned at the distal end of the lateral flow strip to facilitate sample flow *via* capillary action through the detection zone.

As noted above, a capture probe may include an immobilized streptavidin base tetramer embedded in the nitrocellulose surface of a lateral flow strip. This immobilized streptavidin base may be coupled with a biotin-TEG linker that may further be coupled with a ssDNA target capture probe sequence that may be complementary to a target capture region on a hybrid dsDNA probe, and preferably the 5' biotin-reverse oligo. Further, a capture probe may include an immobilized anti-DIG antibody that may be configured to bind to the 5' DIG-reverse oligo. In this configuration, control and infection biomarker amplicons may be bound to their respective locations by their respective capture probes. As noted above, the GNP reporter probes of the invention produce a red color signal in solution or when immobilized on the lateral flow strip. As such, when a certain concentration of complex aggregates are captured in close proximity to one another a visible signal within the detection zone may be generated. This visible signal within the detection zone may indicate a positive result indicating the presence of a target pathogen, or an early-indication of infection in a subject. Notably, this process as generally described above may take less than 10 minutes and, in some instances, less than 3 minutes to run to completion and provide a discernable signal.

As further shown in Figure 1A, any unbound GNP reporter probes not immobilized within the detection zone may continue to flow through the lateral flow strip towards a distal absorbent pad and anneal to an anti-rabbit control capture probe immobilized to a control region on the surface of the lateral flow strip, being configured to capture unbound antibody-conjugated GNP reporter probe. In this manner, the unbound GNP reporter probes immobilized in the control region may also produce a visible signal providing a positive control for the system.

Naturally, the system may be adapted for a variety of practical applications. For example, the system may be modified to detect a plurality of biomarkers RNA transcripts corresponding with a plurality of distinct capture probes at a plurality of detection zones on a lateral flow strip.

Moreover, it should be noted that such probes and their design are exemplary only, as a variety of different probe configurations, as well as probe-generated signals may be interchangeable within the system as generally described herein.

For example, as shown in Figure 4, in one embodiment, the above described lateral flow detection system may be used to detect, with varying degrees of sensitivity, infection of a subject by a known or unknown pathogen. In other embodiments, the above described lateral flow detection system may be used to determine pathogen type, such as bacteria, virus or fungal. In additional embodiments, the above described lateral flow detection system may be used to determine specific pathogens or their serotypes.

In one embodiment the inventive technology may include novel systems, methods, and composition for the detection of pathogen specific infection in a subject in need thereof. In one preferred embodiment, the inventive technology may provide for the detection of infection of a specific pathogen in a human subject. In this preferred embodiment, a biological sample, which may preferably include a saliva sample, may be provided by a subject which may contain one or more biomarkers for infection with a specific pathogen. In this embodiment, a saliva sample, may be further processed, for example by an on-site, or off-site clinical laboratory wherein RNA molecules present in the saliva sample are extracted for further testing. The extracted RNA is then undergoing a qRT-PCR process where the biomarkers of the pathogen. In the embodiment, one or more of the primer sequencers known to be directed to a components of a target pathogen may be used to identify specific biomarkers produced by the target pathogen. In this embodiment, the subject may provide a plurality of biological samples for RNA extraction and qRT-PCT processing so as to generate a time-course of pathogen biomarkers. These plurality of samples may provide a quantified baseline progression of target pathogen biomarkers from an initial point of exposure to the pathogen in a subject. As can be appreciated from the foregoing, such processes may be implemented for multiple target pathogens, and may further be conducted in series using multiple subjects to generate a library of time-course biomarkers of target pathogens.

As noted above the inventive technology may allow the detection of host-derived biomarkers that may be present in a subject's biological sample before the virus can be detected and well before any symptoms of infection may occur. In one preferred embodiment, RNA may be extracted from the biological sample, which in this case is a saliva sample containing host derived biomarkers of infection and further subject to qRT-PCR. In this embodiment, the subject

may provide a plurality of biological samples for RNA extraction and qRT-PCT processing so as to generate a time-course of host-derived biomarkers. Again, multiple samples may provide a quantified baseline progression of host-derived biomarkers, such as RNA biomarkers generated by the hosts innate-immune response in response to the target pathogen from an initial point of exposure to the pathogen and through the incubation period. Again, as can be appreciated from the foregoing, such processes may be implemented for multiple target pathogens, and may further be conducted in series using multiple subjects to generate a library of time-course host-derived biomarkers, and preferably host-derived RNA biomarkers produced in response to a target pathogen. By combining RNA markers from both the host innate-immune response occurring during the incubation period, and from the target pathogen itself, the invention may expand the detection window for infection by various pathogens.

In one preferred embodiment, the inventive technology may provide for the detection of infection of the novel coronavirus SARS-CoV-2 (COVID-19) in a human subject, and in particular host-derived biomarkers of infection generated in response to infection of the novel coronavirus SARS-CoV-2 (COVID-19) in a human subject. As noted above, this example is merely exemplary of a number of different pathogens that may be incorporated in places of the COVID-19 coronavirus. As shown in Figure 15, in this preferred embodiment, a biological sample, which may preferably include a saliva sample, may be provided by a subject which may contain one or more biomarkers for COVID-19 infection. In this embodiment, a saliva sample, may be further processed, for example by an on-site, or off-site clinical laboratory wherein RNA molecules present in the saliva sample are extracted for further testing. The extracted RNA is then undergoing a qRT-PCR process where the biomarkers of the pathogen, in this case the COVID-19 coronavirus are identified. In the embodiment, one or more of the primer sequencers identified in Table 2 (SEQ ID NO. 469-480) below may be used to identify specific biomarkers produced by the COVID-19 coronavirus. In this embodiment, the subject may provide a plurality of biological samples for RNA extraction and qRT-PCT processing so as to generate a time-course of pathogen biomarkers. For example, as shown in Figure 15B, multiple samples may provide a quantified baseline progression of pathogen biomarkers from an initial point of exposure to the pathogen.

As noted above the inventive technology may allow the detection of host-derived biomarkers that may be present in a subject's biological sample the virus can be detected before any symptoms of infection may occur. In one preferred embodiment, RNA may be extracted from

the biological sample, which in this case is a saliva sample containing host derived biomarkers of infection and further subject to qRT-PCR. In this embodiment, the subject may provide a plurality of biological samples for RNA extraction and qRT-PCT processing so as to generate a time-course of host-derived biomarkers. For example, as shown in Figure 15B, multiple samples may provide a quantified baseline progression of host-derived biomarkers, such as RNA biomarkers generated by the hosts innate-immune response in response to the COVID-19 pathogen from an initial point of exposure to the pathogen and through the incubation period. Again, as shown in Figure 15, by combining RNA markers from both the host innate-immune response occurring during the incubation period, and from the COVID-19 coronavirus itself, the invention may expand the detection window for COVID-19 coronavirus infection.

Referring now to Figure 15C, in another embodiment, a lateral flow assay strip may be configured to detect one or more host-derived biomarkers of COVID-19 infection, and preferably host-derived RNA biomarkers of COVID-19 infection, as well as biomarkers of COVID-19 infection. As noted in the Figure 15C, the lateral flow assay strip may be configured to include a plurality host-derived RNA biomarkers of COVID-19 infection positioned sequentially according to their prevalence during the time-course of infection established by qRT-PCR described above. In this manner, the lateral flow assay strip of the invention may be able to not only identify a subject that has been exposed to a pathogen, such as the COVID-19 coronavirus, but may include sequential detection lines embedded with one or more biomarkers that correspond to a selected time-course of infection. In this preferred embodiment, a subject may provide a biological sample, and preferably a saliva sample. The saliva sample is allowed to undergo an amplification reaction to increase the quantity of biomarkers and then applied to the lateral flow assay strip as generally described above. In this embodiment, the host-derived RNA biomarkers of COVID-19 infection may be immobilized by target capture probes forming an immobilized aggregate complex which may in turn produce a visible single, again, as generally described above.

Notably, in this embodiment, COVID-19 biomarkers may also be immobilized by target capture probes forming an immobilized aggregate complex which may in turn produce a visible single separate from the host-derived RNA biomarker visual signal. In this manner, a subject, or health care worker may be able to quickly identify: 1) if the subject has been exposed to, in this case the COVID-19 coronavirus; 2) if the subject is infected with the COVID-19 coronavirus but is still in the incubation period of the virus's infection cycle; 3) the approximate time since

exposure the COVID-19 coronavirus; 4) the approximate time that the infection with the COVID-19 coronavirus biomarkers may be contagious. As can further be appreciated, in additional embodiment, the lateral flow assay strip may further be configured to identify pre-symptomatic subjects, as well as asymptomatic subjects. Most importantly, the results of the lateral flow assay may allow early identification of infection and facilitate proper quarantine and contact tracing protocols.

The invention now being generally described will be more readily understood by reference to the following examples, which are included merely for the purposes of illustration of certain aspects of the embodiments of the present invention. The examples are not intended to limit the invention, as one of skill in the art would recognize from the above teachings and the following examples that other techniques and methods can satisfy the claims and can be employed without departing from the scope of the claimed invention. Indeed, while this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

EXAMPLES

Example 1: Identification of target biomarkers of infection.

In one embodiment the invention may include systems, methods and compositions for the identification and use of one or more RNA transcript biomarkers. As shown in Figure 7, in one preferred embodiment, a first tissue culture experiment (left) can be established and tested to identify target RNA transcripts that may be upregulated during an experimental infection, and that may also be secreted from target cells. RNAs that are upregulated may be used as candidate biomarkers and engineered for compatibility with the lateral flow system as generally described above. In parallel, RNAs from healthy and infected human saliva may be characterized in a clinical trial (right) in order to identify RNA biomarkers of infection in humans. Those biomarkers, if not already identified in the tissue culture experiments, will for compatibility with the lateral flow system as generally describe above.

Example 2: Identification of early host biomarkers.

As generally shown in Figure 8, one embodiment of the invention includes the identification of early host biomarkers for infection using a bioinformatic meta-analysis. In order to identify host nucleic acid biomarkers produced in response to infection at an early stage, the

present inventors searched publicly available transcriptomic datasets. The selected datasets were directed to those generated using various human tissue types that are infected by different viruses at multiple time points. The present inventors analyzed these datasets using a standardized bioinformatic pipeline and identified human coding and non-coding RNA that are upregulated in response to infection. These data summarized the host RNA transcripts that are commonly upregulated across different studies. This list of commonly upregulated RNA transcripts was comprised of exemplary candidate RNA transcript biomarkers. The upregulation of these RNA transcripts signals an ongoing infection (Example in Figure 1).

Concurrently, the present inventors also collected and sequenced RNA purified from saliva samples of healthy and clinical human participant. Through bioinformatic data analysis, the RNA transcripts that are significantly different between healthy participants and infected patients were identified and cataloged. These clinical datasets may then be used to filter out the potential biomarkers. Altogether, the final list of host RNA biomarkers may have the potential to differentiate healthy individuals from subjects that are infected by various pathogens (viruses, bacteria, fungi and protists), using saliva as the non-invasive diagnostic material.

Example 3: Validation of target biomarkers.

As generally shown in Figure 9, one embodiment of the invention includes the validation of target biomarkers using quantitative polymerase chain reaction (PCR) protocols. As biomarkers identified using the methods outlined above may be further confirmed in tissue culture infection experiments. Reverse Transcription quantitative PCR (RT-qPCR) of RNA allows specific quantification of the upregulation of candidate biomarkers as a 'fold change' in infected cells compared to uninfected cells. Such information helps when evaluating detection sensitivity of the lateral flow assay stick with respect to a given biomarker.

While only six exemplary biomarker candidates are being shown here, such list should not be construed as limiting on the number of biomarkers that may be used with the current invention. Indeed, there may be numerous biomarker candidates that may be incorporated into the invention as described herein.

Example 4: Isothermal amplification of infection biomarkers from a bodily fluid sample.

Upon successful validation of RNA biomarkers that are upregulated during infection *in vitro*, the target RNA biomarker may be subjected to one or more optimization processes to ensure

successful isothermal amplification of the biomarker from human saliva and visualization on a lateral flow assay stick.

As generally shown in Figure 10, the presence of a target RNA transcript biomarker in a bodily fluid sample, which in a preferred embodiment may include saliva, is confirmed using an isothermal, one-step reverse transcription and recombinase polymerase amplification (RT-RPA, Piepenburg et al., PLoS Biology 2006) (Fig 10 Step 3.1). The RT-RPA may be customized by combining TwistDX TwistAmp Basic RPA kit with additional RNase inhibitor, reverse transcriptase and oligo dT primers. The use of this customized reagent allows one-step conversion from target RNA to DNA, which can then be amplified to enhance signal at 37° Celsius (approximate body temperature) within 10-20 minutes.

As further shown in Figure 3 Step 3.1, the amplicon may be separated on 2% agarose gel and visualized by ethidium bromide staining. Comparing to the positive control, the RT-RPA amplified the target RNA biomarker using as low as 2 μL human saliva as input, without additional purification. To achieve efficient amplification and detection, multiple primer sets were designed to amplify the target biomarker (Fig 10 Step 3.2). These primer sets vary in length and sequence. While keeping other parameters constant, the efficiency for each primer set to amplify the target RNA is compared based on the intensity of amplicon visualized on 2% agarose gel. In the example shown in Figure 10, while all primer sets where able to amplify the target biomarker, primer set #3 resulted the highest amplification efficiency. Thus, primer set #3 is further integrated into the downstream processes. Finally, based on the test result from Step 3.2, the optimal primer sequences were concatenated with customized adapter sequences on 3' and 5' ends that may be complimentary to probe sequences on a gold nanoparticle-based probe and a target capture probe embedded in the test strip, respectively (Figure 3 Step 3.3). The primers with adapters were then used to amplify the biomarker RNA.

To ensure the adapter sequence remain single-stranded after RPA amplification, the present inventor introduced a tri-carbon chain spacer (C_3) within the primer sequence to prevent DNA polymerase from generating the complementary strand of the adapter sequences. As the result, the end product may include an amplified hybrid DNA probe having with a target dsDNA transcript region, while maintaining the single-stranded adapter sequences for downstream hybridization.

Example 5: Visualization of amplified product using lateral flow assay stick.

As shown in Figure 11, the primary unit of the detection assay is a membrane, which is the substrate through which the solution containing the amplified biomarker(s) and the reporter flow. In one preferred embodiment, a membrane may include one or more embedded capture probes that are able to bind complementary probes in the solution that flows through the membrane. As the capture probes bind their respective amplified biomarker or the reporter, a signal appears that indicates infection or no infection. Multiple variables within this broad description of this assay are tunable to be able to express different types of results.

Colorimetric image of a series of test strips run with 10-fold dilutions of a synthetic RT-RPA product are shown in Figure 12. In this example, a sample contains 2μL amplified biomarker(s), 10μL gold reporter, and 8μL running buffer is applied to the conjugate pad of the test strip. (Concentrations of RT-RPA product are listed along with the visual readout.) The solution flows through the nitrocellulose membrane towards the absorbent pad via capillary action. Samples with amplified biomarkers above the limit of detection will aggregate at the first circle in the detection zone. Excess gold reporter that does not interact with amplified biomarkers, either because they were not present in the initial sample or their concentration is below the limit of detection, will continue to flow down the strip and aggregate at the control zone.

In the example of the strips shown in (A), a negative result will show one circle on the right side and a positive result will show two circles present (even if faint intensity). To enhance intensity of visual signal, additional 10μL gold reporter and 8μL running buffer were combined and applied again to the conjugate pad. (B) Is a color image of the same strips as in (A) shown for comparison. (C) The assay can be assembled to multiplex using different capture probes on the test strip and different adapter primers in the RT-RPA reaction.

Example 6: Materials and Methods.

As shown in the Figures generally, in one embodiment, a lateral flow assay test strip or test strip may be formed of a nitrocellulose membrane which may be a GE Whatman backed nitrocellulose membrane FF120HP; 5cm x 0.4cm. A glass fiber conjugate pad may include a Millipore G041 “SureWick” GFCP103000, 1cm x 0.4cm. A cellulose absorbent pad may include a Millipore C083 “SureWick” cellulose fiber sample pad strips CFSP173000, 1cm x 0.75cm.

As shown in the figures and described generally above, a conjugated GNP probe may include a biotinylated oligo capture probe bound to streptavidin, which may then be embedded on a nitrocellulose membrane. In one example, 600μM oligo capture probes were incubated with

200μM streptavidin for 1 hour at room temperature. With the capture probes now in a complex with streptavidin they may be diluted to a different concentration to optimize binding conditions and signal intensity. In a preferred example, 0.5μL of solution containing this capture probe-streptavidin complex are pipetted onto nitrocellulose membrane in appropriate orientation, with target probe placed nearest the conjugate pad and control probe placed nearest the absorbent pad.

As noted above, a conjugated GNP probe or reporter may be coupled with one or more single-stranded DNA sequences via salt aging method -60nm or 15nm or 12.5nm diameter A running buffer may be mixed with RT-RPA amplified solution product and conjugated gold nanoparticle just prior to running on test strip.

The terminology used herein is for describing embodiments and is not intended to be limiting. As used herein, the singular forms "a," "and" and "the" include plural referents, unless the content and context clearly dictate otherwise. Thus, for example, a reference to "a biomarker" may include a combination of two or more such biomarkers. Unless defined otherwise, all scientific and technical terms are to be understood as having the same meaning as commonly used in the art to which they pertain. As used herein, "about" or "approximately" means within 10% of a stated concentration range or within 10% of a stated time frame.

The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with "and/or" should be construed in the same fashion, i.e., "one or more" of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to "A and/or B", when used in conjunction with open-ended language such as "comprising" can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

Nucleic acids and/or other moieties of the invention may be isolated or "extracted." As used herein, "isolated" means separate from at least some of the components with which it is usually associated whether it is derived from a naturally occurring source or made synthetically, in whole or in part. Nucleic acids and/or other moieties of the invention may be purified. As used

herein, purified means separate from the majority of other compounds or entities. A compound or moiety may be partially purified or substantially purified. Purity may be denoted by weight measure and may be determined using a variety of analytical techniques such as but not limited to mass spectrometry, HPLC, etc.

The term "primer," as used herein, refers to an oligonucleotide capable of acting as a point of initiation of DNA synthesis under suitable conditions. Such conditions include those in which synthesis of a primer extension product complementary to a nucleic acid strand is induced in the presence of four different nucleoside triphosphates and an agent for extension (for example, a DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature.

A primer is preferably a single-stranded DNA. The appropriate length of a primer depends on the intended use of the primer but typically ranges from about 6 to about 225 nucleotides, including intermediate ranges, such as from 15 to 35 nucleotides, from 18 to 75 nucleotides and from 25 to 150 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template nucleic acid but must be sufficiently complementary to hybridize with the template. The design of suitable primers for the amplification of a given target sequence is well known in the art and described in the literature cited herein.

As used herein, a biological marker ("biomarker" or "marker") is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacological responses to therapeutic interventions, consistent with NIH Biomarker Definitions Working Group (1998). Markers can also include patterns or ensembles of characteristics indicative of particular biological processes. The biomarker measurement can increase or decrease to indicate a particular biological event or process. In addition, if the biomarker measurement typically changes in the absence of a particular biological process, a constant measurement can indicate occurrence of that process. In a preferred embodiment a biomarker includes one or more RNA transcripts that may be indicative of infection or other normal or abnormal physiological process.

As referred to herein, the terms "nucleic acid", "nucleic acid molecules" "oligonucleotide", "polynucleotide", and "nucleotides" may interchangeably be used. The terms are directed to polymers of deoxyribonucleotides (DNA), ribonucleotides (RNA), and modified forms thereof in

the form of a separate fragment or as a component of a larger construct, linear or branched, single stranded, double stranded, triple stranded, or hybrids thereof. The term also encompasses RNA/DNA hybrids. The polynucleotides may include sense and antisense oligonucleotide or polynucleotide sequences of DNA or RNA. The DNA molecules may be, for example, but not limited to: complementary DNA (cDNA), genomic DNA, synthesized DNA, recombinant DNA, or a hybrid thereof. The RNA molecules may be, for example, but not limited to: ssRNA or dsRNA and the like. The terms further include oligonucleotides composed of naturally occurring bases, sugars, and covalent internucleoside linkages, as well as oligonucleotides having non-naturally occurring portions, which function similarly to respective naturally occurring portions. The terms "nucleic acid segment" and "nucleotide sequence segment," or more generally "segment," will be understood by those in the art as a functional term that includes both genomic sequences, ribosomal RNA sequences, transfer RNA sequences, messenger RNA sequences, operon sequences, and smaller engineered nucleotide sequences that are encoded or may be adapted to encode, peptides, polypeptides, or proteins. All nucleic acid primers, such as SEQ IN NOs. 445-468, are presented in the 5' to 3' prime direction unless otherwise noted.

As used herein, "complementary" refers to the ability of a single strand of a polynucleotide (or portion thereof) to hybridize to an anti-parallel polynucleotide strand (or portion thereof) by contiguous base-pairing between the nucleotides (that is not interrupted by any unpaired nucleotides) of the anti-parallel polynucleotide single strands, thereby forming a double-stranded polynucleotide between the complementary strands. A first polynucleotide is said to be "completely complementary" to a second polynucleotide strand if each and every nucleotide of the first polynucleotide forms base-paring with nucleotides within the complementary region of the second polynucleotide. A first polynucleotide is not completely complementary (i.e., partially complementary) to the second polynucleotide if one nucleotide in the first polynucleotide does not base pair with the corresponding nucleotide in the second polynucleotide. The degree of complementarity between polynucleotide strands has significant effects on the efficiency and strength of annealing or hybridization between polynucleotide strands. This is of particular importance in amplification reactions, which depend upon binding between polynucleotide strands. An oligonucleotide primer is "complementary" to a target polynucleotide if at least 50%

(preferably, 60%, more preferably 70%, 80%, still more preferably 90% or more) nucleotides of the primer form base-pairs with nucleotides on the target polynucleotide.

As referred to herein, the term "database" is directed to an organized collection of nucleotide sequence information that may be stored in a digital form. In some embodiments, the database may include any sequence information. In some embodiments, the database may include the genome sequence of a subject or a microorganism. In some embodiments, the database may include expressed sequence information, such as, for example, an EST (expressed sequence tag) or cDNA (complementary DNA) databases. In some embodiments, the database may include non-coding sequences (that is, untranslated sequences), such as, for example, the collection of RNA families (Rfam) which contains information about non-coding RNA genes, structured cis-regulatory elements and self-splicing RNAs. In exemplary embodiments, the databases may be selected from redundant or non-redundant GenBank databases (which are the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences). Exemplary databases may be selected from, but not limited to: GenBank CDS (Coding sequences database), PDB (protein database), SwissProt database, PIR (Protein Information Resource) database, PRF (protein sequence) database, EMBL Nucleotide Sequence database, and the like, or any combination thereof.

As used herein, the term "detection" refers to the qualitative determination of the presence or absence of a microorganism in a sample. The term "detection" also includes the "identification" of a microorganism, i.e., determining the genus, species, or strain of a microorganism according to recognized taxonomy in the art and as described in the present specification. The term "detection" further includes the quantitation of a microorganism in a sample, e.g., the copy number of the microorganism in a microliter (or a milliliter or a liter) or a microgram (or a milligram or a gram or a kilogram) of a sample. The term "detection" also includes the identification of an infection in a subject or sample.

As used herein the term "pathogen" refers to an organism, including a microorganism, which causes disease in another organism (e.g., animals and plants) by directly infecting the other organism, or by producing agents that causes disease in another organism (e.g., bacteria that produce pathogenic toxins and the like). As used herein, pathogens include, but are not limited to bacteria, protozoa, fungi, nematodes, viroids and viruses, or any combination thereof, wherein each pathogen is capable, either by itself or in concert with another pathogen, of eliciting disease

in vertebrates including but not limited to mammals, and including but not limited to humans. As used herein, the term "pathogen" also encompasses microorganisms which may not ordinarily be pathogenic in a non-immunocompromised host.

The term "infection," or "infect" as used herein is directed to the presence of a microorganism within a subject body and/or a subject cell. For example, a virus may be infecting a subject cell. A parasite (such as, for example, a nematode) may be infecting a subject cell/body. In some embodiments, the microorganism may comprise a virus, a bacteria, a fungi, a parasite, or combinations thereof. According to some embodiments the microorganism is a virus, such as, for example, dsDNA viruses (such as, for example, Adenoviruses, Herpesviruses, Poxviruses), ssDNA viruses (such as, for example, Parvoviruses), dsRNA viruses (such as, for example, Reoviruses), (+) ssRNA viruses (+) sense RNA (such as, for example, Picornaviruses, Togaviruses), (−) ssRNA viruses (−) sense RNA (such as, for example, Orthomyxoviruses, Rhabdoviruses), ssRNA-RT viruses (+) sense RNA with DNA intermediate in life-cycle (such as, for example, Retroviruses), dsDNA-RT viruses (such as, for example, Hepadnaviruses). In some embodiments, the microorganism is a bacteria, such as, for example, a gram negative bacteria, a gram positive bacteria, and the like. In some embodiments, the microorganism is a fungi, such as yeast, mold, and the like. In some embodiments, the microorganism is a parasite, such as, for example, protozoa and helminths or the like. In some embodiments, the infection by the microorganism may inflict a disease and/or a clinically detectable symptom to the subject. In some embodiments, infection by the microorganism may not cause a clinically detectable symptom. In some embodiments, the microorganism is a symbiotic microorganism. In additional embodiments, the microorganism may comprise archaea, protists; microscopic plants (green algae), plankton, and the planarian. In some embodiments, the microorganism is unicellular (single-celled). In some embodiments, the microorganism is multicellular.

As used herein, the term "asymptomatic" refers to an individual who does not exhibit physical symptoms characteristic of being infected with a given pathogen, or a given combinations of pathogens.

The target biomarkers of this invention may be used for diagnostic and prognostic purposes, as well as for therapeutic, drug screening and patient stratification purposes (e.g., to group patients into a number of "subsets" for evaluation), as well as other purposes described herein.

Some embodiments of the invention comprise detecting in a sample from a patient, a level of a biomarker, wherein the presence or expression levels of the biomarker are indicative of infection or possible infection by one or more pathogens. As used herein, the term "biological sample" or "sample" includes a sample from any bodily fluid or tissue. Biological samples or samples appropriate for use according to the methods provided herein include, without limitation, blood, serum, urine, saliva, tissues, cells, and organs, or portions thereof. A "subject" is any organism of interest, generally a mammalian subject, and preferably a human subject.

Any isothermal amplification protocol can be used according to the methods provided herein. Exemplary types of isothermal amplification include, without limitation, nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP), strand displacement amplification (SDA), helicase-dependent amplification (HDA), nicking enzyme amplification reaction (NEAR), signal mediated amplification of RNA technology (SMART), rolling circle amplification (RCA), isothermal multiple displacement amplification (EVIDA), single primer isothermal amplification (SPIA), recombinase polymerase amplification (RPA), and polymerase spiral reaction (PSR, available at nature.com/articles/srepl2723 on the World Wide Web). In some cases, a forward primer is used to introduce a T7 promoter site into the resulting DNA template to enable transcription of amplified RNA products via T7 RNA polymerase. In other cases, a reverse primer is used to add a trigger sequence of a toehold sequence domain.

As used herein, the term "amplified" refers to polynucleotides that are copies of a particular polynucleotide, produced in an amplification reaction. An amplified product, according to the invention, may be DNA or RNA, and it may be double-stranded or single-stranded. An amplified product is also referred to herein as an "amplicon". As used herein, the term "amplicon" refers to an amplification product from a nucleic acid amplification reaction. The term generally refers to an anticipated, specific amplification product of known size, generated using a given set of amplification primers.

Table 1. Comparison of gold standard tests to invention's lateral flow assay stick

Diagnostic Test Type	**Sensitivity**	**Specificity**	**Avg. time to detection post-exposure**	**Able to detect unknown pathogens?**	**Laboratory?**	**Trained personnel?**	**Cost**
Serology-based	High	Moderate	Late	No	Yes – most cases	Yes – most cases	$$
Cultures	Moderate	Moderate	Late	Only if clinically suspected & able to be cultured	Yes	Yes	$$
PCR	High	High	Mid	No	Yes	Yes	$$$
Our Product	High*	Moderate*	Earliest	Yes	No	No	$

Table 2: Primers used for the detection of SARS-CoV-2 (COVID-19)

Name	Description	Oligonucleotide sequence (5'->3')	Label	Conc.	SEQ ID NO.
2019-nCoV_N1-F	2019-nCoV_N1 Forward Primer	5'-GAC CCC AAA ATC AGC GAA AT-3'	None	20 µM	469
2019-nCoV_N1-R	2019-nCoV_N1 Reverse Primer	5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'	None	20 µM	470
2019-nCoV_N1-P	2019-nCoV_N1 Probe	5'-FAM-ACC CCG CAT TAC GTT TGG ACC-BHQ1-3'	FAM, BHQ-1	5 µM	471
2019-nCoV_N2-F	2019-nCoV_N2 Forward Primer	5'-TTA CAA ACA TTG GCC GCA AA-3'	None	20 µM	472
2019-nCoV_N2-R	2019-nCoV_N2 Reverse Primer	5'-GCG CGA CAT TCC GAA GAA-3'	None	20 µM	473
2019-nCoV_N2-P	2019-nCoV_N2 Probe	5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3'	FAM, BHQ-1	5 µM	474
2019-nCoV_N3-F	2019-nCoV_N3 Forward Primer	5'-GGG AGC CTT GAA TAC ACC AAA A-3'	None	20 µM	475
2019-nCoV_N3-R	2019-nCoV_N3 Reverse Primer	5'-TGT AGC ACG ATT GCA TTG-3'	None	20 µM	476
2019-nCoV_N3-P	2019-nCoV_N3 Probe	5'-FAM-AYC ACA TTG GCA CCC GCA ATC CTG-BHQ1-3'	FAM, BHQ-1	5 µM	477
RP-F	RNAse P Forward Primer	5'-AGA TTT GGA CCT GCG AGC G-3'	None	20 µM	478
RP-R	RNAse P Reverse Primer	5'-GAG CGG CTG TCT CCA CAA GT-3'	None	20 µM	479
RP-P	RNAse P Probe	5'-FAM – TTC TGA CCT GAA GGC TCT GCG CG – BHQ-1-3'	FAM, BHQ-1	5 µM	480

CLAIMS

What is claimed is:

1. A method of detecting a host RNA transcript biomarker comprising the step of:
   - collecting a bodily fluid sample from a subject containing an RNA transcript biomarker;
   - converting said RNA transcript biomarker into a DNA probe, such as a double stranded DNA (dsDNA), single stranded DNA (ssDNA), or and a hybrid double stranded DNA (dsDNA) probe having:
     - a dsDNA target sequence;
     - a single stranded DNA (ssDNA) annealing region; and
     - a ssDNA target capture region;
   - introducing said hybrid dsDNA probe to a DNA conjugated reporter probe, wherein said ssDNA annealing region on hybrid dsDNA probe is complementary to a ssDNA annealing region of said DNA conjugated reporter probe such that the two probes are coupled together in a solution;
   - introducing the hybrid dsDNA probe and DNA conjugated reporter probe solution to a lateral flow assay test strip;
   - passing the solution through at least one detection zone on said lateral flow assay test strip, wherein said detection zone contains a plurality of embedded target capture probes having a ssDNA region that is complementary to said ssDNA target capture region on said hybrid dsDNA probe;
   - forming an immobilized complex aggregate comprising said hybrid dsDNA probe, said DNA conjugated reporter probe, and said target capture probe by annealing the complementary target capture region on said hybrid dsDNA probe with the target capture region on said target capture probe;
   - allowing a plurality of immobilized complex aggregates to form in said detection zone such that a detectable signal is produced.

2. The method of claim 1 wherein said bodily fluid sample comprises a saliva sample.

3. The method of claim 1 wherein said step of converting comprises the step of converting said RNA transcript biomarker into DNA probe through an isothermal reverse transcription recombinase polymerase amplification (RT-RPA) reaction.

4. The method of claim 3 wherein the reagents necessary to produce an isothermal reverse transcription recombinase polymerase amplification (RT-RPA) reaction are pre-loaded into a reaction cylinder.

5. The method of claim 1 wherein said dsDNA target sequence is coupled with said ssDNA annealing region and said ssDNA target capture region through a linker.

6. The method of claim 5 wherein said linker comprises a tri-carbon chain spacer (C_3) linker.

7. The method of claim 1 wherein said DNA conjugated reporter probe comprises a conjugated gold nanoparticle (GNP) probe.

8. The method of claim 7 wherein said conjugated (GNP) probe comprises a GNP coupled to said ssDNA annealing region through a thiol, PEG_{18}, and PolyA construct.

9. The method of claim 1 wherein said target capture probe comprises a target capture probe having an immobilized streptavidin base tetramer coupled with a biotin-TEG linker that may further be coupled with said ssDNA target capture probe sequence that is complementary to said target capture region on said hybrid streptavidin.

10. The method of any of claims 1 and 8 wherein said lateral flow assay test strip further comprises:

- a conjugate pad in fluid communication with a membrane that allows said solution to flow towards an absorbent pad via capillary action, wherein said absorbent pad is positioned distal to said detection zone.
- a control zone that may immobilize unbound conjugated gold nanoparticle (GNP) probe

11. The method of claim 10 wherein said membrane comprises a nitrocellulose membrane.

12. The method of claim 1 wherein said RNA transcript biomarker comprises at least one RNA transcript biomarker encoded by at least one nucleotide sequence selected from the group consisting of: SEQ ID NO. 1-444, and 657-815.

13. A lateral flow assay for the early detection of RNA transcript biomarkers comprising:

- a bodily fluid sample having a host RNA transcript biomarker from a subject;
- a reaction cylinder configured to receive the saliva sample and further configured to generate an amplified sample through an isothermal reverse transcription recombinase polymerase amplification (RT-RPA) reaction wherein said amplified sample comprises a hybrid dsDNA probe coupled with a DNA conjugated reporter probe;
- a conjugate pad configured to receive the amplified sample;

- a membrane in fluid communication with said conjugate pad and further configured to allow said solution to flow through said membrane via capillary action;
- a detection zone containing a plurality of embedded target capture probes configured to bind and immobilize said hybrid dsDNA probe;
- a control zone configured to bind and immobilize one or more unbound DNA conjugated reporter probes; and
- an absorbent pad positioned distal to said detection zone and said control zone.

14. The lateral flow assay of claim 13 wherein said bodily fluid sample comprises a saliva sample.

15. The lateral flow assay of claim 13 wherein the reagents necessary to produce said isothermal RT-RPA reaction are pre-loaded into said reaction cylinder.

16. The lateral flow assay of claim 13 wherein said membrane comprises a nitrocellulose membrane.

17. The lateral flow assay of claim 13 wherein said hybrid dsDNA probe comprises:

- a dsDNA target sequence;
- a ssDNA annealing region; and
- a ssDNA target capture region.

18. The lateral flow assay of claim 17 wherein said ssDNA annealing region on hybrid dsDNA probe is complementary to a ssDNA annealing region of said DNA conjugated reporter probe, such that the two probes are coupled together in said amplified solution.

19. The lateral flow assay of claim 18 wherein said dsDNA target sequence is coupled with said ssDNA annealing region and said ssDNA target capture region through a linker.

20. The lateral flow assay of claim 19 wherein said linker comprises a tri-carbon chain spacer (C_3) linker.

21. The lateral flow assay of claim 13 wherein said DNA conjugated reporter probe comprises a conjugated gold nanoparticle (GNP) probe.

22. The lateral flow assay of claim 21 wherein said conjugated GNP probe comprises a GNP coupled to said ssDNA annealing region through a thiol, PEG_{18}, and PolyA construct.

23. The lateral flow assay of any of claims 13 and 17 wherein said target capture probes comprise a target capture probe having an immobilized streptavidin base tetramer coupled with a biotin-TEG linker that may further be coupled with said ssDNA target capture probe sequence that is complementary to said target capture region on said hybrid dsDNA probe.

24. The lateral flow assay of claim 13 wherein said host RNA transcript biomarker comprises at least one RNA transcript biomarker encoded by at least one nucleotide sequence selected from the group consisting of: SEQ ID NO. 1-444, and 657-815.

25. A antibody-based lateral flow assay for the early detection of RNA transcript biomarkers comprising:

- a bodily fluid sample having a host RNA transcript biomarker from a subject;
- a reaction cylinder configured to receive the saliva sample and further configured to generate an amplified sample through an isothermal reverse transcription recombinase polymerase amplification (RT-RPA) reaction wherein said amplified sample comprises a hybrid dsDNA probe coupled with an antibody conjugated reporter probe;
- a conjugate pad configured to receive the amplified sample;
- a membrane in fluid communication with said conjugate pad and further configured to allow said amplified sample to flow through said membrane via capillary action;
- a detection zone containing a plurality of embedded antibody target capture probes configured to bind and immobilize said hybrid dsDNA probe;
- a control zone containing a plurality of embedded antibody target capture probes configured to bind and immobilize said hybrid dsDNA probe;
- a capture zone having an antibody configured to bind and immobilize one or more antibody DNA conjugated reporter probes.

26. The antibody-based lateral flow assay of claim 25 wherein said bodily fluid sample comprises a saliva sample.

27. The antibody-based lateral flow assay of claim 25 wherein the reagents necessary to produce said isothermal RT-RPA reaction are pre-loaded into said reaction cylinder.

28. The antibody-based lateral flow assay of claim 25 wherein said membrane comprises a nitrocellulose membrane.

29. The antibody-based lateral flow assay of claim 25 wherein said hybrid dsDNA probe comprises:

- a dsDNA target sequence;
- a 5’ forward ssDNA oligo; and
- a 5’ reverse ssDNA oligo.

30. The antibody-based lateral flow assay of claim 29 wherein said 5' forward ssDNA oligo comprises a 5' FITC forward oligo.

31. The antibody-based lateral flow assay of claim 25 wherein said 5' reverse ssDNA oligo comprises a 5' DIG reverse oligo, or a 5' Biotin reverse oligo.

32. The antibody-based lateral flow assay of claim 30 wherein said conjugated reporter probe comprises a gold nanoparticle (GNP) coupled with an antibody forming an antibody conjugated reporter probe.

33. The antibody-based lateral flow assay of claim 32 wherein said antibody comprises an anti-FITC antibody.

34. The antibody-based lateral flow assay of claims 30 and 33 wherein said FITC antibody binds to said 5' FITC forward oligo of said hybrid dsDNA probe.

35. The antibody-based lateral flow assay of claim 25 wherein said target capture probe of said detection zone comprises an anti-DIG antibody.

36. The antibody-based lateral flow assay of claims 31 and 35 wherein said anti-DIG antibody binds to the 5' DIG reverse oligo of said hybrid dsDNA probe.

37. The antibody-based lateral flow assay of claim 25 and 31 wherein said target capture probe of said control zone comprises a target capture probe having an immobilized streptavidin base tetramer coupled with a biotin-TEG linker that may further be coupled with said 5' Biotin reverse oligo.

38. The antibody-based lateral flow assay of claim 30 wherein said target capture probe of said detection zone comprises an anti-rabbit antibody.

39. The antibody-based lateral flow assay of claim 25 wherein said host RNA transcript biomarker comprises at least one RNA transcript biomarker encoded by at least one nucleotide sequence selected from the group consisting of: SEQ ID NO. 1-444, and 657-815.

40. A method of early-pathogen detection comprising the step of:

- collecting a bodily fluid sample from a first subject;
- extracting host-derived biomarkers of infection and a pathogen biomarkers from said bodily fluid sample;
- quantifying said host-derived biomarkers of infection and a pathogen biomarkers through PCR, real time PCR (RT-PCR), or quantitative real-time polymerase chain reaction (qRT-PCR);

- establishing a time-course of the levels of host-derived biomarkers of infection and optionally correlating said host-derived biomarkers of infection with said levels of pathogen biomarkers in said bodily fluid sample;
- optionally repeating the four above steps at different time-points;
- collecting a bodily fluid sample from a second subject containing a host-derived biomarker of infection;
- detecting one or more host-derived biomarkers of infection that correlate to infection with said pathogen.

41. The method of claim 40 wherein said bodily fluid sample comprises a saliva sample.

42. The method of claim 41 wherein said host-derived biomarkers of infection comprise host-derived RNA biomarkers of infection.

43. The method of claim 42 wherein said pathogen biomarkers comprises pathogen biomarkers selected from the group consisting of: viral pathogen biomarkers, bacterial pathogen biomarkers, and pathogen fungal biomarkers.

44. The method of claim 43 wherein said viral pathogen biomarkers comprise viral pathogen biomarkers from novel coronavirus SARS-CoV-2.

45. The method of claim 40 wherein said viral pathogen biomarkers from novel coronavirus SARS-CoV-2 comprises one or more biomarkers that may be amplified in a PCR reaction by the nucleotide primers according to SEQ ID NOs. 469-480.

46. The method of claim 40 wherein said host-derived biomarker of infection comprises host-derived RNA biomarkers of infection and further comprising the step of converting said host-derived RNA biomarkers of infection into a hybrid double stranded DNA (dsDNA) probe through an isothermal reverse transcription recombinase polymerase amplification (RT-RPA) reaction.

47. The method of claims 1 wherein said step of detecting comprises the method of claims 1-12.

48. A method of detecting an infection in a subject in need thereof, comprising the step of detecting at least one host-derived RNA biomarker of infection from a biological sample provided by said subject, wherein said at least one host-derived RNA biomarker of infection is selected from the group consisting of: a host-derived RNA biomarker of infection encoded by the nucleotide sequence according to SEQ ID NOs. 1-444, and 657-815.

49. The method of claim 48 wherein said step of detecting comprises the method of claims 1-12.

50. The method of claim 48 wherein said step of detecting comprises the step of detecting said host-derived RNA biomarker of infection comprises detecting a host-derived RNA biomarker of infection using PCR, RT-PCR, or qRT-PCR.

51. A lateral flow assay configured to detect at least one host-derived RNA biomarker from a biological sample provided by a subject, wherein said at least one host-derived RNA biomarker is selected from the group consisting of: a host-derived RNA biomarker encoded by the nucleotide sequence according to SEQ ID NOs. 1-444, and 657-815.

52. An assay configured to detect at least one host-derived RNA biomarker from a biological sample provided by a subject, wherein said at least one host-derived RNA biomarker is selected from the group consisting of: a host-derived RNA biomarker encoded by the nucleotide sequence according to SEQ ID NOs. 1-444, and 657-815, wherein said assay is a PCR assay, RT-PCR assay, or qRT-PCR assay.

53. A microarray assay configured to detect least one host-derived RNA biomarker from a biological sample provided by a subject, wherein said at least one host-derived RNA biomarker is selected from the group consisting of: a host-derived RNA biomarker encoded by the nucleotide sequence according to SEQ ID NOs. 1-444, and 657-815.

54. A lateral flow assay configured to detect at least one host-derived RNA biomarker indicative for a viral infection from a biological sample provided by a subject, wherein said at least one host-derived RNA biomarker indicative for a viral infection is selected from the group consisting of: IFIT2, ICAM1, ERG1, IFIH1, ISG15, CFB, CXCL10, DDX58, and IRAK2.

55. An assay configured to detect at least one host-derived RNA biomarker indicative for a viral infection from a biological sample provided by a subject, wherein said at least one host-derived RNA biomarker indicative for a viral infection is selected from the group consisting of IFIT2, ICAM1, ERG1, IFIH1, ISG15, CFB, CXCL10, DDX58, and IRAK2, wherein said assay is a PCR assay, RT-PCR assay, or qRT-PCR assay.

56. A microarray assay configured to detect least one host-derived RNA biomarker indicative for a viral infection from a biological sample provided by a subject, wherein said at least one host-derived RNA biomarker indicative for a viral infection is selected from the group consisting of: IFIT2, ICAM1, ERG1, IFIH1, ISG15, CFB, CXCL10, DDX58, and IRAK2.

57. A method of detecting a viral infection in a subject in need thereof, comprising detecting least one host-derived RNA biomarker indicative in a biological sample provided by a subject, wherein

said at least one host-derived RNA biomarker indicative for a viral infection is selected from the group consisting of IFIT2, ICAM1, ERG1, IFIH1, ISG15, CFB, CXCL10, DDX58, and IRAK2, and said biological sample is saliva.

58. A lateral flow assay configured to detect at least one host-derived RNA biomarker indicative for a SARS-CoV-2 infection from a biological sample provided by a subject, wherein said at least one host-derived RNA biomarker indicative for a viral infection is selected from the group consisting of: MX1, PARP12, IFITM2, CD68, and SERINB3.

59. An assay configured to detect at least one host-derived RNA biomarker indicative for a SARS-CoV-2 infection from a biological sample provided by a subject, wherein said at least one host-derived RNA biomarker indicative for a viral infection is selected from the group consisting of MX1, PARP12, IFITM2, CD68, and SERINB3, wherein said assay is a PCR assay, RT-PCR assay, or qRT-PCR assay.

60. A microarray assay configured to detect least one host-derived RNA biomarker indicative for a SARS-CoV-2 infection from a biological sample provided by a subject, wherein said at least one host-derived RNA biomarker indicative for a viral infection is selected from the group consisting of: MX1, PARP12, IFITM2, CD68, and SERINB3.

61. A method of detecting a SARS-CoV-2 infection in a subject in need thereof, comprising detecting least one host-derived RNA biomarker in a biological sample provided by a subject, wherein said at least one host-derived RNA biomarker is indicative for a SARS-CoV-2 infection is selected from the group consisting of MX1, PARP12, IFITM2, CD68, and SERINB3, and said biological sample is saliva.

62. A lateral flow assay configured to detect at least one host-derived RNA biomarker indicative for an influenza infection from a biological sample provided by a subject, wherein said at least one host-derived RNA biomarker indicative for a viral infection is selected from the group consisting of: PLRG1, MSC, NKG7, NME8, and MMP12.

63. An assay configured to detect at least one host-derived RNA biomarker indicative for an influenza infection from a biological sample provided by a subject, wherein said at least one host-derived RNA biomarker indicative for a viral infection is selected from the group consisting of PLRG1, MSC, NKG7, NME8, and MMP12, wherein said assay is a PCR assay, RT-PCR assay, or qRT-PCR assay.

64. A microarray assay configured to detect least one host-derived RNA biomarker indicative for an influenza infection from a biological sample provided by a subject, wherein said at least one host-derived RNA biomarker indicative for a viral infection is selected from the group consisting of: PLRG1, MSC, NKG7, NME8, and MMP12.

65. A method of detecting an influenza infection in a subject in need thereof, comprising detecting least one host-derived RNA biomarker in a biological sample provided by a subject, wherein said at least one host-derived RNA biomarker is indicative for an influenza infection is selected from the group consisting of PLRG1, MSC, NKG7, NME8, and MMP12, and said biological sample is saliva.

66. The method of any of claims 51-65, wherein said RNA biomarker is selected from the group consisting of: a host-derived RNA biomarker encoded by the nucleotide sequence according to SEQ ID NOs. 1-444, and 657-815.

67. A nucleotide sequence encoding a host-derived RNA biomarker used to detect an infection in a subjected in need thereof, wherein said RNA biomarker is selected from the group consisting of: a nucleotide sequence according to SEQ ID NOs. 1-444, and 657-815.

68. A method of detecting a host-derived RNA biomarker comprising:

- collecting a bodily fluid sample potentially containing a host-derived RNA biomarker and optionally a biomarker of a viral, bacterial, or fungal infection;
- identifying a transcript of said host-derived RNA biomarker in the sample, and optionally a biomarker of a viral, bacterial, or fungal infection using a method selected from the group consisting of: PCR, RT-PCR, qPCR, transcript sequencing, a lateral flow assay, hybridization assay, microarray, nucleic acid detection assay.

69. The method of claim 68, wherein said bodily fluid sample comprises a saliva sample.

70. The method of claim 69, wherein said host-derived biomarkers of infection comprise host-derived RNA biomarkers of infection.

71. The method of claim 70, wherein said host-derived RNA biomarkers of infection comprises pathogen biomarkers selected from the group consisting of: viral pathogen biomarkers, bacterial pathogen biomarkers, and pathogen fungal biomarkers.

72. The method of claim 71, wherein said viral pathogen biomarkers comprise viral pathogen biomarkers from novel coronavirus SARS-CoV-2.

73. The method of claim 72, wherein said viral pathogen biomarkers from novel coronavirus SARS-CoV-2 comprises one or more biomarkers that may be amplified in a PCR reaction by the nucleotide primers according to SEQ ID NOs. 469-480.

74. The method of claim 70, wherein said host-derived biomarker of infection comprises host-derived RNA biomarkers of infection and further comprising the step of converting said host-derived RNA biomarkers of infection into a hybrid double stranded DNA (dsDNA) probe through an isothermal reverse transcription recombinase polymerase amplification (RT-RPA) reaction.

75. The method of claim 70, wherein said host-derived biomarker of infection comprises a host-derived RNA biomarker of infection is selected from the group consisting of: a host-derived RNA biomarker of infection encoded by the nucleotide sequence according to SEQ ID NOs. 1-444, and 657-815.

76. The method of claim 70, wherein said biomarker of a viral, bacterial, or fungal infection comprises an RNA biomarker of a viral, bacterial, or fungal infection.

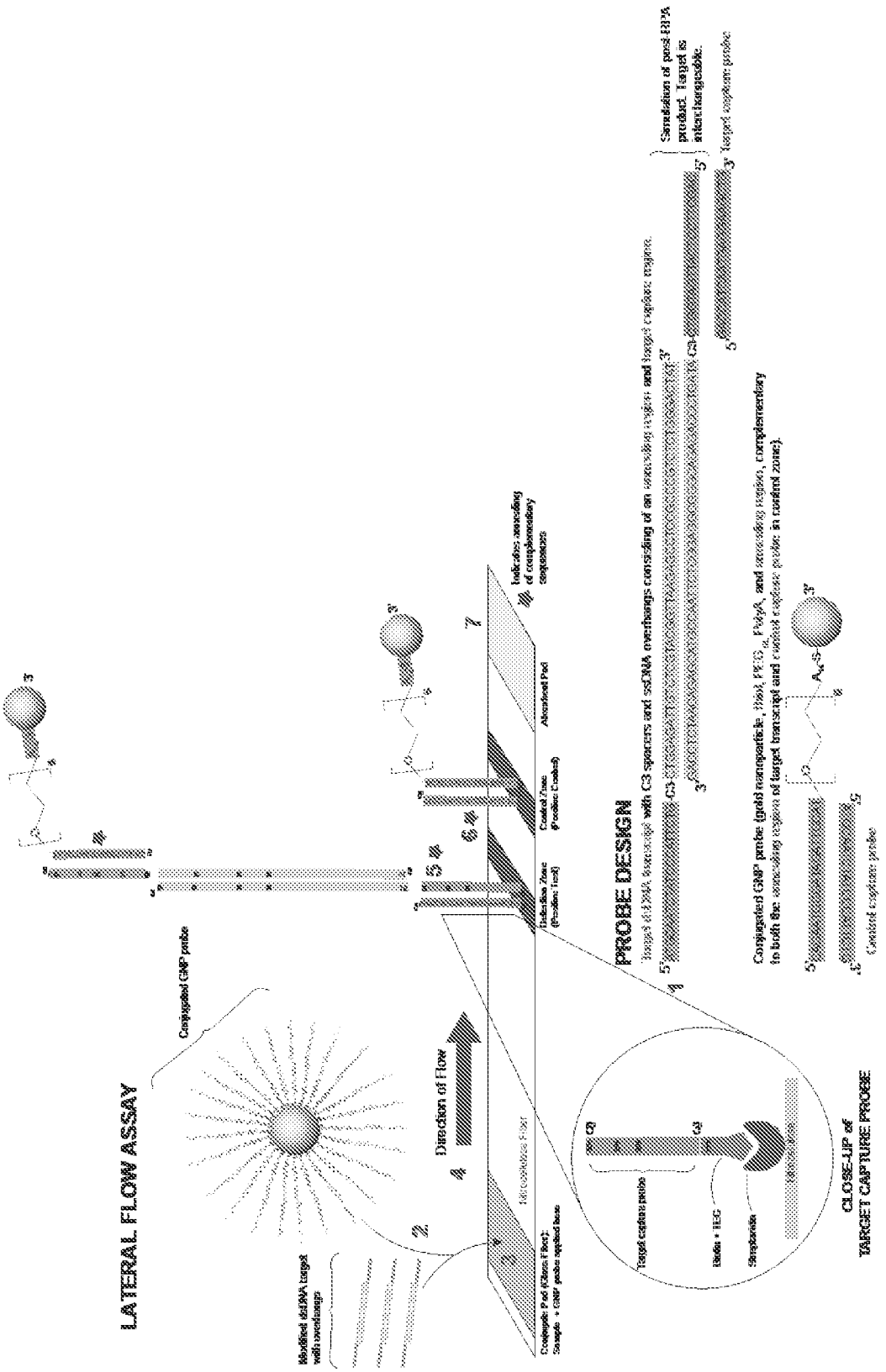

LATERAL FLOW ASSAY
Conjugated GNP probe
Direction of Flow
Modified ssRNA target with overhangs
Conjugate Pad (Glass Fiber): Sample + GNP probe applied here
Nitrocellulose Fiber
Detection Zone (Positive Test)
Control Zone (Positive Control)
Absorbent Pad
Indicates absence of complementary sequences
PROBE DESIGN
Target ssRNA transcript with C3 spacers and ssDNA overhangs consisting of an unencoding region and target capture region.
Cannulation of post-PBPA product. Target is intact/aggregable.
Target capture probe
Conjugated GNP probe (gold nanoparticle, Thiol, PEG$_{12}$, PolyA_{30} and unencoding region, complementary to both the unencoding region of target transcript and control capture probe in control zone).
Control capture probe
CLOSE-UP of TARGET CAPTURE PROBE
Biotin + TEG
Streptavidin

FIGURE 1A

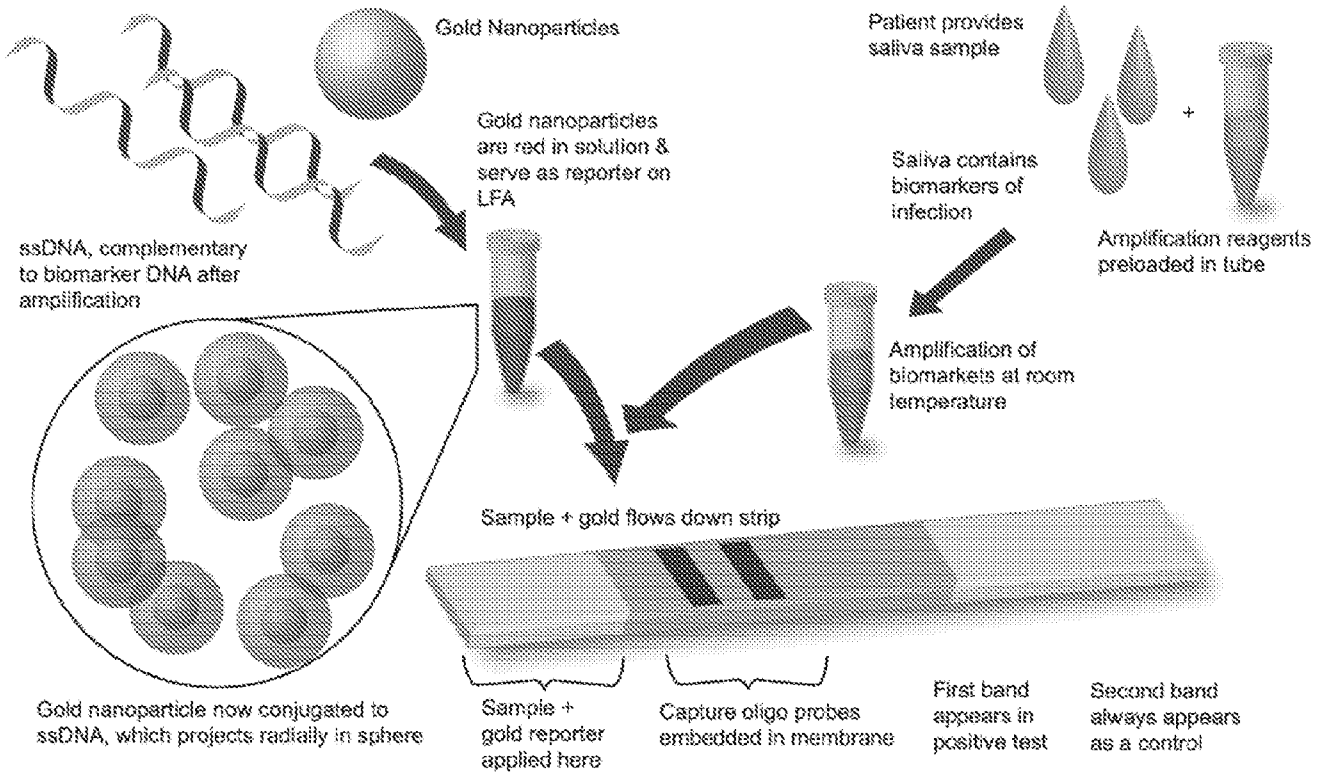

Gold Nanoparticles
Gold nanoparticles are red in solution & serve as reporter on LFA
ssDNA, complementary to biomarker DNA after amplification
Patient provides saliva sample
+
Saliva contains biomarkers of infection
Amplification reagents preloaded in tube
Amplification of biomarkets at room temperature
Sample + gold flows down strip
Gold nanoparticle now conjugated to ssDNA, which projects radially in sphere
Sample + gold reporter applied here
Capture oligo probes embedded in membrane
First band appears in positive test
Second band always appears as a control

FIGURE 1B

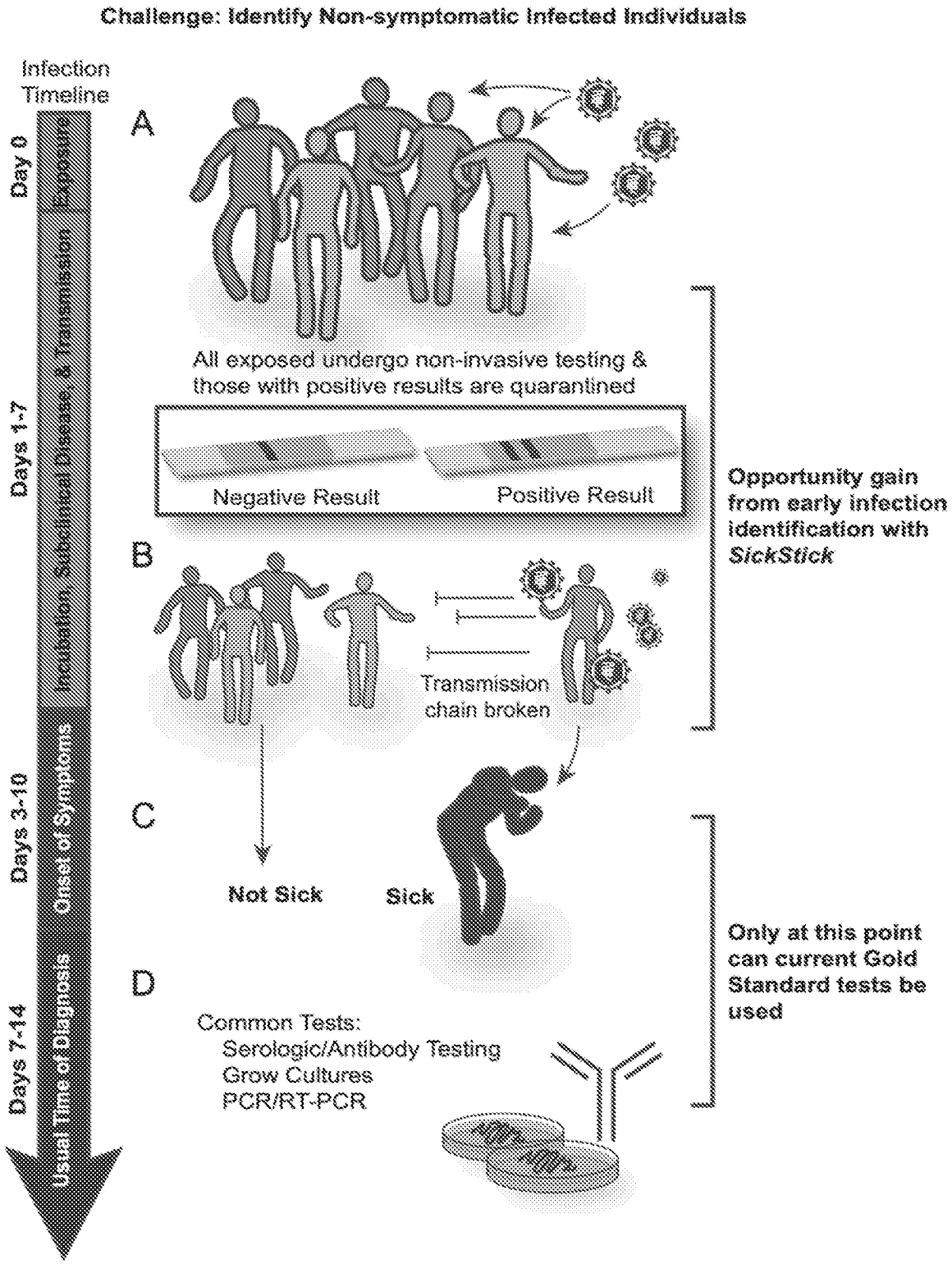

Challenge: Identify Non-symptomatic Infected Individuals
Infection Timeline
Day 0
Days 1-7
Days 3-10
Days 7-14
Exposure
Incubation Subclinical Disease & Transmission
Symptomatic Disease
Death or Full Recovery
A
All exposed undergo non-invasive testing & those with positive results are quarantined
Negative Result
Positive Result
B
Transmission chain broken
C
Not Sick
Sick
D
Common Tests:
Serologic/Antibody Testing
Grow Cultures
PCR/RT-PCR
Opportunity gain from early infection identification with SickStick
Only at this point can current Gold Standard tests be used

FIGURE 2

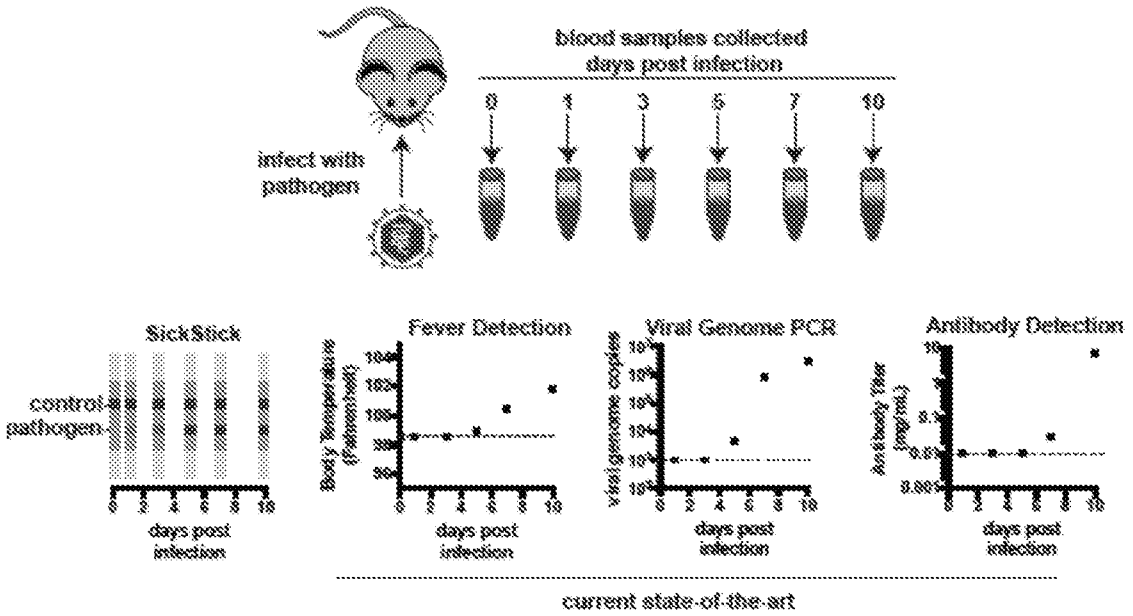


FIGURE 3A

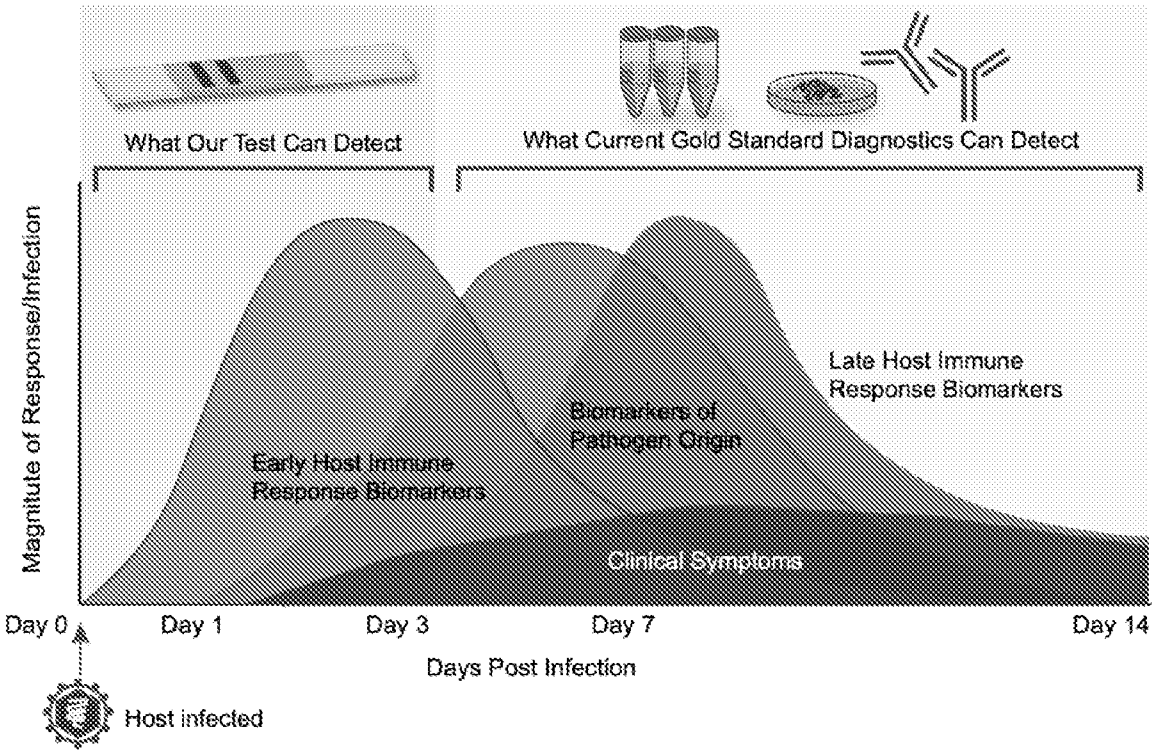


FIGURE 3B

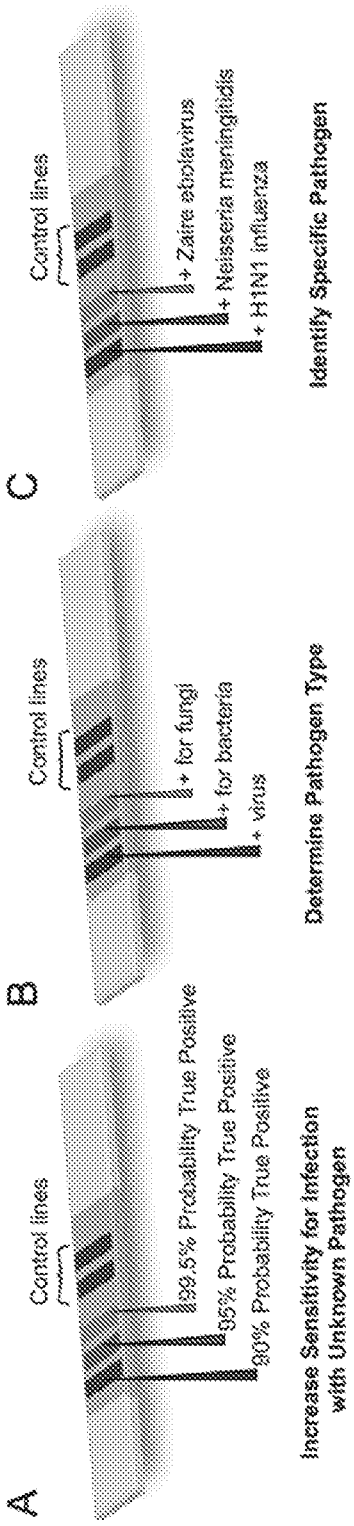

A
Control lines
99.5% Probability True Positive
95% Probability True Positive
80% Probability True Positive
Increase Sensitivity for Infection with Unknown Pathogen
B
Control lines
+ for fungi
+ for bacteria
+ virus
Determine Pathogen Type
C
Control lines
+ Zaire ebolavirus
+ Neisseria meningitidis
+ H1N1 influenza
Identify Specific Pathogen

FIGURE 14

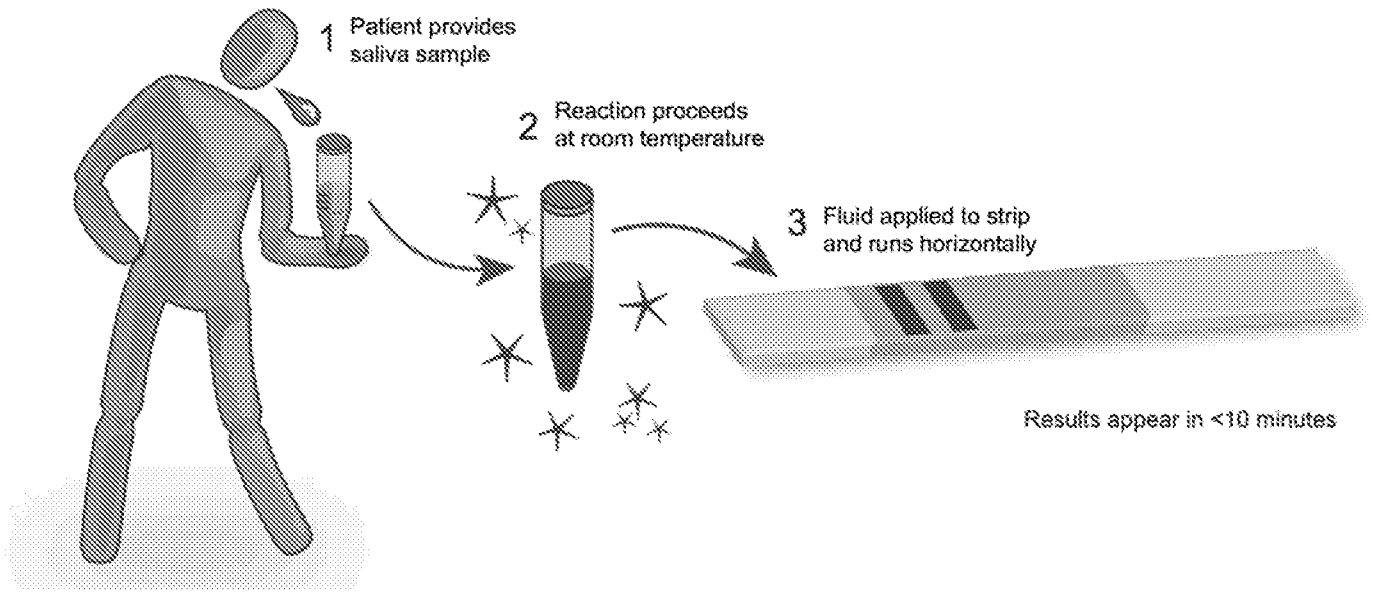

1 Patient provides saliva sample
2 Reaction proceeds at room temperature
3 Fluid applied to strip and runs horizontally
Results appear in <10 minutes

FIGURE 5

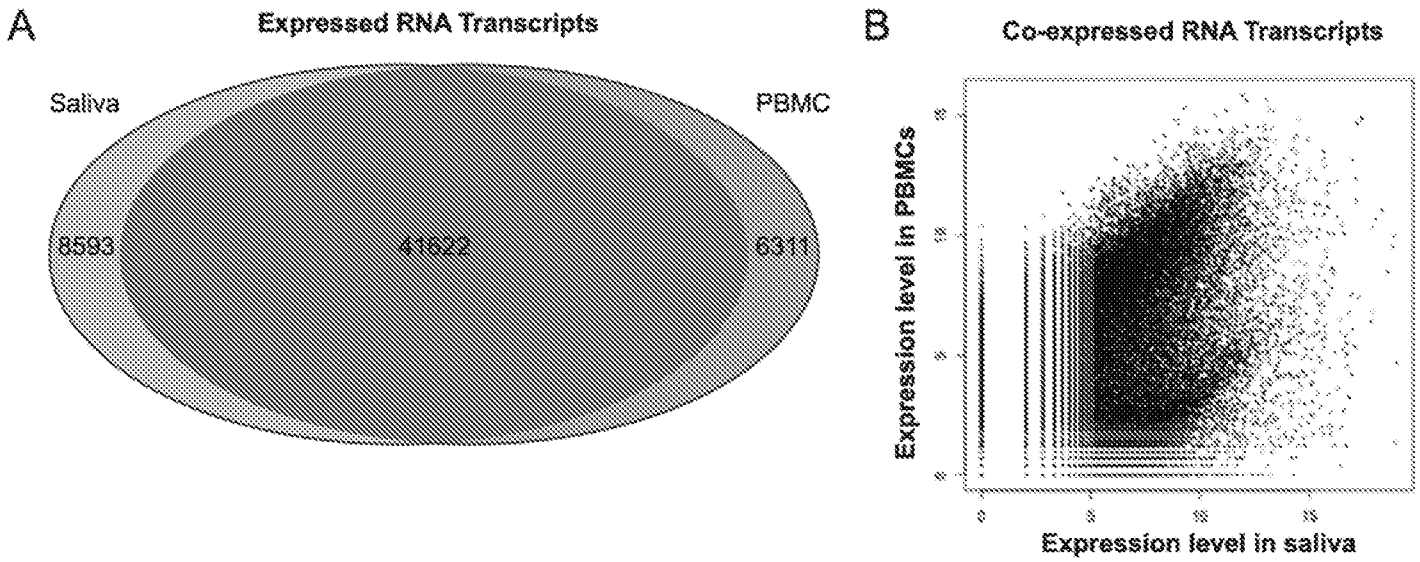

A
Expressed RNA Transcripts
Saliva
PBMC
8593
41622
6311
B
Co-expressed RNA Transcripts
Expression level in PBMCs
Expression level in saliva

FIGURE 6


RNA made in infected cells
Secreted from cells
RNA found in healthy saliva
RNA found in sick saliva

FIGURE 7

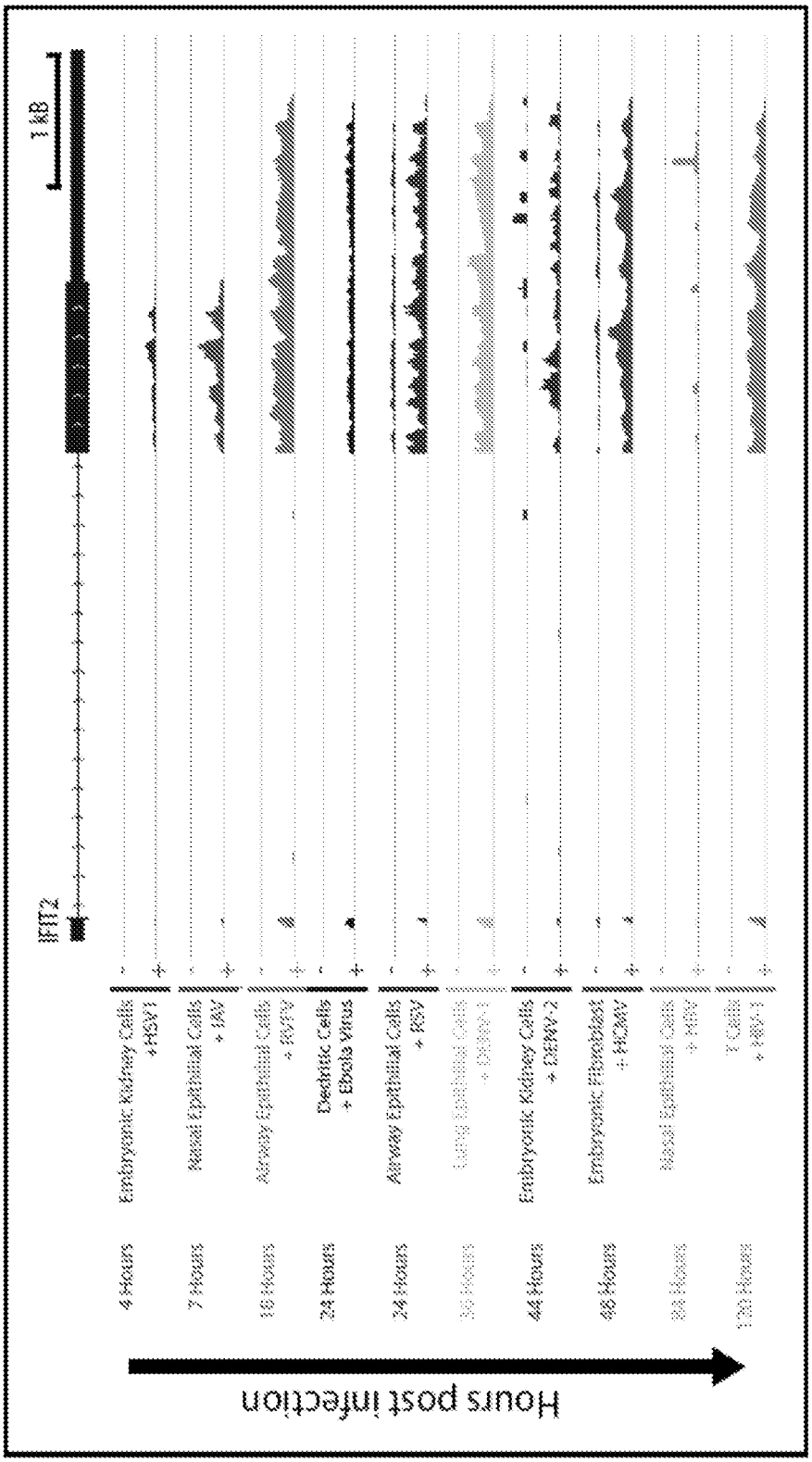

1 kB
IFIT2
Hours post infection
4 Hours
Embryonic Kidney Cells + HSV1
7 Hours
Renal Epithelial Cells + IAV
16 Hours
Airway Epithelial Cells + RSV
24 Hours
Dendritic Cells + Ebola Virus
24 Hours
Airway Epithelial Cells + RSV
30 Hours
Lung Epithelial Cells + CMV-1
44 Hours
Embryonic Kidney Cells + DENV-2
48 Hours
Embryonic Fibroblast + HCMV
84 Hours
Renal Epithelial Cells + HIV
120 Hours
T Cells + HIV-1

FIGURE 8

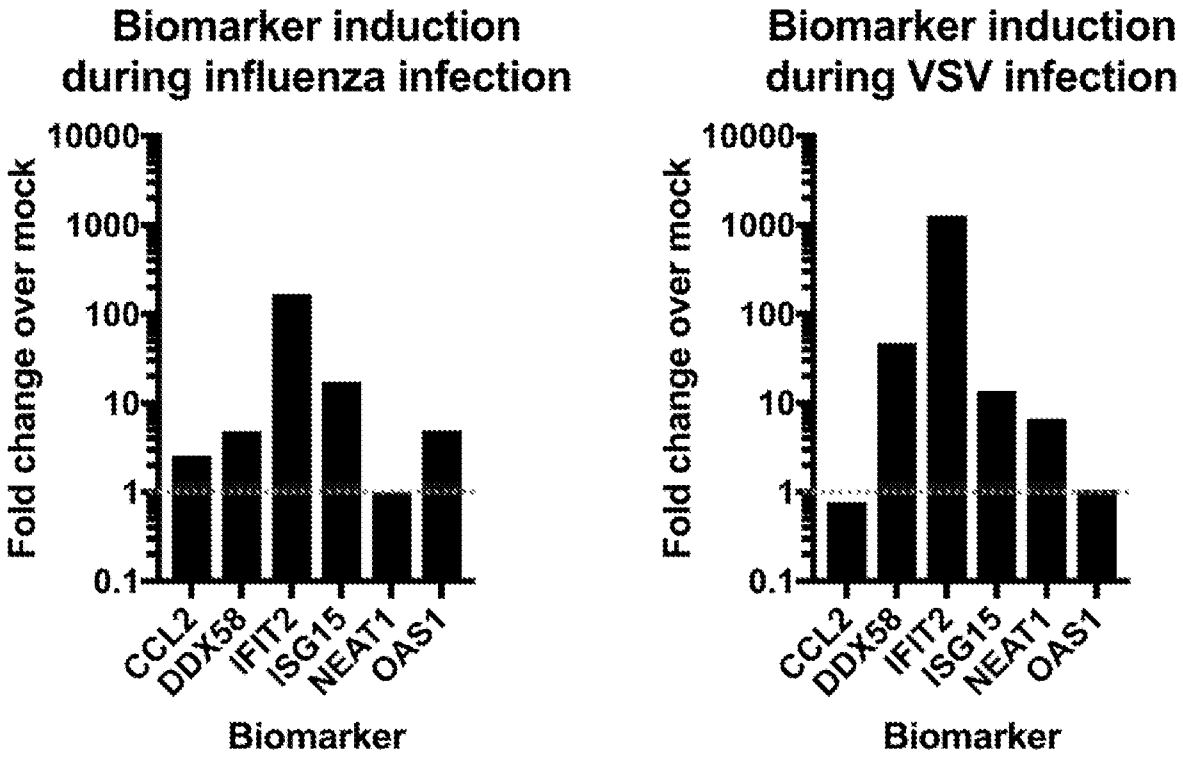

Biomarker induction during influenza infection
Fold change over mock
10000
1000
100
10
1
0.1
CCL2
DDX58
IFIT2
ISG15
NEAT1
OAS1
Biomarker
Biomarker induction during VSV infection
Fold change over mock
10000
1000
100
10
1
0.1
CCL2
DDX58
IFIT2
ISG15
NEAT1
OAS1
Biomarker

FIGURE 9

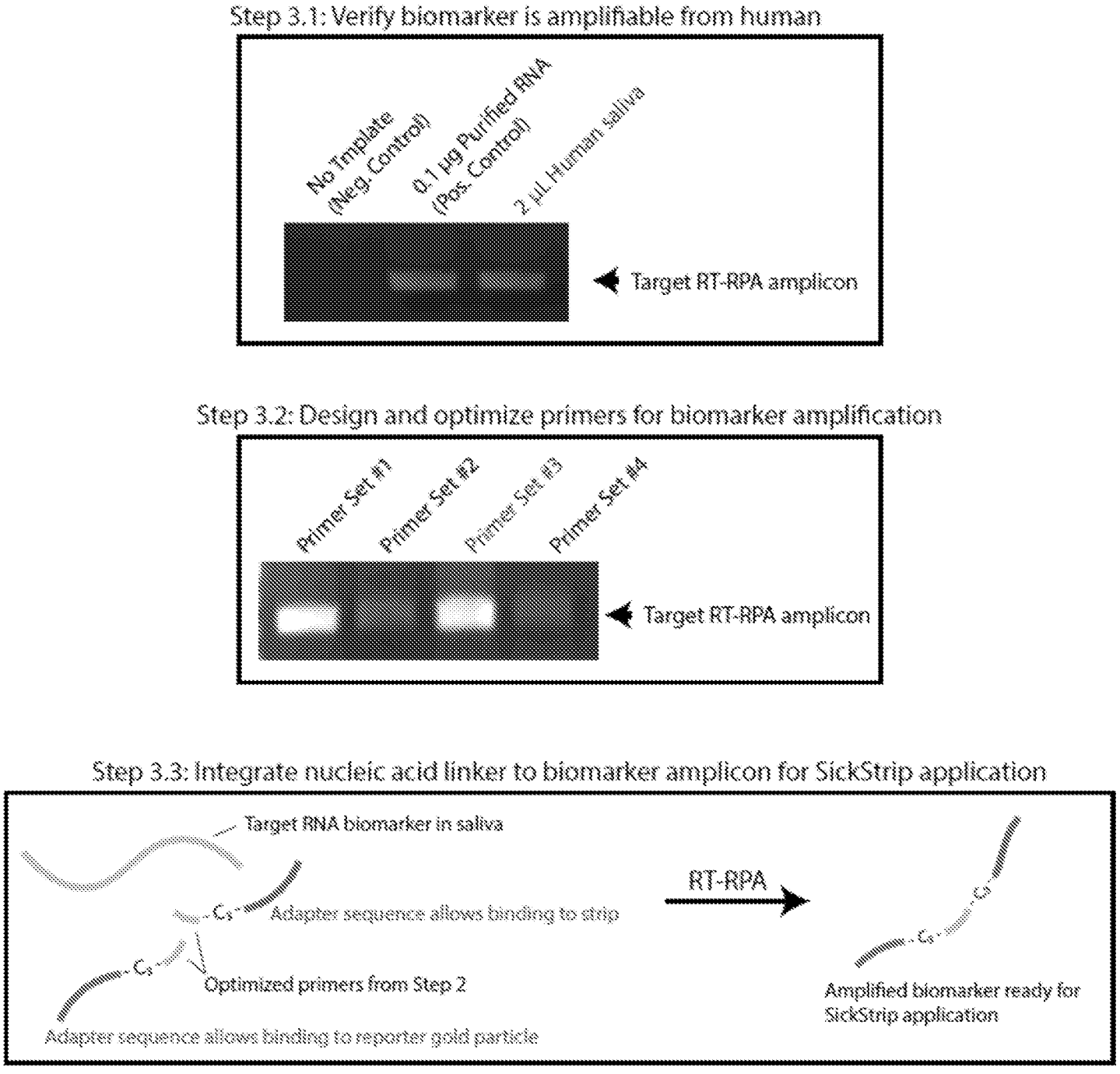

Step 3.1: Verify biomarker is amplifiable from human
No Template (Neg. Control)
0.1 µg Purified RNA (Pos. Control)
2 µL Human saliva
Target RT-RPA amplicon
Step 3.2: Design and optimize primers for biomarker amplification
Primer Set #1
Primer Set #2
Primer Set #3
Primer Set #4
Target RT-RPA amplicon
Step 3.3: Integrate nucleic acid linker to biomarker amplicon for SickStrip application
Target RNA biomarker in saliva
C3
Adapter sequence allows binding to strip
C3
Optimized primers from Step 2
Adapter sequence allows binding to reporter gold particle
RT-RPA
C3
C3
Amplified biomarker ready for SickStrip application

FIGURE 10

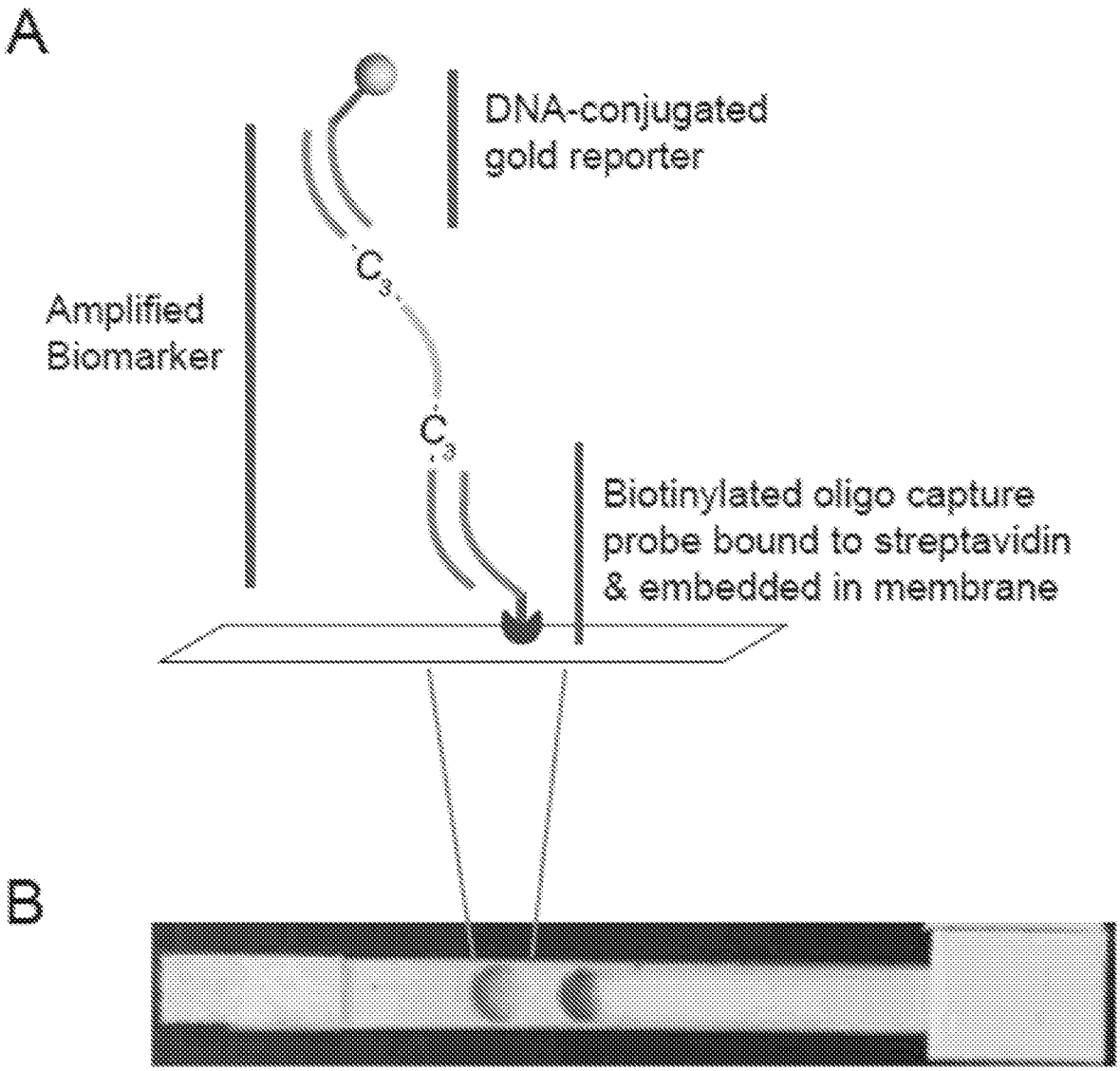

A
DNA-conjugated
gold reporter
Amplified
Biomarker
C3
C3
Biotinylated oligo capture
probe bound to streptavidin
& embedded in membrane
B

FIGURE 11

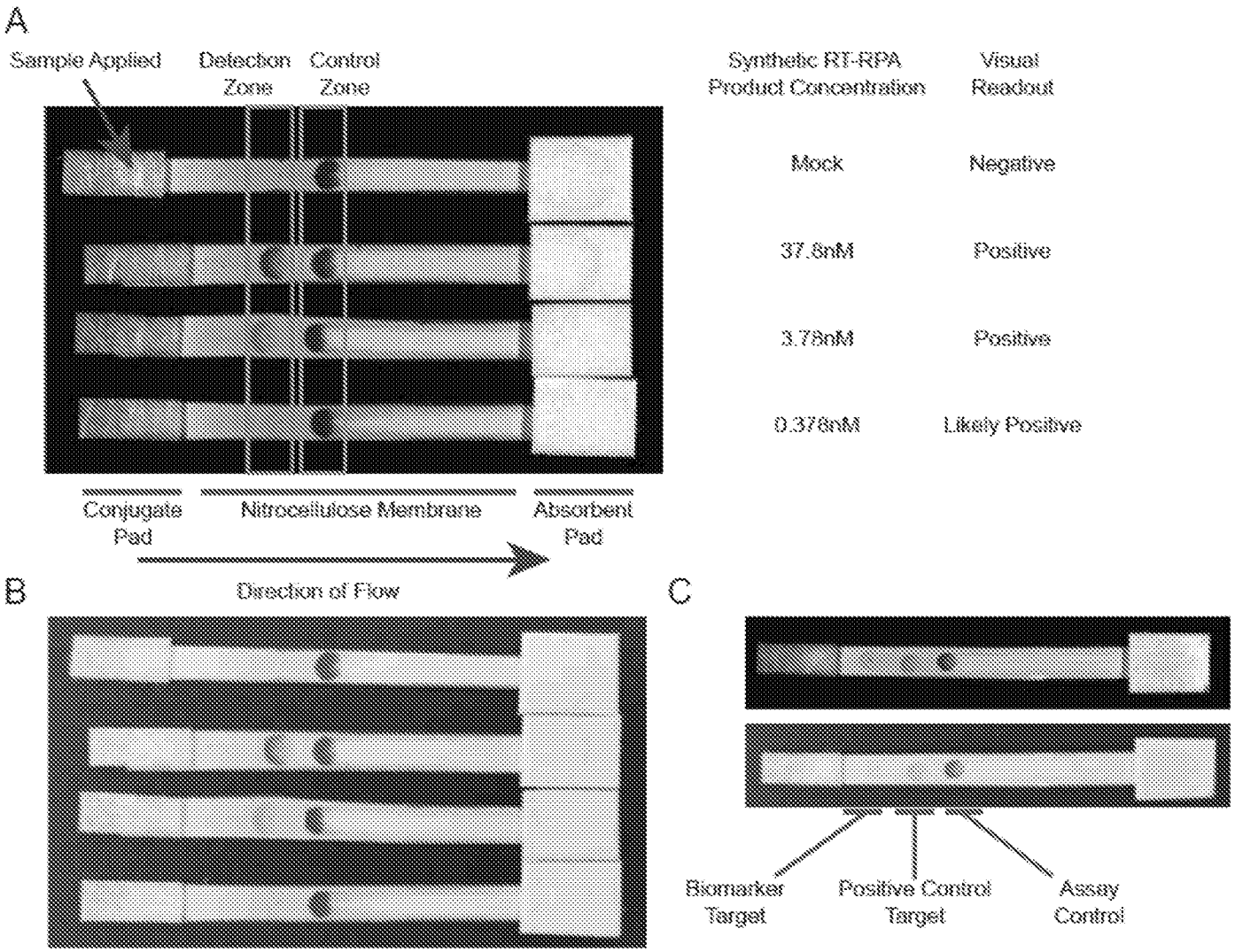

A
Sample Applied
Detection Zone
Control Zone
Synthetic RT-RPA Product Concentration
Visual Readout
Mock
Negative
37.8nM
Positive
3.78nM
Positive
0.378nM
Likely Positive
Conjugate Pad
Nitrocellulose Membrane
Absorbent Pad
Direction of Flow
B
C
Biomarker Target
Positive Control Target
Assay Control

FIGURE 12

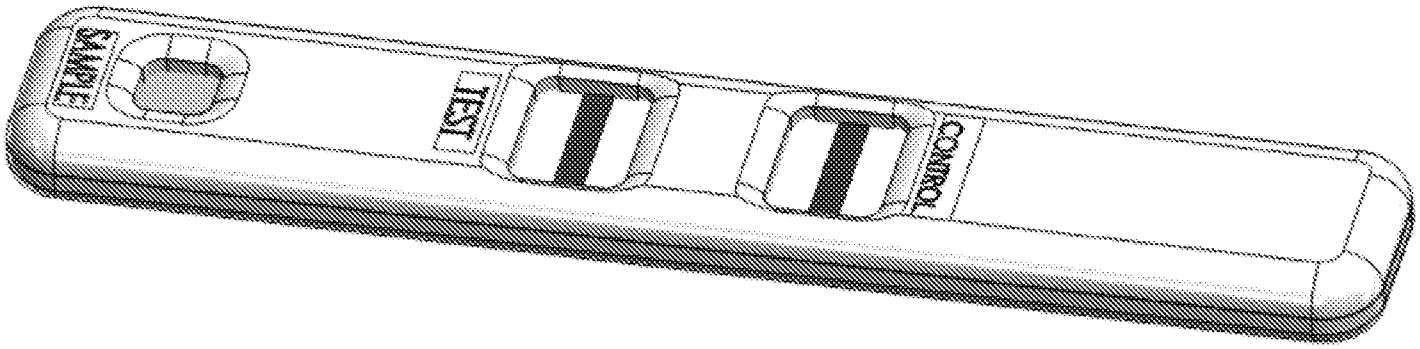


FIGURE 13A

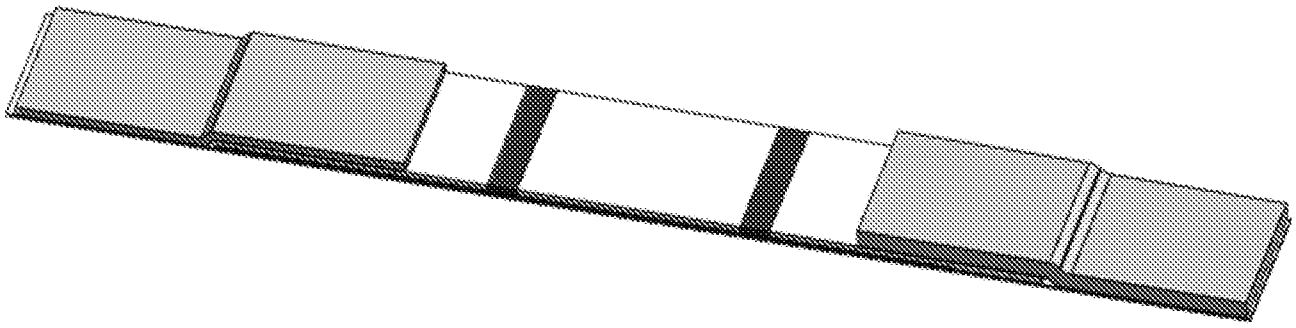


FIGURE 13B

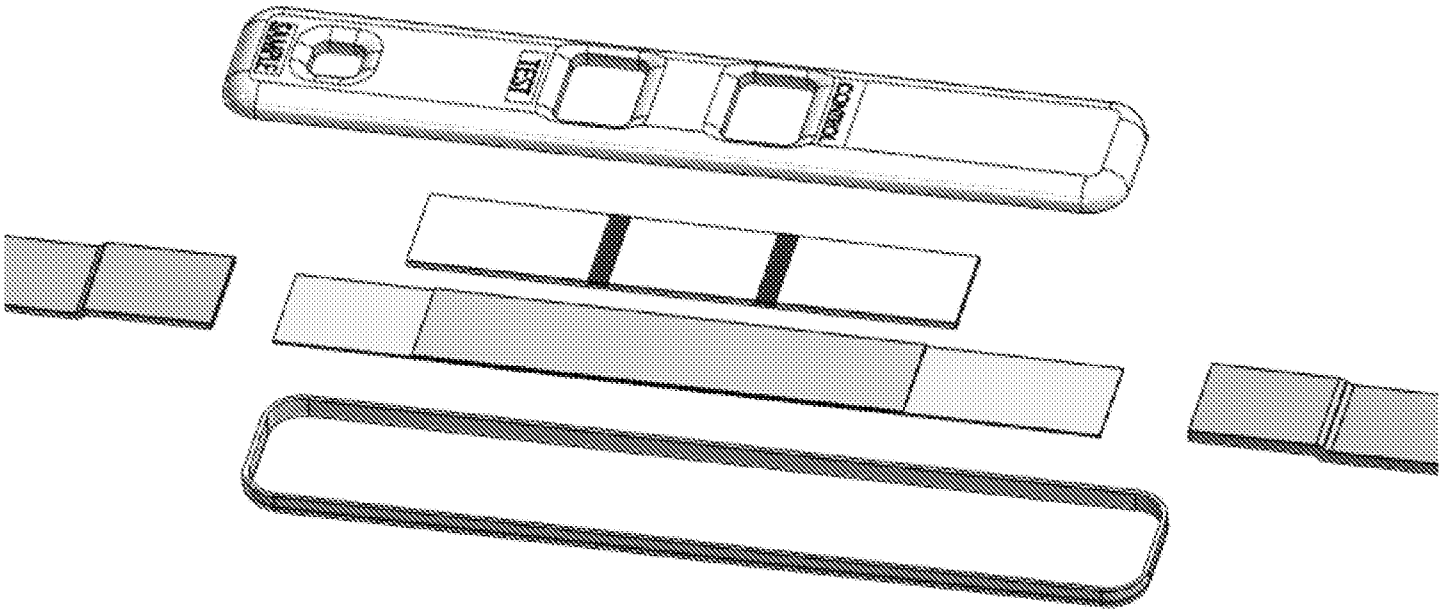


FIGURE 13C

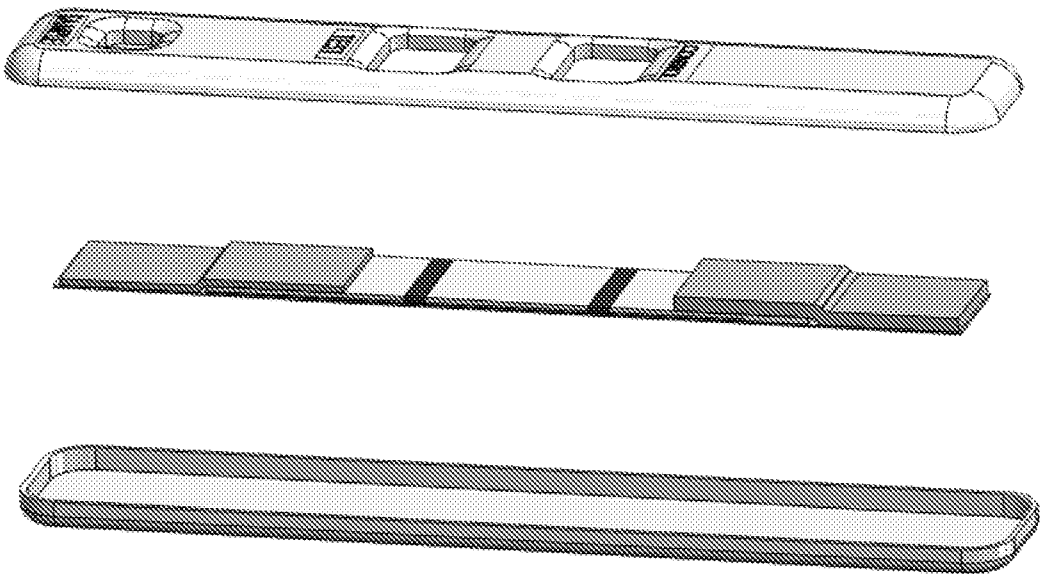


FIGURE 13D

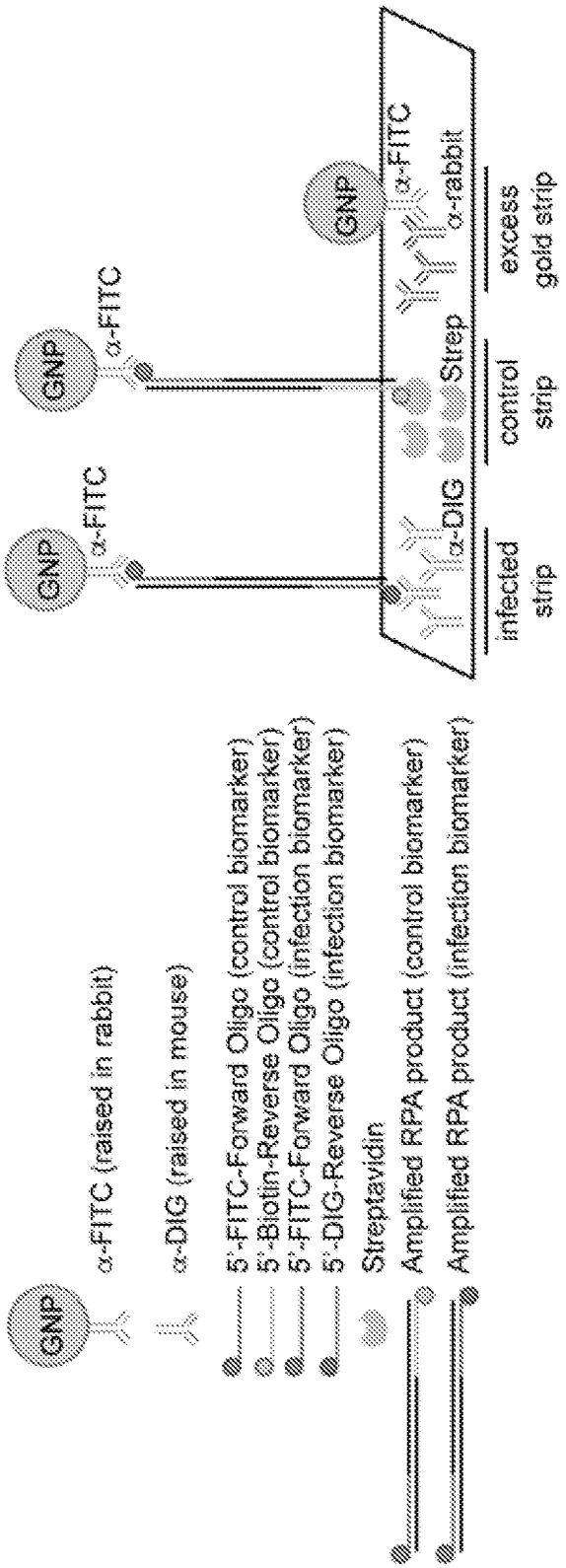

GNP
α-FITC (raised in rabbit)
α-DIG (raised in mouse)
5'-FITC-Forward Oligo (control biomarker)
5'-Biotin-Reverse Oligo (control biomarker)
5'-FITC-Forward Oligo (infection biomarker)
5'-DIG-Reverse Oligo (infection biomarker)
Streptavidin
Amplified RPA product (control biomarker)
Amplified RPA product (infection biomarker)
GNP
α-FITC
GNP
α-FITC
α-DIG
Strep
α-FITC
α-rabbit
infected strip
control strip
excess gold strip

FIGURE 14

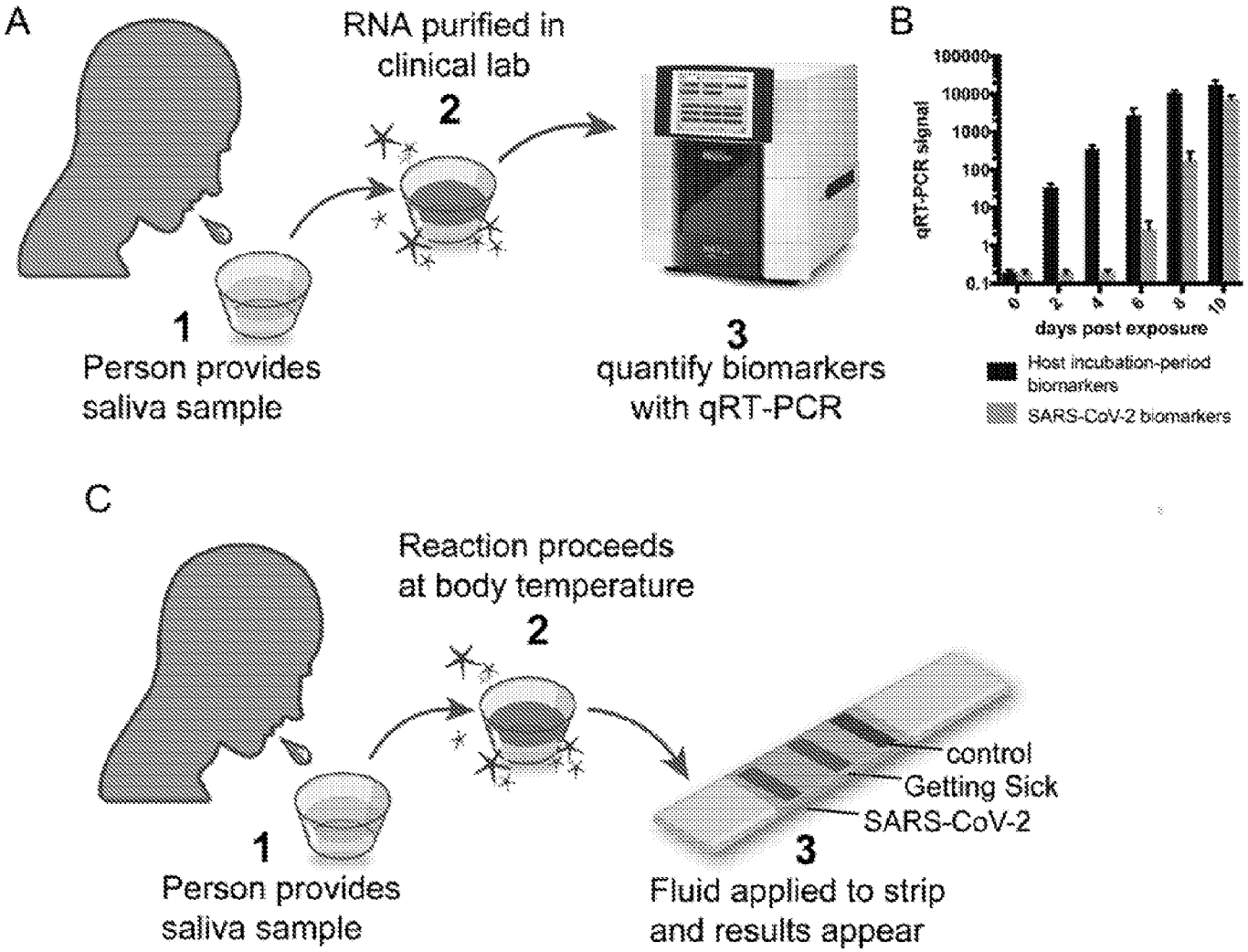

A
RNA purified in clinical lab
2
1
Person provides saliva sample
3
quantify biomarkers with qRT-PCR
B
qRT-PCR signal
100000
10000
1000
100
10
1
0.1
0
2
4
6
8
10
days post exposure
Host incubation-period biomarkers
SARS-CoV-2 biomarkers
C
Reaction proceeds at body temperature
2
1
Person provides saliva sample
control
Getting Sick
SARS-CoV-2
3
Fluid applied to strip and results appear

FIGURE 15

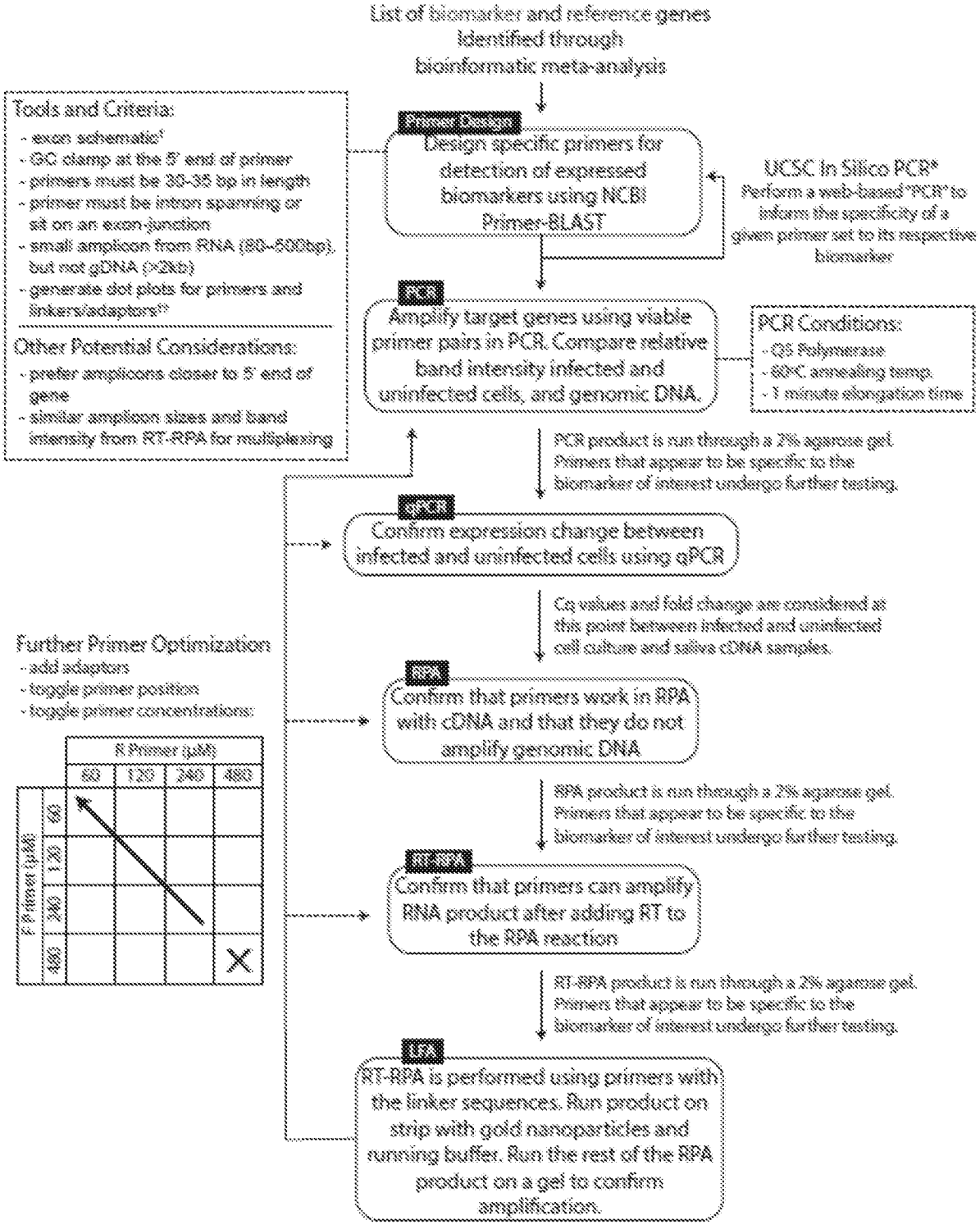

List of biomarker and reference genes identified through bioinformatic meta-analysis
Tools and Criteria:
- exon schematic[1]
- GC clamp at the 5' end of primer
- primers must be 30-35 bp in length
- primer must be intron spanning or sit on an exon-junction
- small amplicon from RNA (80–500bp), but not gDNA (>2kb)
- generate dot plots for primers and linkers/adapters[2]
Other Potential Considerations:
- prefer amplicons closer to 5' end of gene
- similar amplicon sizes and band intensity from RT-RPA for multiplexing
Step 1 - 2
Design specific primers for detection of expressed biomarkers using NCBI Primer-BLAST
UCSC In Silico PCR*
Perform a web-based "PCR" to inform the specificity of a given primer set to its respective biomarker
Step 3
Amplify target genes using viable primer pairs in PCR. Compare relative band intensity infected and uninfected cells, and genomic DNA.
PCR Conditions:
- OS Polymerase
- 60°C annealing temp.
- 1 minute elongation time
PCR product is run through a 2% agarose gel. Primers that appear to be specific to the biomarker of interest undergo further testing.
Step 4
Confirm expression change between infected and uninfected cells using qPCR
Cq values and fold change are considered at this point between infected and uninfected cell culture and saliva cDNA samples.
Further Primer Optimization
- add adapters
- toggle primer position
- toggle primer concentrations:
R Primer (µM)
60
120
240
480
F Primer (µM)
60
120
240
480
Step 5A
Confirm that primers work in RPA with cDNA and that they do not amplify genomic DNA
RPA product is run through a 2% agarose gel. Primers that appear to be specific to the biomarker of interest undergo further testing.
Step 5B
Confirm that primers can amplify RNA product after adding RT to the RPA reaction
RT-RPA product is run through a 2% agarose gel. Primers that appear to be specific to the biomarker of interest undergo further testing.
Step 6
RT-RPA is performed using primers with the linker sequences. Run product on strip with gold nanoparticles and running buffer. Run the rest of the RPA product on a gel to confirm amplification.

FIGURE 16

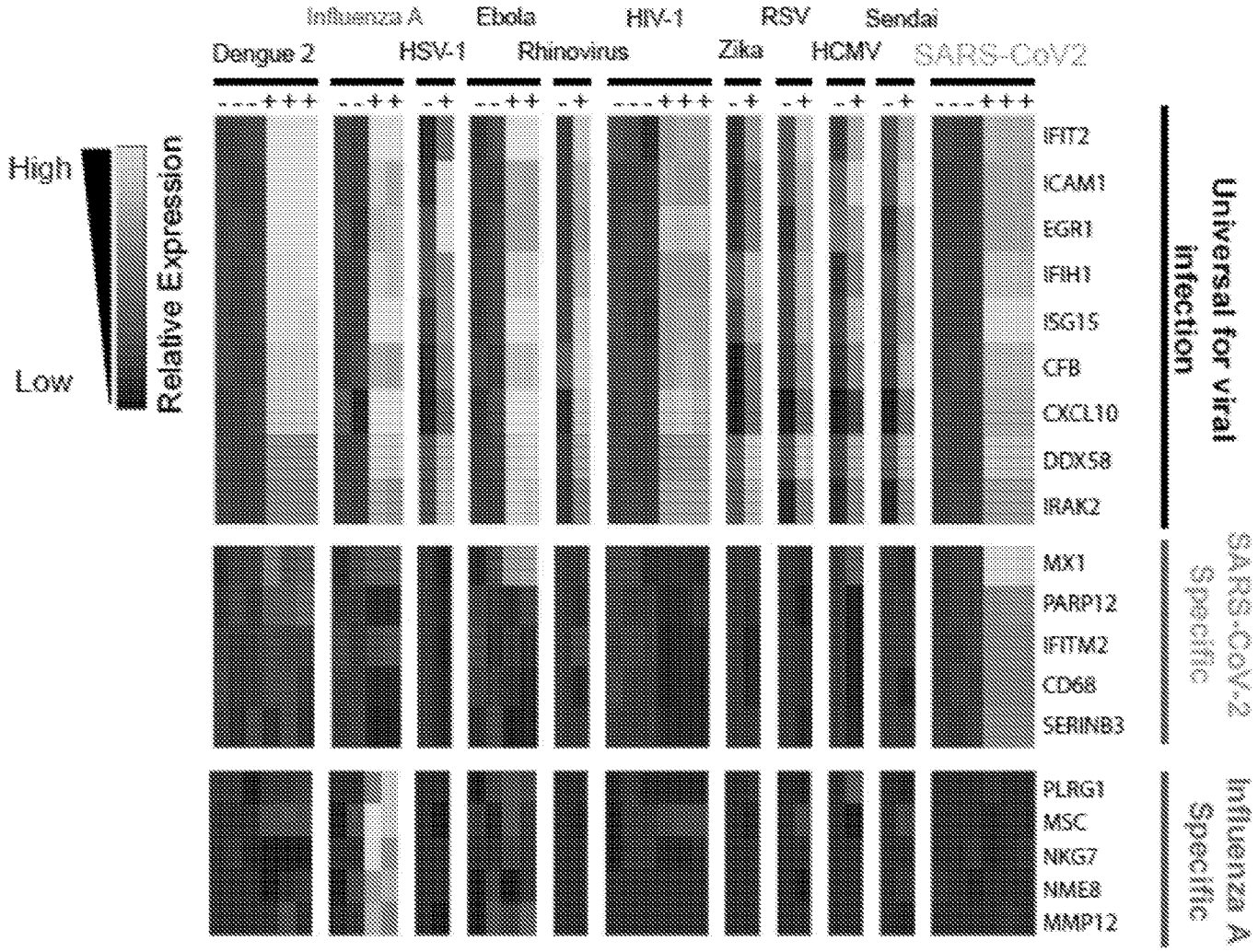

Influenza A
Ebola
HIV-1
RSV
Sendai
Dengue 2
HSV-1
Rhinovirus
Zika
HCMV
SARS-CoV2
High
Low
Relative Expression
IFIT2
ICAM1
EGR1
IFIH1
ISG15
CFB
CXCL10
DDX58
IRAK2
MX1
PARP12
IFITM2
CD68
SERINB3
PLRG1
MSC
NKG7
NME8
MMP12
Universal for viral infection
SARS-CoV-2 Specific
Influenza A Specific

FIGURE 17

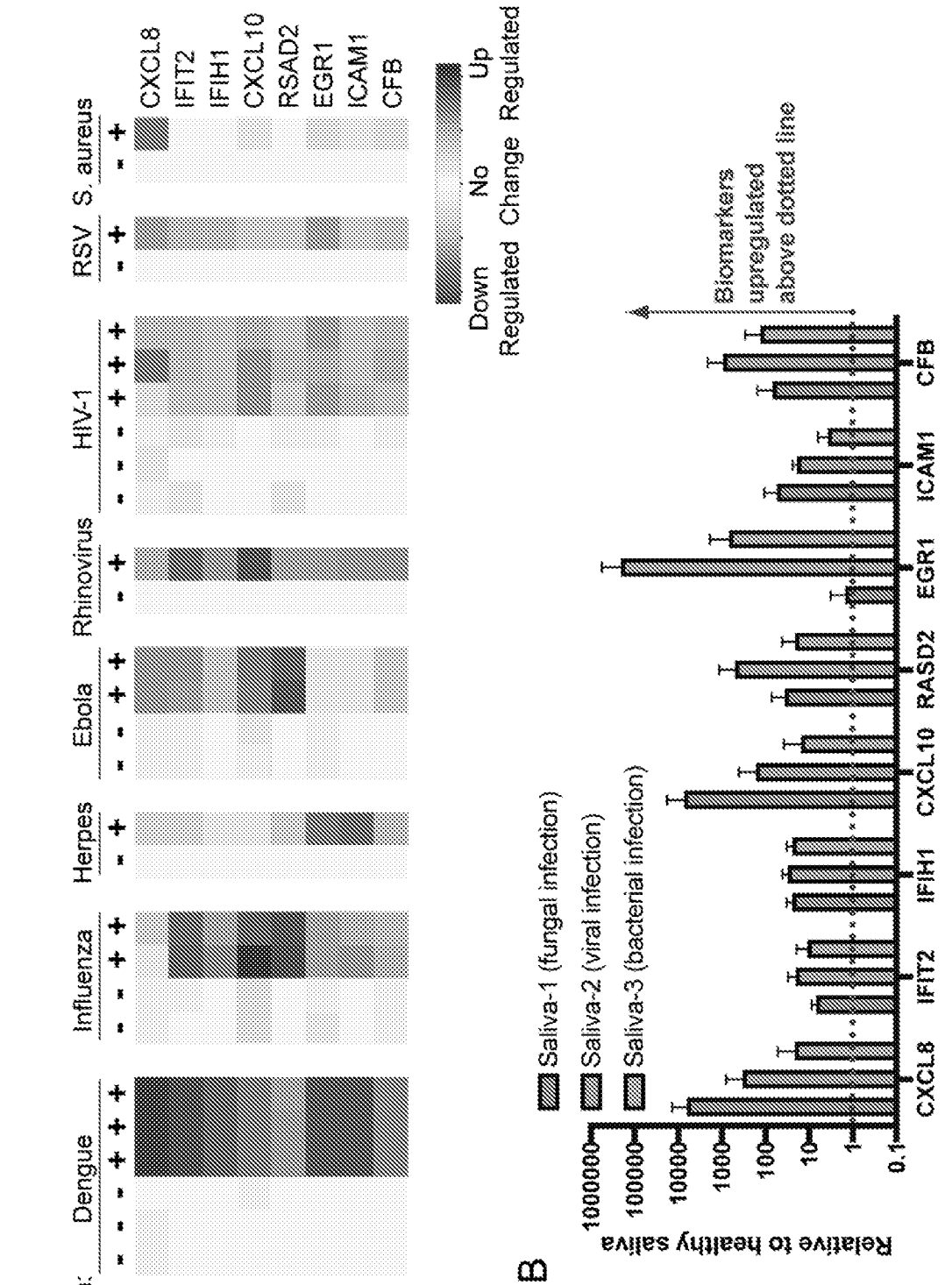
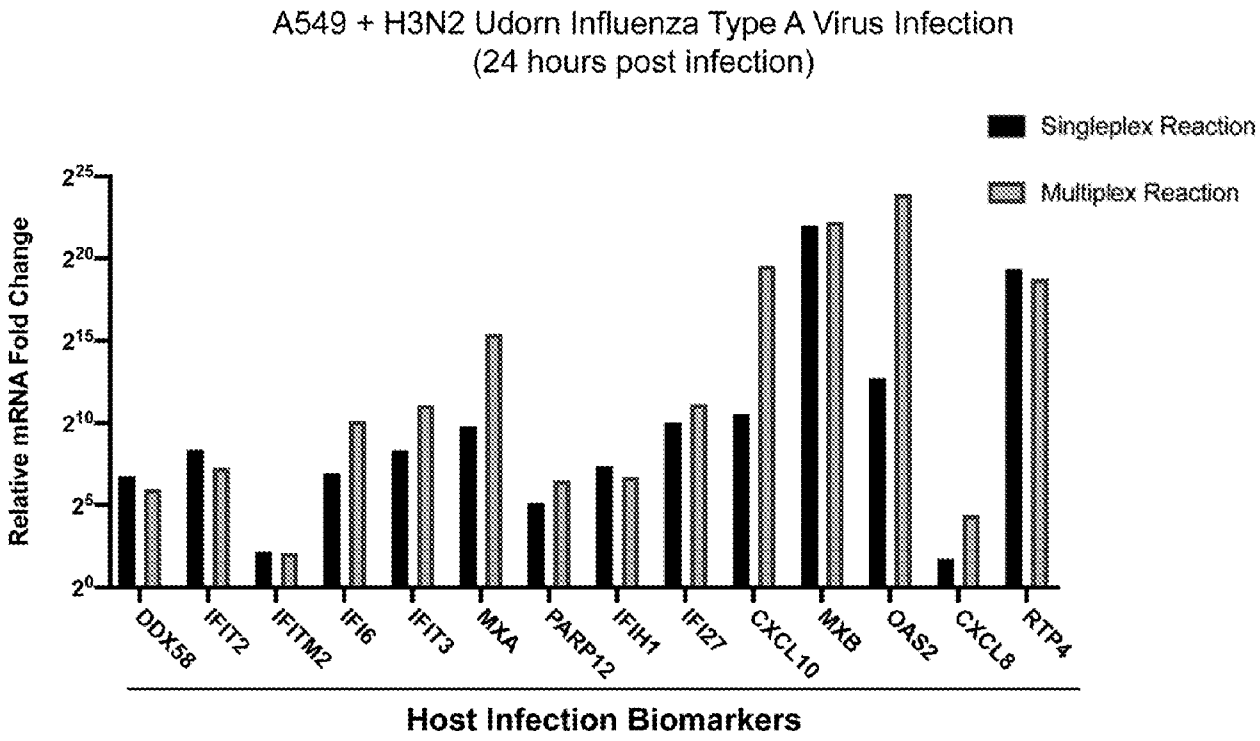

A
Pathogen:
Dengue
Influenza
Herpes
Ebola
Rhinovirus
HIV-1
RSV
S. aureus
CXCL8
IFIT2
IFIH1
CXCL10
RSAD2
EGR1
ICAM1
CFB
Down Regulated
No Change
Up Regulated
B
Saliva-1 (fungal infection)
Saliva-2 (viral infection)
Saliva-3 (bacterial infection)
Relative to healthy saliva
1000000
100000
10000
1000
100
10
1
0.1
Biomarkers upregulated above dotted line
CXCL8
IFIT2
IFIH1
CXCL10
RASD2
EGR1
ICAM1
CFB
Biomarkers

FIGURE 18



*mRNA relative expression level measured using customized TaqMan RT-qPCR primers and probes

FIGURE 19

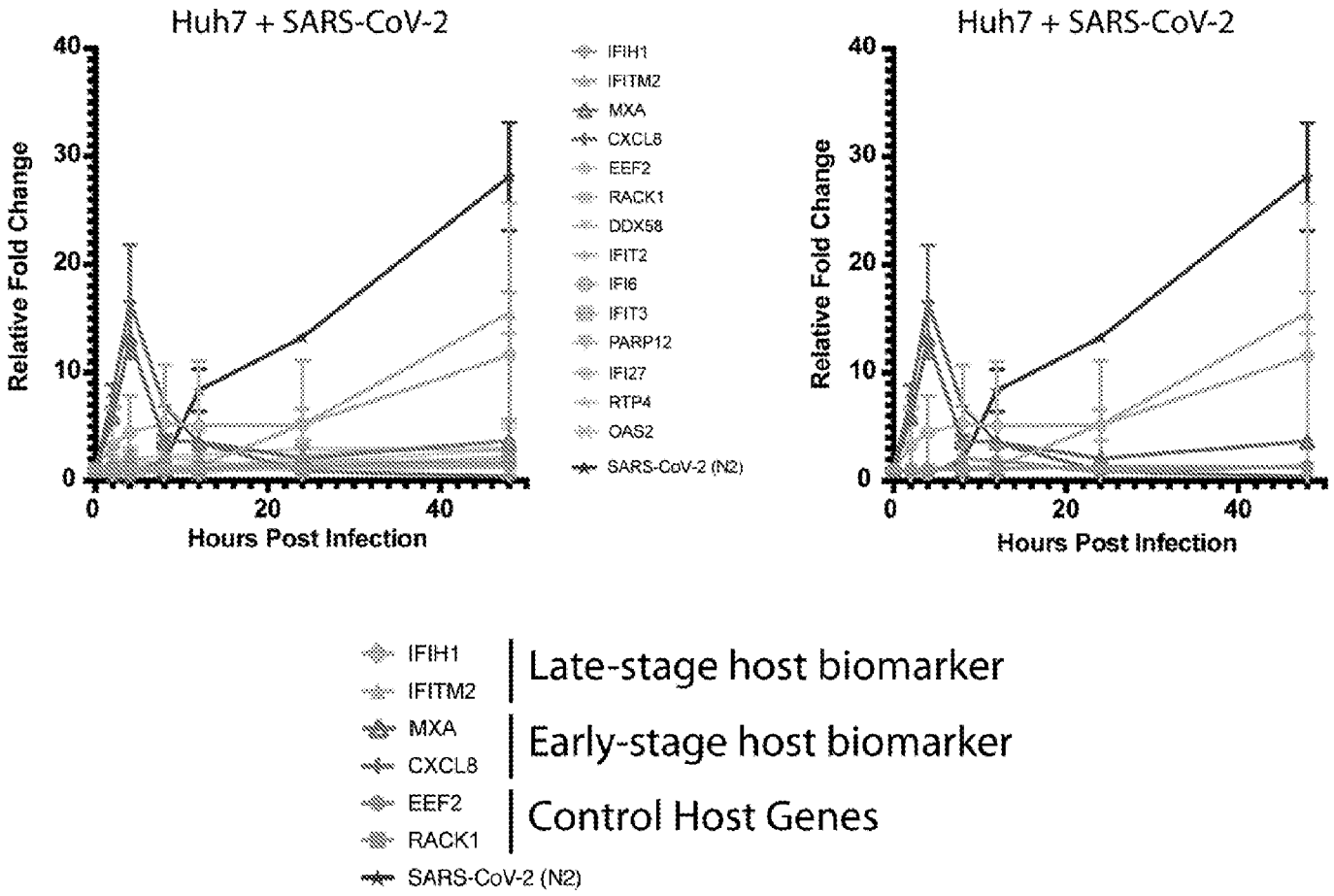

Huh7 + SARS-CoV-2
Relative Fold Change
Hours Post Infection
IFIH1
IFITM2
MXA
CXCL8
EEF2
RACK1
DDX58
IFIT2
IFI6
IFIT3
PARP12
IFI27
RTP4
OAS2
SARS-CoV-2 (N2)
Huh7 + SARS-CoV-2
Relative Fold Change
Hours Post Infection
IFIH1
IFITM2
Late-stage host biomarker
MXA
CXCL8
Early-stage host biomarker
EEF2
RACK1
Control Host Genes
SARS-CoV-2 (N2)

FIGURE 20

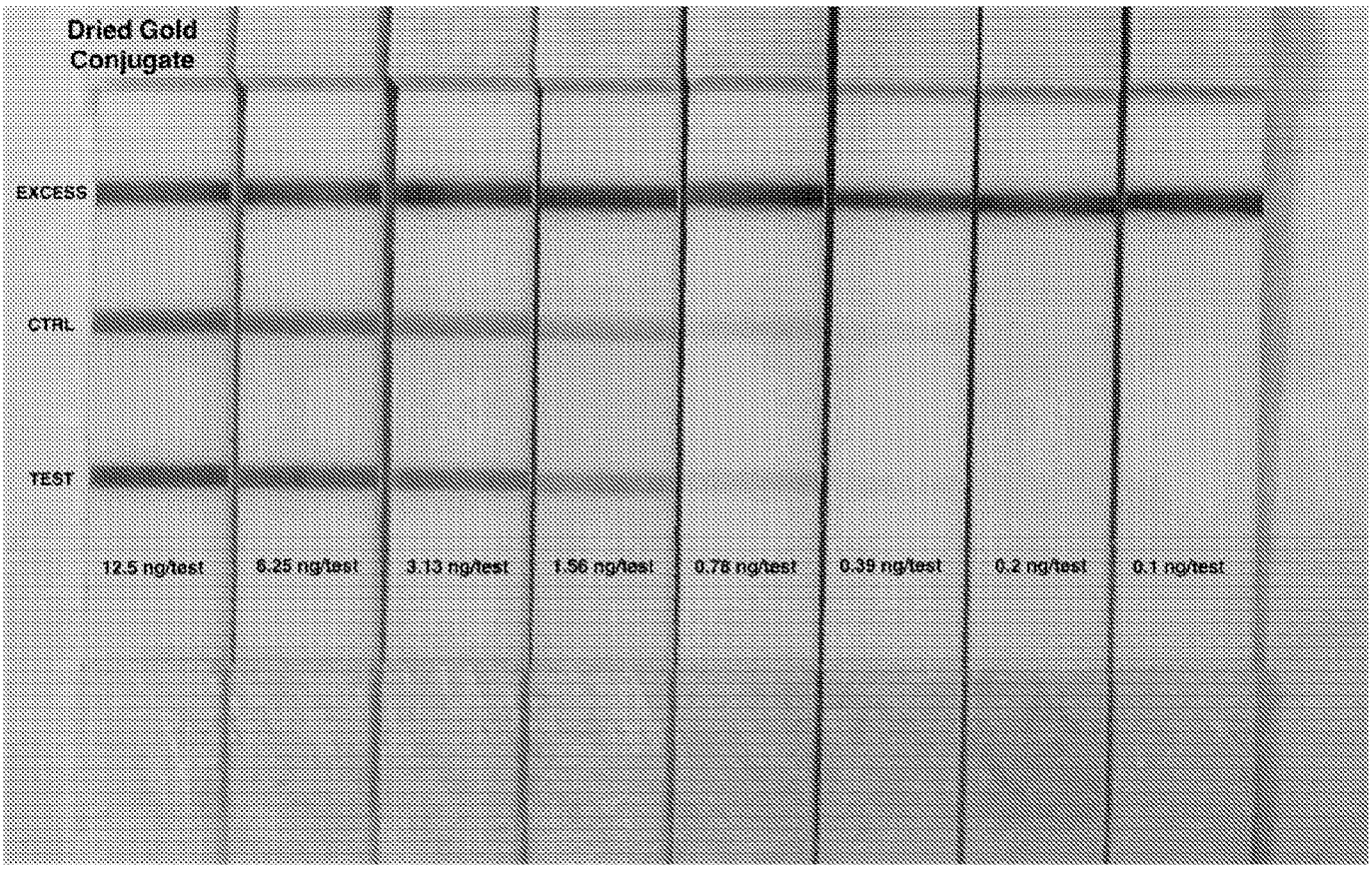

Dried Gold Conjugate
EXCESS
CTRL
TEST
12.5 ng/test
6.25 ng/test
3.13 ng/test
1.56 ng/test
0.78 ng/test
0.39 ng/test
0.2 ng/test
0.1 ng/test

FIGURE 21

Primer Pair	FORWARD PRIMER SEQUENCE (5'->3')	SEQ ID NO.	REVERSE PRIMER SEQUENCE (5'->3')	SEQ ID NO.	Template	primer efficiency slope (m)	(1/10) Cq	Melting curve	logFC +/- Flu (RACK1)	logFC +/- Flu (CALR)
CALR (1F+1R)	GAGIAATCTCCCTGATC CCAGTATTTATGCC	481	ATTTGTTTCTCTGCT GCCTTTGTTACGCCC	516	A549 +flu	3.567	20.1	OK	na	na
NCL (2F+2R)	AGGAAAAGACAGTAAG AAAGAGCGAGATGC	482	TACCCTTTAGATTTG CCATTTTGGTTCTGG	517	A549 +flu	4.073	18.7	OK	na	na
RACK1 (2F+1R)	TCCCACTTTGTTATATG AIGCIGGTTATCTTT	483	CAAATCGACTCTGTGG TGGTGCCTCTTGTGA G	518	A549 +flu	3.219	18	OK	na	na
IFITM2 (For1/Rev1)	ATAGCATTCGCGTACT CCGT	484	TGATGCCTCCTGATC TATCGC	519	A549+ flu	3.479	20	OK	6.011	6.747
IFI27 (For1/Rev1)	GCCACGGAATTAACCC GAGC	485	GCCACAACTCCTCCA ATCACA	520	A549+ flu	3.337	22.1	OK	959.828	1077.512
CD68 (For1/Rev1)	GCAGCACTAGTTGATAT ICTCG	486	TGTTACTGGTGCAAG AGAAA	521	A549+ flu	3.538	22.8	OK	6.131	6.883
OAS3 (For1/Rev1)	AAATGGGAGAATCCCC GCGT	487	CCGCATTGCTGTAGC TGTGG	522	A549+ flu	3.415	22.3	OK	3.936	4.632
OAS1 (For2/Rev2)	GGGTGGTGGAGACCCA AAGG	488	TCGGTCTCTATCTGTCT GCATTG	523	A549+ flu	3.453	23.3	OK	16.423	21.422
C3 (1F+1R)	CAAAGATCAACTCACC TGTAATAAATTCGACC	489	TTGTATTGAAGCAATT CTCCTCAGCACAGCG GC	524	A549 + IFN	3.354	20.8	OK	0.475	1.328
IFIT3 (For2/Rev2)	ACAGCAGAGACACAGA GGGCA	490	AGCTGTGGAAGGATT TTCTCCAGG	525	A549+ flu	3.38	21.9	OK	227.935	268.26
DDX60 (For1/Rev1)	GATACCTACCTGTCTTG AGTC	491	GCTTCTAACTCTGAAC TTGCA	526	A549+ flu	3.084	25.1	OK	11.962	14.578
CFB (1F+1R)	ATTTGTCTCCCCTGCA CCGA	492	GCCAGAATCACCTCT GCAAGTAT	527	A549 + IFN	3.344	23.5	OK	4.511	5.785
IFIH1 (For2/Rev2)	ACAGCTTCAACTGGTG TTGGA	493	ATGGCAAACTTCCTG AGTGGCT	528	A549+ flu	3.258	22.1	OK	75.156	86.098
ZFP36 (1F+1R)	GGATCTGACTTCTCATC TATGAGAGCCTCCTG	494	CAGTGGGCGAAGTGG GTGAGGGTGACAGCT C	529	A549 + IFN	3.619	21.2	OK	0.597	1.343
DDX58 (For1/Rev1)	CCTGTAAGACCCCTGGAC CCTA	495	AGGGCATCCAAAAAG CCACG	530	A549+ flu	3.458	23.8	OK	65.895	75.199
NFKBIA (2F+2R)	CAACAGAGTTACCTAT CAGGGCTATTCTCC	496	CCAAACATCACATCA TCATAGGGCAGCTCG T	531	A549 + IFN	3.845	25.1	OK	1.938	3.932
TNFAIP (For1/Rev1)	TGTTGAGCAGCCCCTAA CAGG	497	CTCTGTGGTACACAC CGGCT	532	A549+ flu	2.974	27.7	OK	0.617	0.726
IFIT2 (For1/Rev1)	CCCTGCCGAACAGCTG AGAA	498	AGTTGCCATAGACTG CTCTC	533	A549+ flu	3.334	19.7	OK	1871.222	2202.26
ICAM1 (1F+1R)	ACCATCTACAGCTTTC CGGC	499	ACTATCTACAGCTTT CCGGC	534	A549 + IFN	3.259	24.3	OK	4.356	6.245
CXCL8 (2F+2R)	CCAGGAAGAAACCACC GGAA	500	CTTGGCAAAACTGCA CCTTCAC	535	A549 + IFN	3.64	26.2	OK	13.09	18.228
IRAK2 (1F+1R)	GTGGAATAGTGTTGGC CGACGT	501	GCAGTGCTGCTTGGA ATATC	536	A549 + IFN	3.392	24.8	OK	0.804	1.054
BIRC3 (2F+2R)	AGTGCGGTTTTTTATT ATGTGGGTAACAGTG	502	TTTTCATCTCCTGGG CTGTCTGATGTGGAT AG	537	A549 + IFN	3.041	20.7	OK	0.675	1.97
CXCL10 (For2/Rev2)	TGCAAGCCAATTTTGT CCACG	503	GCCTCTGTGTGGTCC ATCCT	538	A549+ flu	3.351	25.6	OK	1907.262	2244.676
OAS2 (For2/Rev2)	TGAGCCAGTTCCATAA AACCA	504	GTGTCTGTCATTGTC GCACTT	539	A549+ flu	3.039	25.9	OK	1489.376	1752.861
OAS3 (For3/Rev3)	CCTTGGTGTTGGCATC TTCTG	505	TGCATTGTCGGCACT TTCC	540	A549+ flu	3.269	26.1	OK	415090.8	465955.8
RTP4 (For2/Rev2)	AACCTTCAGCTAGAGT GCCTGG	506	GCAGAGAATCTGCAC TTGGGC	541	A549+ flu	2.959	28.2	OK	2201.367	2471.122
RTP4 (For4/Rev4)	TGGATGCTTGAAGTTGG ATGGC	507	CAACTTCGCTGGCAG GAGGAA	542	A549+ flu	3.468	28.3	OK	2162.919	2427.962
DUSP2 (For2/Rev2)	AGGTCAACAGTGTGTT TCGTC	508	GGACCTTTTCTCTGG AGGGGC	543	A549+ flu	3.311	23.1	OK	281.755	363.295
CDP1 (For2/Rev2)	GTGCTAGAAGCCAATG CTCGT	509	TGGGCCTGTCATATG AGATCTC	544	A549+ flu	3.337	25.8	OK	87.725	88.339
IFI44 (For1/Rev1)	GGCCTGTCTACTGGATC ACATA	510	TCTTTCAGTGATGTG GAATCAA	545	A549+ flu	3.502	25.1	OK	1175.169	1380.644
IFI44L (For2/Rev2)	TCTGTCTCCAAACCGT GGCT	511	AGCAGCTTGCGCAGA TGATTT	546	A549+ flu	3.016	27.6	OK	1093557.667	1287918.571
ISG15 (For2/Rev2)	ATGCGACGAACCTCTG AGCA	512	GGTCAGCCACAGAAG CTCGT	547	A549+ flu	3.337	22.7	OK	264.193	331.563
ISG15 (For4/Rev4)	AGGCAGTGAACTTCATC TTTGC	513	ACATCTGATCTTCTGG GTGA	548	A549+ flu	3.396	22.3	OK	335.316	376.406
MxB (For3/Rev3)	CATGATTGTGAAGTGC CGGG	514	CAACGGGAGCGATTT TTGAA	549	A549+ flu	3.135	25.7	OK	1177.107	1321.349
SEPPING1 (For3/Rev3)	ACCAGAAGTTTCGAGT CCG	515	GCATTTGGATTTGAG GAGCGT	550	A549+ flu	3.312	29.7	OK	5.788	6.279

FIGURE 22

Gene Target	Primer Designation	SEQ ID NO.	Primer Sequence (5'-3')	TaqMan Probe Designation	SEQ ID NO.	TaqMan Probe Sequence (5'-3')	(1:10) Cq From A549 Flu RNA	Multiplex Group	TaqMan Quencher	TaqMan 5' Dye	
CALR	WTF_CALR_1F	551	GAGTAATTCTCCCGATCCCAGTATCTATGCC	QY_CALR_TM1	587	ATGAGGCATACGCTGAGGAGTTTGG	20.1	1	QSY	ABY	Control biomarkers
	WTF_CALR_1R	552	ATTTGTTTCTCTGCTGCCTTTGTTACGCCC								
RACK1	WTF_RACK1_2F	553	TCCCACTTTGTTAGTGATGTGGTTATCTCC								
	WTF_RACK1_1R	554	CAAATCGCCTCGTGGTGGTGCCGTTGTGAG	QY_RACK1_TM1	588	CAGTTTGCCCTCTCAGGCTCCT	18.0	1	MGBNFQ	VIC	
EEF2	TM_EEF2_1F	555	ATCGAGGAGTCGGGAGACATATCATCGCGG	TM_EEF2_1P	589	ACGGTCAGTGAAGAGTCGG	18.7	1	MGBNFQ	6FAM	
	TM_EEF2_1R	556	ACCCTTGGTGATGTCGGTGAGGATGTTGGG								
DDX58	TM_DDX58_1F	557	CCGGAAGACCCTCGGACCTCTA	TM_DDX58_1P	590	TTAGGGAGGAAGAGGTGCAG	21.8	2	QSY	ABY	Infection biomarkers
	TM_DDX58_1R	558	AGGACATCCCAAAAGCCAGG								
IFIT2	IFIT2_For1	559	CCCTGCCGAACAGCTGAGAA	QY_IFIT2_TM1	591	CTGCAACCATGAGTGAGAAC	19.7	2	MGBNFQ	6FAM	
	IFIT2_Rev1	560	AGTTGCCGTAGGCTGTCTC								
IFITM2	TM_IFITM2_1F	561	ACAGCATTCGCCGTCTCCT	TM_IFITM2_1P	592	TGCCTCCACCGCCAAGTGC	20.0	2	MGBNFQ	VIC	
	TM_IFITM2_1R	562	TGATGCCTCCTCTGACCTACTGC								
MXA	MxA_For5	563	TAGAGAGTGCCAGGCTTTG	QY_MXA_TM1	593	TACACACCGTGACGGATATG	21.6	3	MGBNFQ	VIC	Infection biomarkers
	MxA_Rev5	564	ATCTGTGAAAGCAAGCCGGA								
IFI6	TM_IFI6_1F	565	TCGCCTGCTGTGCCCAACTATC	TM_IFI6_1P	594	CTGCTGCTCTTCACTTGC	18.9	3	QSY	ABY	
	TM_IFI6_1R	566	TTCTTTACCTCGCTCCCACCCAC								
IFIT3	TM_IFIT3_1F	567	ACAGCAGAGATACAGAGGGCA	TM_IFIT3_1P	595	TCATGAGTGAGGTCACCAAG	21.9	3	MGBNFQ	6FAM	
	TM_IFIT3_1R	568	AGCTGTGCAAGGATTTTCTCCAGG								
IFI27	IFI27_For1	569	GCCACGGAATTAACCCGAGC	QY_IFI27_TM1	596	CATCAGCAGTGACCAGTGTG	22.1	4	MGBNFQ	6FAM	Infection biomarkers
	IFI27_Rev1	570	GCCACAAGTCCTCCAATCACA								
IFIH1	TM_IFIH1_1F	571	ACAGCTTCACCTCGTCGTGGA	TM_IFIH1_1P	597	CGAAGCAAGCCAAAGCTGAAG	22.1	4	MGBNFQ	VIC	
	TM_IFIH1_1R	572	AGGGCAATTTCTCCCAGTGGT								
PARP12	TM_PARP12_1F	573	ACCATGCAAACCTGCAATACC	TM_PARP12_1P	598	TCCCAGGCCCGAAGAGCATC	23.7	4	QSY	ABY	
	TM_PARP12_1R	574	GCAGCGTGCCGTTAAAGAG								
IRF9	TM_IRF9_1F	575	GCTCTTCAGAACCGCCTACTTC	TM_IRF9_1P	599	CTCCAGCCATACTCCACACGAATC	23.9	5	MGBNFQ	6FAM	Infection biomarkers
	TM_IRF9_1R	576	CTCCAGCAAGTATCTGGGCAA								
CXCL10	CXCL10_For2	577	TGCAAGCCAATTTTGTCCACG	QY_CXCL10_TM1	600	AGCAGTTAGCAAGGAAAGGTC	25.6	5	MGBNFQ	VIC	
	CXCL10_Rev2	578	GCCTCTCTGTGTGGTCCATCCT								
MxB	TM_MxB_1F	579	CATGATTTGTGAAGTGCCGGG	TM_MxB_1P	601	CTGAGCTTGGCAGAGGCAAC	25.7	5	QSY	ABY	
	TM_MxB_1R	580	CAACGGGAGCGATTTTTGGA								
OAS2	TM_OAS2_1F	581	CGTTGTGTTGGGCATCTCTG	TM_OAS2_1P	602	CCAGTCCCATCCTTGAAGCAG	26.1	6	MGBNFQ	VIC	Infection biomarkers
	TM_OAS2_1R	582	TGCATTGTCGGCACTTTCC								
CXCL8	TM_CXCL8_1F	583	CCAGGAGAAACACCACCGAA	TM_CXCL8_1P	603	TGGCCGTGGCTCTCTTG	26.2	6	MGBNFQ	6FAM	
	TM_CXCL8_1R	584	CTTGGCAAACTGTGCCTTTCAC								
RTP4	TM_RTP4_1F	585	TCGACCCTCAAGTTGGACTTGGC	TM_RTP4_1P	604	CTCTCTGTTGGTATTGCTTC	28.3	6	QSY	ABY	
	TM_RTP4_1R	586	CAACTTCCTCCTGGCAAGGAAGAA								

FIGURE 23

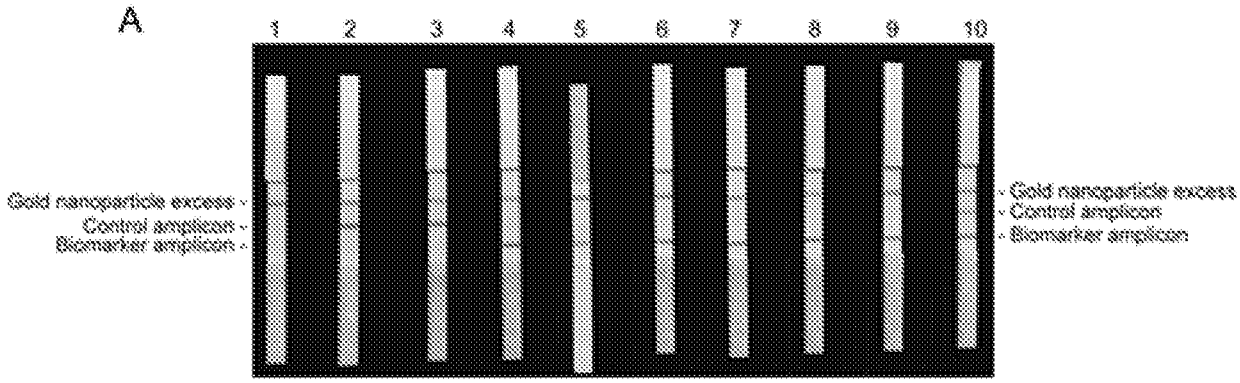
A

Biomarker	Forward Primer	SEQ ID NO.	Sequence (5' --> 3')	Reverse Primer	SEQ ID NO.	Sequence (5' --> 3')
CALR	WTF_CALR_1F	605	GAGTATTCTCCCGATCCCAGTATCTATGCC	WTF_CALR_1R	623	ATTTGTTTCTCTGCTGCCTTTGTTACGCCC
RACK1	WTF_RACK1_2F	606	TCCCACTTTGTTAGTGATGTGGTTATCTCC	WTF_RACK1_1R	624	CAAATCGCCTCGTGGTGGTGCCCGTTGTGAG
EEF2	WTF_EEF2_1F	607	ATCGAGGAGTCGGGAGAGCATATCATCGCGG	WTF_EEF2_1R	625	ACCCTTGGTGATGTCGGTGAGGATGTTGGG
DDX58	RPA_DDX58_1F	608	CAGGATTATATCCGGAAGACCCTGGACCCTA	RPA_DDX58_1R	626	TGCATGGTCTAGGGCATCCAAAAAGCCACG
IFIT2	RPA_IFIT2_1F	609	TGAACCGAGCCCTGCCGAACAGCTGAGAA	RPA_IFIT2_1R	627	ATGGCATTTTAGTTGCCGTAGGCTGCTCTC
IFITM2	RPA_IFITM2_1F	610	CCTGGGCTTCATAGCATTCGCGTACTCCGT	RPA_IFITM2_1R	628	TGGCCTCAATGATGCCTCCTGATCTATCGC
MxA	RPA_MxA_1F	611	AGTATCGTGGTAGAGAGCTGCCAGGCTTTG	RPA_MxA_1R	629	TTATCGAAACATCTGTGAAAGCAAGCCGGA
IFI6	RPA_IFI6_1F	612	ACCGTTTACTCGCTGCTGTGCCCATCTATC	RPA_IFI6_1R	630	AGCACTTTTTCTTACCTGCCTCCACCCCAC
IFIT3	RPA_IFIT3_1F	613	CTTTTCGGAACAGCAGAGACACAGAGGGCA	RPA_IFIT3_1R	631	GGCATTTCAGCTGTGGAAGGATTTTCTCCAGG
IFI27	RPA_IFI27_1F	614	TACTCTCTAGGCCACGGAATTAACCCGAGC	RPA_IFI27_1R	632	ATGGGCACAGCCACAACTCCTCCAATCACA
IFIH1	RPA_IFIH1_1F	615	CTGGGACTAACAGCTTCACCTGGTGTTGGA	RPA_IFIH1_1R	633	GCATCTGCAATGGCAAACTTCTTGCATGGCT
PARP12	RPA_PARP12_1F	616	GATGTGACGACCATGCAAACCTGCAATACC	RPA_PARP12_1R	634	CAAAGTAGAAAGGCAGCGTGCGGTTAAAGAG
IRF9	RPA_IRF9_1F	617	TGCGTGGAGCTCTTCAGAACCGCCTACTTC	RPA_IRF9_1R	635	TGGAGTCTGCTCCAGCAAGTATCGGGCAA
CXCL10	RPA_CXCL10_1F	618	AATTATTCCTGCAAGCCAATTTTGTCCACG	RPA_CXCL10_1R	636	ATGGGAGAGGCAGCCTCTGTGTGGTCCATCCT
MxB	RPA_MxB_1F	619	AGAAGGGCTACATGATTGTGAAGTGCCGGG	RPA_MxB_1R	637	GTCCTTCTAACAACGGGAGCGATTTTTGGA
OAS2	RPA_OAS2_1F	620	TGGAGGGGACCGTTGGTGTTGGCATCTTCTG	RPA_OAS2_1R	638	TTCCTGGTGTCTGCATTGTCGGCACTTTCC
CXCL8	RPA_CXCL8_1F	621	AGGACAAGAGCCAGGAAGAAACCACCGGAA	RPA_CXLC8_1R	639	TAGCACTCCTTGGCAAAACTGCACCTTCAC
RTP4	RPA_RTP4_1F	622	CGGGCCACATGGACGCTGAAGTTGGATGGC	RPA_RTP4_1R	640	GCGGAAGCCCAACTTCGCTGGCAGGAGGAA

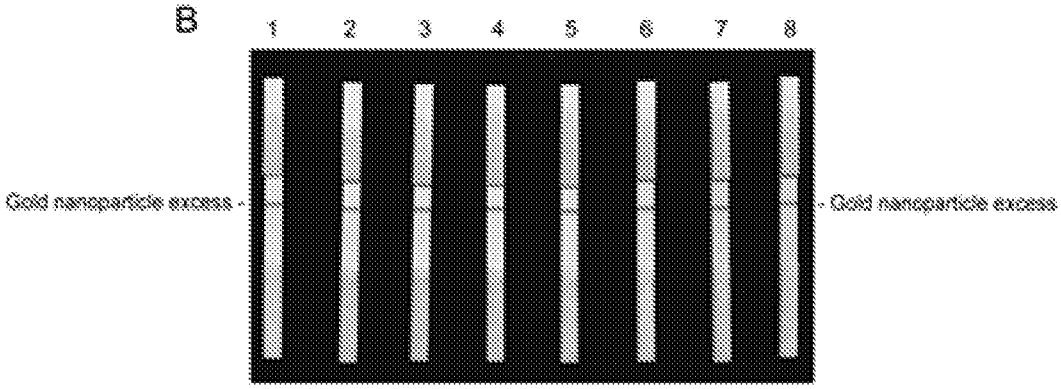
B

Biomarker	RT-RPA primers with 5' modifications for Assay compatibility	SEQ ID NO.
IFI6_1F_FITC	/5FluorT/ACCGTTTACTCGCTGCTGTGCCCATCTATC	641
IFI6_1R_DIG	/5DigN/AGCACTTTTTCTTACCTGCCTCCACCCCAC	642
IRF9_1F_FITC	/5FluorT/TGCGTGGAGCTCTTCAGAACCGCCTACTTC	643
IRF9_1R_DIG	/5DigN/TGGAGTCTGCTCCAGCAAGTATCGGGCAA	644
IFIT2_1F_FITC	/5FluorT/TGAACCGAGCCCTGCCGAACAGCTGAGAA	645
IFIT2_1R_DIG	/5DigN/ATGGCATTTTAGTTGCCGTAGGCTGCTCTC	646
CXCL8_1F_FITC	/5FluorT/AGGACAAGAGCCAGGAAGAAACCACCGGAA	647
CXCL8_1R_DIG	/5DigN/TAGCACTCCTTGGCAAAACTGCACCTTCAC	648
MxA_1F_FITC	/5FluorT/AGTATCGTGGTAGAGAGCTGCCAGGCTTTG	649
MxA_1R_DIG	/5DigN/TTATCGAAACATCTGTGAAAGCAAGCCGGA	650
OAS2_1F_FITC	/5FluorT/TGGAGGGGACCGTTGGTGTTGGCATCTTCTG	651
OAS2_1R_DIG	/5DigN/TTCCTGGTGTCTGCATTGTCGGCACTTTCC	652
IFIT3_1F_FITC	/5FluorT/CTTTTCGGAACAGCAGAGACACAGAGGGCA	653
IFIT3_1R_DIG	/5DigN/GGCATTTCAGCTGTGGAAGGATTTTCTCCAGG	654
RACK1_2F_FITC	/5FluorT/TCCCACTTTGTTAGTGATGTGGTTATCTCC	655
RACK1_1R_Biot	/5BiodT/CAAATCGCCTCGTGGTGGTGCCCGTTGTGAG	656

FIGURE 24



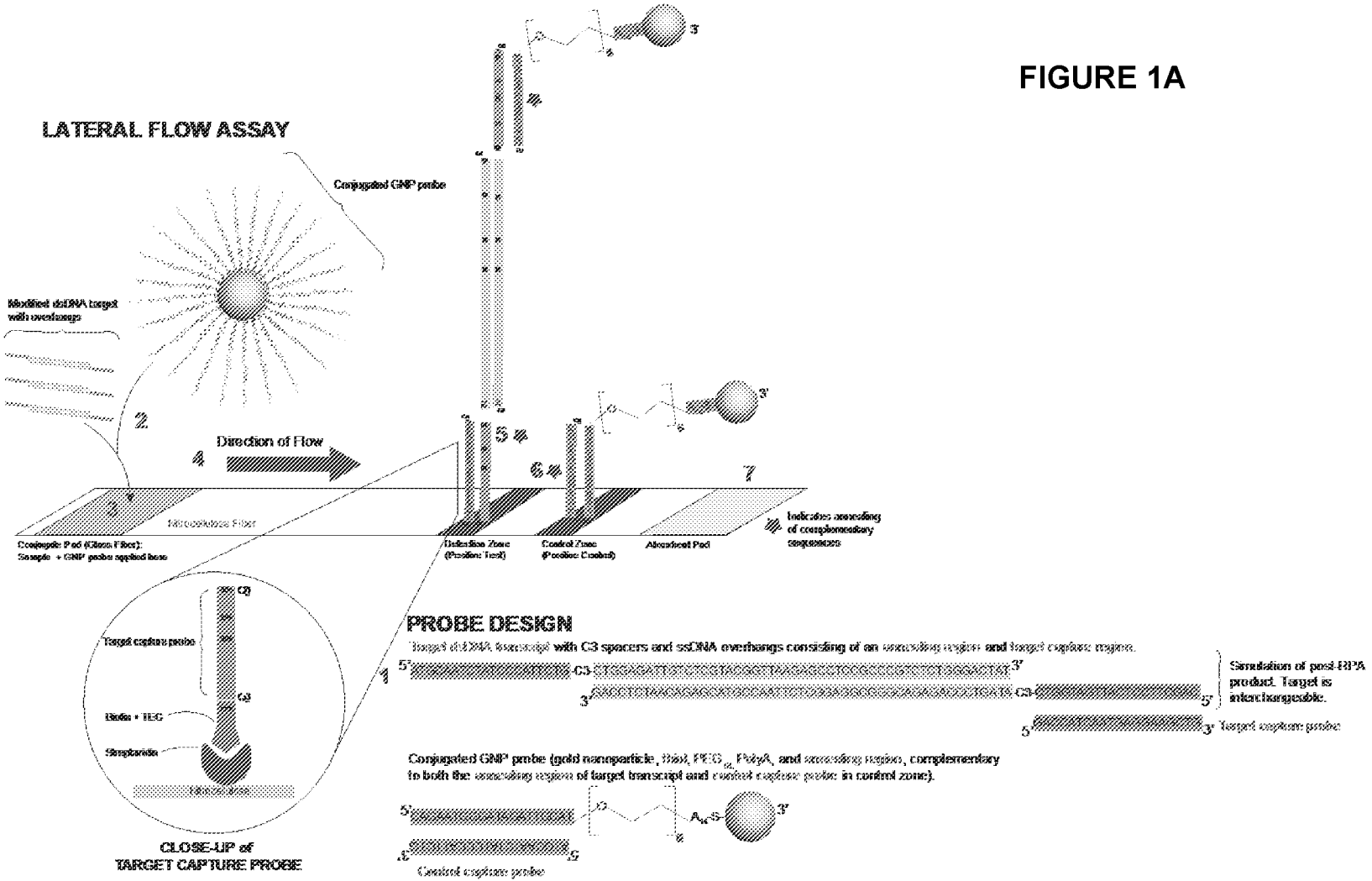
1. PBS only
2. PBS mixed with RT-RPA reaction containing NCL (host control) primers with 5' FITC or Biotin modifications
3. PBS mixed with RT-RPA reaction containing RACK1 (host control) primers with 5' FITC or Biotin modifications
4. PBS mixed with RT-RPA reaction containing IFIT2 (host biomarker) primers with 5' FITC or DIG modifications
5. PBS mixed with RT-RPA reaction containing MxA (host biomarker) primers with 5' FITC or DIG modifications
6. PBS mixed with RT-RPA reaction containing IFI6 (host biomarker) primers with 5' FITC or DIG modifications
7. PBS mixed with RT-RPA reaction containing IFIT3 (host biomarker) primers with 5' FITC or DIG modifications
8. PBS mixed with RT-RPA reaction containing IRF9 (host biomarker) primers with 5' FITC or DIG modifications
9. PBS mixed with RT-RPA reaction containing OAS2 (host biomarker) primers with 5' FITC or DIG modifications
10. PBS mixed with RT-RPA reaction containing RACK1 (host control) primers with 5' FITC or Biotin modifications, and an RT-RPA reaction containing MxA (host biomarker) primers with 5' FITC or DIG modifications



1. PBS mixed with NCL (host control) primers with 5' FITC or Biotin modifications
2. PBS mixed with RACK1 (host control) primers with 5' FITC or Biotin modifications
3. PBS mixed with IFIT2 (host biomarker) primers with 5' FITC or DIG modifications
4. PBS mixed with MxA (host biomarker) primers with 5' FITC or DIG modifications
5. PBS mixed with IFI6 (host biomarker) primers with 5' FITC or DIG modifications
6. PBS mixed with IFIT3 (host biomarker) primers with 5' FITC or DIG modifications
7. PBS mixed with IRF9 (host biomarker) primers with 5' FITC or DIG modifications
8. PBS mixed with OAS2 (host biomarker) primers with 5' FITC or DIG modifications

FIGURE 25

FIGURE 1A


LATERAL FLOW ASSAY
Conjugated GNP probe
Modified dsDNA target with overhangs
2
Direction of Flow
4
5
6
7
Nitrocellulose Fiber
Conjugate Pad (Glass Fiber): Sample + GNP probe applied here
Detection Zone (Positive Test)
Control Zone (Positive Control)
Absorbent Pad
Indicates annealing of complementary sequences
Target capture probe
Biotin + TEG
Streptavidin
Glass Bead
CLOSE-UP of TARGET CAPTURE PROBE
PROBE DESIGN
Simulation of post-RPA product. Target is interchangeable.
Target capture probe
Conjugated GNP probe (gold nanoparticle, thiol, PEG, PolyA, and annealing region, complementary to both the annealing region of target transcript and control capture probe in control zone).
Control capture probe