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(71) Applicant: **THE ARIZONA BOARD OF REGENTS on behalf of THE UNIVERSITY OF ARIZONA** [US/US]; The University of Arizona, Tech Transfer Arizona, University Services Annex, 4th Floor, P.O. Box 210300A, Tucscon, AZ 85721 (US).

(72) Inventors: **WOROBEY, Michael**; The University of Arizona, PO Box 210088, Room 334, Tucson, AZ 85721 (US). **WATTS, Thomas**; The University of Arizona, PO Box 210088, Room 334, Tucson, AZ 85721 (US).

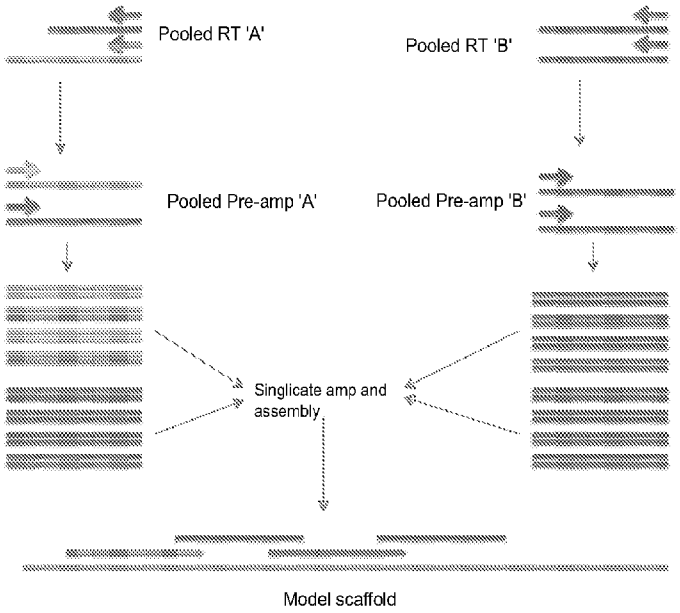
(74) Agent: **NGUYEN, Quang**; Nguyen & Tarbet, 4199 Campus Drive, Suite 550, Irive, CA 92612 (US).

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(54) Title: METHODS AND SYSTEMS FOR RNA OR DNA DETECTION AND SEQUENCING

FIG. 1



(57) Abstract: Disclosed are methods and systems for detecting RNA and sequencing RNA in a wide range of samples such as samples with low concentrations of nucleic acid, samples with degraded nucleic acid, samples that would not otherwise be amenable to conventional sequencing or RNA detection methods, poor quality samples, high quality samples in which rare mutations are sought, formalin-fixed paraffin-embedded samples, blood samples, etc. The methods of the present invention may use paired, large panels of primers to amplify many short fragments that overlap between but not within each panel. Each panel's amplicon set may fill the gaps between those of the opposing panel, thereby providing complete gene or genomic coverage. A preliminary, multiplex amplification step amplifies target nucleic acid for all downstream reactions such as Sanger sequencing, cloning, and Next Generation Sequencing (NGS).

[Continued on next page]

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METHODS AND SYSTEMS FOR RNA OR DNA DETECTION AND SEQUENCING

FIELD OF THE INVENTION

[0001] The present invention relates to detection and sequencing of RNA or DNA, more particularly to detection and sequencing of RNA or DNA in samples with low concentrations of nucleic acid or poor quality samples. The present invention also features methods of detection and sequencing of viral RNA of viruses such as but not limited to human immunodeficiency virus (HIV), hepatitis C virus, hepatitis B virus, influenza virus, etc.

GOVERNMENT SUPPORT

[0002] This invention was made with government support under Grant No. R01 AI084691 awarded by NIH. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] In repositories around the world there are countless medical, veterinary, and museum specimens that would be valuable for the elucidation of the history and evolution of pathogens if (a) the pathogen can be detected and (b) genomic material of the pathogen can be amplified in sufficient length to be of phylogenetic use. For example, there are thousands of serum/plasma samples in storage worldwide from putative AIDS cases that go back to the 1970s and 1980s and that could elucidate both the epidemiological and evolutionary history of HIV. There are also thousands of samples that pre-date the recognition of AIDS, such as from hepatitis B studies that can yield valuable data regarding HIV, hepatitis C and other pathogens.

[0004] However, samples such as those describe above may have a low titer, e.g., the samples are degraded and/or may not be of the pathogen's target tissue. Some samples may have a low functional titer, e.g., there may be nucleic acids in the sample that represent the complete pathogen genome, but because of degradation there may be no nucleic acids long enough to complete a given PCR reaction, thus giving the sample an effective titer of zero (PCR-negative). Or, a given aliquot of sample nucleic acids used for testing may not contain the target sequence even though the sequence is present in the nucleic acid pool. In some cases, samples may have "cryptic" sequences (e.g., sequences that differ at some sites compared to known strains). For example, particularly with RNA viruses, there may be an issue

related to sequence evolution. If one wants to investigate a sample that is 50 years old, and only modern sequences are available for primer design, then any given primer pair designed is only a rough guess as to the actual target sequence. Without wishing to limit the present invention to any theory or mechanism, it is believed that it in cases such as these, a single RT-PCR assay is liable to a miss, and designing around 'conserved regions' may also be inadequate. Another issue for samples is a limited quantity of the sample. In some cases, antiquated samples cannot be replicated and are often quite small.

[0005] Current or traditional strategies for detecting RNA and/or for phylogenetically viable amplification are discussed herein. Qpcr or single step RT and PCR assays are commercially available for many pathogens. They are designed to amplify a relatively short stretch of conserved sequence and so work well with some, but not all, samples of low functional titer and cryptic sequence. Because of their design, they do not yield useful phylogenetic data. Conventional RT-PCR (e.g., long form PCR) can be used for generating phylogenetically useful data. Attempts are often made to amplify PCR fragments of greater than 1000bp. This method faces problems with poor sample quality since in such samples no such long template molecules may survive intact. Next Generation Sequencing (NGS) can develop long sequences, (often) whole genomes, from highly fractured specimens, and because amplification is not sequence based, has no issues with cryptic sequence. However, there is a minimum RNA titer that can be successfully amplified by NGS library technology (e.g., Ovation RNA Seq FFPE requires 100 to 200 ng RNA, see User Guide of Ovation RNA-Seq FFPE, pg. 8, "Total FFPE-derived RNA input must be in the range of 100 to 200 ng…input under 100 ng may result in insufficient yield for analysis."). There can also be problems with NGS if there is a low pathogen titer amongst a high titer of background (e.g. host) RNA.

[0006] Inventors surprisingly discovered methods and systems for detecting RNA and sequencing RNA in a wide range of samples, e.g., samples with low concentrations of nucleic acid, samples with degraded nucleic acid, samples that would not otherwise be amenable to conventional sequencing or RNA detection methods, poor quality samples, high quality samples in which rare mutations are sought (e.g. drug resistant clones), formalin-fixed paraffin-embedded (FFPE) samples, blood samples (e.g., serum or plasma samples), etc. For example, despite

samples having no quantifiable RNA (thus being unusable according to NGS methods), Inventors were able to detect RNA and sequence RNA in said samples using methods of the present invention.

[0007] Briefly, methods of the present invention may use paired, large panels of primers to amplify many short fragments that overlap between (but not within) each panel. In some embodiments, each panel's amplicon set fills the gaps between those of the opposing panel, thereby providing complete gene or genomic coverage. A preliminary, multiplex amplification step, moreover, amplifies target RNA for all downstream reactions (including but not limited to Sanger sequencing, cloning, and NGS).

[0008] Inventors have tried conventional methods for recovering RNA in archival serum, plasma, or FFPE samples. For example, 53 serum samples from putative HIV infected individuals (1978, 1979) were tested with conventional PCR, which failed. RNA from samples was unquantifiable, thus it was not possible to use NGS. Using methods of the present invention, near full-length sequences were generated from 9 samples. The results of this study were published in the leading science journal *Nature* in 2016 to wide acclaim (Nature 539(7627) · October 2016). Additionally, forty-eight plasma or serum samples taken from patients displaying AIDS symptoms in Kinshasa in 1983 were serologically HIV-1-positive, but no HIV-1 RNA was recoverable using conventional PCR approaches at NIH, and the samples were deemed to be of no value. An initial screening run using methods of the present invention (based on non-overlapping 200bp PCR fragments) designed to produce approximately 1300bp of data generated greater than 75% of sequence target on the first run through in 46 samples (96%). Subsequently, a randomly selected 10 of these samples were subjected to a full panel of primer pools designed to cover the entire HIV coding region. Average yield after a first pass was 79% of total target sequence (high of 91%, low of 69%). It should be noted that these samples represent a wide variety of HIV subtypes including heretofore unknown subtypes indicating that this method is quite universal for HIV. These results indicate that it may be possible not only to recover HIV-1 RNA but also to generate near full-length sequences in the vast majority of such samples, even though they may appear to contain no recoverable RNA by previous methods. It should also be noted here that while this process is being done manually, currently available robotics,

DNA quantification technology and automatic sequencing makes this technology highly scalable and automatable. FFPE samples from DRC, circa 1958-1966 were screened using an 8 primer assay (70-110bp target length). HIV was detected in two samples (DRC60, DRC66). NGS sequencing of DRC66 has generated poor results to date. Approximately 450bp of non-overlapping sequence was generated from DRC60 after extensive efforts using standard methods. Re-screening of the sample pool using methods of the present invention revealed an additional positive result, one of which was PCR-negative using previously applied conventional methods. Moreover, methods of the present invention with 192 primer pairs generated a near full-length sequence from DRC66 (>7000bp).

[0009] The methods of the present invention may be used for a variety of applications. Applications may include but are not limited to: detecting RNA or DNA, detecting viral RNA (e.g., HIV RNA, etc.), detecting the presence (or absence) of a particular RNA, sequencing RNA, sequencing of RNA in a historical or poor quality sample, sequencing a plurality of variants of a particular RNA, e.g., viral RNA variants within a single sample, including rare, drug-resistant variants, etc. In some embodiments, the methods and systems of the present invention may be used for determining a range of hosts and vectors that may be susceptible to a virus infection; early and ongoing detection of viruses in emerging geographic ranges via rapid RT-PCR and sequencing of human and mosquito samples; bulk screening of travellers; and screening novel sources, for purposes of virus surveillance and tracking, e.g., municipal waste water; screening protocols allowing for screening of pools samples containing large numbers of individual organisms/mosquitos. Other applications may include but are not limited to: testing or screening whole blood samples (e.g., donated blood), e.g., providing higher sensitivity as compared to traditional methods, being capable of detecting presence of viruses earlier than serological testing or currently available nucleic acid testing; RNA detection, sequencing, or screening in cases where resources may be limited, since methods such as NGS may be too expensive; clinical diagnostics (e.g., HCV detection); vaccine research (e.g., for testing old, rare samples); kits for sequencing whole genomes; liquid biopsies (e.g., cancer biopsies); FFPE sample testing, etc.

[0010] The present invention is not limited to detection and/or sequencing of RNA. For example, the present invention also features methods for detecting and/or

sequencing DNA. For example, the pre-amplification step may be used for detecting DNA viruses or other DNA targets.

[0011] As used herein, the term "conventional" may refer to Next Generation sequencing protocols that typically involve an RT-PCR amplification step of a sizeable viral genomic fragment, prior to Next Gen sequencing.

SUMMARY OF THE INVENTION

[0012] The present invention features methods for detecting a target (e.g., RNA, DNA) in a sample, or methods for sequencing a target (e.g., RNA, DNA) in a sample. In some embodiments, the method comprises subjecting the sample to reverse transcription (RT) using reverse transcriptase and one primer from each of one or more pairs of primers. The method may further comprise subjecting the sample from the previous step to polymerase chain reaction (PCR) amplification using Taq polymerase and the other primer of each of the one or more pairs of primers. The method may further comprise subjecting a portion of the sample from the previous step to PCR amplification using Taq polymerase and both of the primers from each of the one or more pairs of primers. The method may further comprise making detectable the amplified product of the PCR amplification (e.g., second PCR amplification with both of the primers from each of the pairs of primers. Detectable amplified product may be indicative of the presence of the target (e.g., RNA, DNA) in the sample.

[0013] The method may comprise subjecting the sample to reverse transcription using reverse transcriptase and a first primer from each of two or more pairs of primers comprising the first primer and a second primer, wherein, the first primers are non-overlapping with respect to each other, and the second primers are non-overlapping with respect to each other. The method may further comprise the sample from the previous step to polymerase chain reaction (PCR) amplification using Taq polymerase and the second primers of each of the pairs of primers. The method may further comprise subjecting a portion of the sample from the previous step to PCR amplification using Taq polymerase and both of the primers from each of the pairs of primers. The method may further comprise sequencing amplified products from the previous step.

[0014] The sample may be a sample of low quality. For example, the sample may not have quantifiable nucleic acid (e.g., RNA). In some embodiments, the sample comprises a low concentration of nucleic acid. In some embodiments, the sample comprises degraded nucleic acid. In some embodiments, the sample has low viral density. In some embodiments, the sample comprises a formalin-fixed paraffin-embedded (FFPE) sample. In some embodiments, the sample comprises serum or plasma.

[0015] In some embodiments, the primers have the same annealing temperature or are within 5 degrees of an average annealing temperature. The pairs of primers may each be adapted to amplify a fragment of the target from 60 nt to 300 nt (e.g., 60 to 70 nt, 70 to 80 nt, 80 to 100 nt, 100 to 150 nt, 150 to 200 nt, 200 to 300 nt, etc.) in length. In some embodiments, the fragment of the target from 60 nt to 300 nt in length includes the primer. The pairs of primers may each be adapted to amplify a fragment of the target from 60 nt to 600 nt (e.g., 60 to 70 nt, 70 to 80 nt, 80 to 100 nt, 100 to 150 nt, 150 to 200 nt, 200 to 300 nt, 300 to 400 nt, 400 to 500 nt, 500 to 600 nt, etc.) in length. In some embodiments, the fragment of the target from 60 nt to 600 nt in length includes the primer.

[0016] In some embodiments, the primers used in the reverse transcription (RT) step are non-overlapping. In some embodiments, the Taq polymerase is proofreading Taq polymerase.

[0017] In some embodiments, the target is a Retrovirus, e.g., human immunodeficiency virus (e.g. HIV-1, HIV-2). In some embodiments, the target is a Hepadnavirus (e.g. hepatitis B virus (HBV)). In some embodiments, the target is a Hepacivirus (e.g. hepatitis C virus (HCV)). In some embodiments, the target is a Flavivirus (e.g. yellow fever virus, west nile virus, dengue fever virus, Zika virus (ZIKV), etc.) In some embodiments, the target a Filovirus, e.g., an Ebolavirus (e.g., EBOV), Marbug virus (MARV). In some embodiments, the target is an Orthomyxovirus, e.g., an influenza virus (e.g., influenza virus A, influenza virus B, influenza virus C). In some embodiments, the target is a Paramyxovirus (e.g., Mumps virus (MuV), measles virus (MeV)). In some embodiments, the target is a Pneumovirus (e.g., a respiratory syncytial virus (RSV)). In some embodiments, the target is a Bunyavirus. In some embodiments, the target is a Togavirus (e.g., rubella

virus). The present invention is not limited to the aforementioned targets or viruses since the methods of the present invention may be applied to any appropriate RNA detection application.

[0018] In some embodiments, the method further comprises isolating RNA from the sample prior to performing the RT step. In some embodiments, the method further comprises subjecting the sample to DNAse prior to the RT step.

[0019] The present invention also features kits for detecting or sequencing a target (e.g., RNA, DNA) in a sample. In some embodiments, the kit comprises two or more pairs of primers as described herein. For example, the pairs of primers comprise a first primer and a second primer. The pairs of primers may each be adapted to amplify a fragment of the target (e.g., RNA, DNA) from 60 nt to 600 nt (e.g., 60 to 70 nt, 70 to 80 nt, 80 to 100 nt, 100 to 150 nt, 150 to 200 nt, 200 to 300 nt, 300 to 400 nt, 400 to 500 nt, 500 to 600 nt, etc.). The first primers may be non-overlapping with respect to each other, and the second primers may be non-overlapping with respect to each other. The kit may further comprise reverse transcriptase. The kit may further comprise Taq polymerase. The kit may comprise 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, 100 or more, etc., pairs of primers.

[0020] Any feature or combination of features described herein are included within the scope of the present invention provided that the features included in any such combination are not mutually inconsistent as will be apparent from the context, this specification, and the knowledge of one of ordinary skill in the art. Additional advantages and aspects of the present invention are apparent in the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] FIG. 1 shows a schematic view of an example of nucleic acid amplification methods of the present invention (and alignment of the amplicons).

[0022] FIG. 2 (PRIOR ART) shows a schematic view of conventional amplification methods used for the detection and amplification of a target RNA molecule in an old, degraded, low titer sample. Tube 1 (left tube): As an example, in this tube there are 10^{13} RNA molecules, but only one molecule that is both capable of being primed by

primer KG1R and long enough to form an 80bp product when amplified with primers KG1F/KG1R. RT is performed with 10^7 molecules of KG1R. There is one usable product (Tube 2, middle tube). PCR amplification is performed on a subsample (aliquot) of Tube B (e.g., 10% of Tube B) using primers KG1F and KG1R. There is a large chance (e.g., about a 90% chance) that this (Tube 3, right tube) will be a negative sample.

[0023] FIG. 3 shows a schematic view of the methods of the present invention used for the detection and amplification of a target molecule in an old, degraded, low titer sample. Note that this schematic view is a simplified schematic. For example, instead of 1 pair of non-overlapping primers, there may be 10 or more (and 10 or more final amplifications). (Note the present invention is not limited to 1 pair, or 10 or more pairs of primers.) Tube 1: As a example, in this tube there are 10^{13} RNA molecules, but only one molecule that is both capable of being primed by primer KG1R and long enough to form an 80bp product when amplified with primers KG1F/KG1R. In Tube 1, RT is performed with 10^7 molecules of KG1R. There is one usable product. Tube 2 shows RT is then performed with 10^7 molecules of KG1R. There is one usable product. Tube 3 shows amplification with 10^7 molecules of KG1F and proofreading Taq. After 20 amplification cycles, there are 10^5 usable products. An aliquot (e.g., 10% of the product) is transferred to Tube 4 and amplified in a PCR reaction with primers KG1F and KG1R. There is essentially a 100% chance that this will be a positive sample. As an example, with a 10 primer pool and 10 final reactions, there is potential to get 10 amplicons for sequencing.

DETAILED DESCRIPTION OF THE INVENTION

[0024] Referring now to FIG. 1 and FIG. 3, the present invention features methods and systems for detecting RNA or DNA in a wide range of samples, e.g., samples with low concentrations of nucleic acid, samples with degraded nucleic acid, samples that would not otherwise be amendable to conventional sequencing or RNA detection methods, poor quality samples, high quality samples, formalin-fixed paraffin-embedded (FFPE) samples, blood samples (e.g., serum or plasma samples), breast milk samples, archival serum/plasma, etc. The present invention also features methods and systems for sequencing of RNA in said samples. The present invention also features methods of detecting or sequencing viral nucleic acid.

[0025] Methods of the present invention may be used for detection and phylogenetically relevant amplification of pathogens (e.g., RNA viruses) for which sequence data can only be guessed at, and are difficult, fractured and low titer samples. For example, when attempting to detect and amplify sequence from, for example, fifty-year-old HIV samples, modern sequences can be of only limited use in designing primers.

[0026] Methods of the present invention feature using panels of primer pairs. For example, in some embodiments, the methods of the present invention use panels (e.g., 8-10) of primer pairs (e.g., degenerate primers, non-degenerate primers) for detection. In some embodiments, the methods of the present invention use panels (e.g., 8-10) of non-degenerate primer (or a mix of degenerate and non-degenerate primers) for detection. In some embodiments, the methods of the present invention use 50-200 primer pairs (e.g., degenerate, non-degenerate, a combination thereof) for near full-length sequencing. Methods of the present invention also feature a pre-amplification step of RT products so as to increase titer prior to final PCR. This helps allow for adequate sequence yield from some samples that have immeasurably low yields of RNA and/or are highly fractured. Because methods of the present invention may be designed to amplify 1000-1500 bp of sequence from a given aliquot of native RNA, sample size may be much less a factor than if each PCR fragment was generated from a separate RT reaction. In the case of the 1978, 1979 serum samples mentioned above, near full-length sequence could be generated using only 30ul of 50ul serum eluate generated from 50-100ul of serum.

[0027] The present invention also features methods of detecting or sequencing genetic variations of RNA (e.g., variations of the RNA within the same sample), e.g., detecting within-host viral genetic variation with phenotypic consequences (e.g., on drug resistance, pathogenesis, cell/tissue tropism, transmissibility to secondary hosts, etc.). Without wishing to limit the present invention to any theory or mechanism, it is believed that the methods of the present invention may help to detect genetic variations that are not necessarily apparent when large amplicons are generated. For example, variations in primer regions in some clones may prevent them from being amplified. Also, the effective template number of large amplicons may be much smaller than small amplicons (e.g., there may only be one or a few template molecules 1000 nt in length in a degraded sample whereas there may be

more template molecules shorter in length). In the present invention, if several 100 nt regions within that same region are separately assayed, there may be orders of magnitude more template molecules and thus a chance to observe within-patient diversity that may otherwise be invisible when aiming for a larger fragment.

[0028] As previously discussed, the methods of the present invention feature targeting several small fragments of the target RNA. For example, in some embodiments, the fragment that is targeted is about 70 nt in length. In some embodiments, the fragment is about 80 nt in length. In some embodiments, the fragment is about 90 nt in length. In some embodiments, the fragment is about 100 nt in length. In some embodiments, the fragment is about 110 nt in length. In some embodiments, the fragment is from 65 to 90 nt in length. In some embodiments, the fragment is from 70 to 100 nt in length. In some embodiments, the fragment is from 70 to 110 nt in length. In some embodiments, the fragment is from 80 to 100 nt in length. In some embodiments, the fragment is from 100 to 200 nt in length. In some embodiments, the fragment is from 70 to 200 nt in length. The present invention is not limited to the aforementioned examples. For example, in some embodiments, the fragment is more than 200 nt in length, e.g., from 200 to 250 nt, from 200 to 300 nt, from 300 to 400 nt, from 400 to 500 nt, etc. Fragment size may depend on the quality of the sample.

[0029] In some embodiments, at least 2 primer pairs are used. In some embodiments, at least 3 primer pairs are used. In some embodiments, at least 4 primer pairs are used. In some embodiments, at least 5 primer pairs are used. In some embodiments, at least 6 primer pairs are used. In some embodiments, at least 7 primer pairs are used. In some embodiments, at least 8 primer pairs are used. In some embodiments, at least 9 primer pairs are used. In some embodiments, at least 10 primer pairs are used. In some embodiments, at least 20 primer pairs are used. In some embodiments, at least 30 primer pairs are used. In some embodiments, at least 40 primer pairs are used. In some embodiments, at least 50 primer pairs are used. In some embodiments, at least 75 primer pairs are used. In some embodiments, at least 100 primer pairs are used. In some embodiments, at least 150 primer pairs are used. In some embodiments, at least 200 primer pairs are used. The present invention is not limited to the aforementioned examples, and more than 200 primer pairs may be used.

[0030] In some embodiments, 2 primer pairs are used. In some embodiments, 3 primer pairs are used. In some embodiments, 4 primer pairs are used. In some embodiments, 5 primer pairs are used. In some embodiments, 6 primer pairs are used. In some embodiments, 7 primer pairs are used. In some embodiments, 8 primer pairs are used. In some embodiments, 9 primer pairs are used. In some embodiments, 10 primer pairs are used. In some embodiments, 11 primer pairs are used. In some embodiments, 12 primer pairs are used. In some embodiments, 13 primer pairs are used. In some embodiments, 14 primer pairs are used. In some embodiments, 15 primer pairs are used. In some embodiments, 16 primer pairs are used. In some embodiments, 16 primer pairs are used. In some embodiments, 18 primer pairs are used. In some embodiments, 19 primer pairs are used. In some embodiments, 20 primer pairs are used. In some embodiments, 21 primer pairs are used. In some embodiments, 22 primer pairs are used. In some embodiments, 23 primer pairs are used. In some embodiments, 24 primer pairs are used. In some embodiments, 25 primer pairs are used. In some embodiments, 26 primer pairs are used. In some embodiments, 27 primer pairs are used. In some embodiments, 28 primer pairs are used. In some embodiments, 29 primer pairs are used. In some embodiments, 30 primer pairs are used. In some embodiments, 40 primer pairs are used. In some embodiments, 50 primer pairs are used. In some embodiments, 100 primer pairs are used. In some embodiments, 200 primer pairs are used. As previously discussed, the present invention is not limited to the aforementioned examples, and more than 200 primer pairs may be used.

[0031] The primer pairs may be divided into at least 2 pools. In some embodiments, 2 pools are used. In some embodiments, 3 pools are used. In some embodiments, 4 pools are used. In some embodiments, 5 pools are used. In some embodiments, 2 pools are used. In some embodiments, 6 pools are used. In some embodiments, 7 pools are used. In some embodiments, 8 pools are used. In some embodiments, 9 pools are used. In some embodiments, 10 pools are used. In some embodiments, more than 10 pools are used. In some embodiments, pools have about 10 primers each.

[0032] For example, in some embodiments, when attempting to detect the presence of a particular RNA, about 8 to 10 primer pairs may be used. In some embodiments, when attempting to sequence a particular RNA, about 50 primer pairs may be used.

However, the present invention is not limited to the aforementioned examples.

[0033] As previously discussed, the present invention may help sequence RNAs in historical samples of unknown subtypes, as well as sequence variants of an RNA. Without wishing to limit the present invention to any theory or mechanism, it is believed that the use of multiple, small fragments may be advantageous for sequencing unknown subtypes or RNAs with polymorphisms.

EXAMPLE 1 – Plasma/Serum Samples

[0034] Example 1 describes an example of a protocol for plasma/serum samples. The present invention is not limited to the details of Example 1. This example describes a procedure for working up a sample using 40 overlapping primer pairs designed to generate products of approximately 200bp. This will provide 6000+ bp of sequence. Actual amount of sequence is a function of how much overlap designed into the primers. For FFPE samples or severely degraded liquid samples, 70-100bp primer pairs may be designed. High quality proof-reading Taq is used. This protocol uses Promega Goscript and RNAsin plus for RT. Taq is Accustart.

[0035] Make up 4 forward primer pools (A, B, C, D) and 4 matching reverse primer pools (A, B, C, D). The following schematic may be used (for both forward group and reverse group):

	Pool A	Pool B	Pool C	Pool D
Primer #	1	2	3	4
Primer #	5	6	7	8
Primer #	9	10	11	12
Primer #	13	14	15	16
Primer #	17	18	19	20
Primer #	21	22	23	24
Primer #	25	26	27	28
Primer #	29	30	31	32
Primer #	33	34	35	36
Primer #	37	38	39	40

[0036] *Reverse Transcription*: For each sample to be amplified, the following may

be used: Primer/dNTP: Make up 4 reverse primer/dntp pools, one for each of A,B,C,D. (1) Primer- 9ul, (2) dNTP- 3ul. Add 4ul of 'A' to wells 1 and 5 of an 8 place strip. Add 'B' to wells 2 and 6 and etc. Then add 6ul sample to each of wells 1-4 and 6ul control to wells 5-8. Mix well. Incubate at 70C for 5'. Ice. RT mix: (1) 5X buffer- 36ul; (2) 25mM Mg- 36ul; (3) RNasin- 9ul; (4) Goscript- 9ul. Add 10ul per sample, mix well and incubate at 42C for 2 hours, followed by 10' at 85C.

[0037] Preliminary amplification: The following may be used: Add 4ul 'A' forward primer pool to wells 1 and 5 of an 8 place strip, and 4ul of 'B' to wells 2 and 6 and etc. Master mix: 10X- 45ul; 50mM Mg- 18ul; dNTP- 9ul; Taq- 4ul; Water- 252ul. 36ul mix into each well. Add 10ul individual RT reaction the appropriate well. Mix well. Amplify for 30 cycles in a standard PCR program at the appropriate annealing temperature (e.g., primers may be designed around 52 degrees C).

[0038] Final amplification: The following may be used: Make up 5 x 8 well strips with individual primer pairs in numerical order as in schema below (e.g., 2ul primer total per well). One may want one set for the sample and one for the control.

	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8
Strip 1	Primer 1	Primer 2	Primer 3	Primer 4	Primer 5	Primer 6	Primer 7	Primer 8
Strip 2	Primer 9	Primer 10	Primer 11	Primer 12	Primer 13	Primer 14	Primer 15	Primer 16
Strip 3	Primer 17	Primer 18	Primer 19	Primer 20	Primer 21	Primer 22	Primer 23	Primer 24
Strip 4	Primer 25	Primer 26	Primer 27	Primer 28	Primer 29	Primer 30	Primer 31	Primer 32
Strip 5	Primer 33	Primer 34	Primer 35	Primer 36	Primer 37	Primer 38	Primer 39	Primer 40

[0039] Make up 8 Master mixes, four for samples (A,B,C,D) and four for controls (A,B,C,D). Master mix: 10X- 27.5ul; 50mM Mg- 11ul; dNTP- 5.5ul; Taq- 1ul; Pre-amp product- 22ul; Water- 187ul. Add 23ul of Sample 'A' master mix to primer wells 1 and 5 of each strip, Master mix Sample 'B' to wells 2 and 6 and etc. as per schema above. Mix well. Amplify for 40 cycles at appropriate annealing temperature.

[0040] In some embodiments, this protocol, e.g., when used in conjunction with appropriately designed primers, may generate 70-90% of the target sequence on the first go through. Note that some products may be double banded with a contaminating human band, so some gel clipping may be required unless going to

NGS sequencing. Note that when the first go round is done, there is 10ul leftover RT reaction for each pool of each sample. This can be used to pre-amp and amp on an alternative cycling program such as a Touchdown program to generate bands that cover some of the blank spots. In some embodiments, this step is done before going to sequencing. Once sequencing is done and the sequences are lined up with the primers, new primers may be designed to match missing sequence. Note also that this protocol may help ensure that no given amplification is exposed to confounding internal primers from the primer pairs immediately upstream or downstream of the amplification. This may help ensure that mis-incorporation of primers doesn't compromise the data.

EXAMPLE 2 – Recovery of HIV Sequences from Degraded Archival Samples

[0041] Methods of the present invention for recovering viral RNA were used test 1970s HIV serum samples (degraded archival samples). Example 2 describes the recovery of eight near-full-length genomes from US serum samples from 1978-79 – eight of the nine oldest HIV-1 group M genomes to date. Example 2 also describes recovery of the HIV-1 genome from the individual known as 'Patient 0' (Auerbach et al, 1984, Am J Med 76: 487-492) (showing there is neither biological nor historical evidence he was the primary case in the US or for subtype B as a whole).

[0042] *HIV-1 serological screening of serum samples from San Francisco from 1978.* 2231 samples collected from the cohort of gay and bisexual men in San Francisco in 1978 (Jaffe et al., 1985, Ann Intern Med 103: 210-214) were tested, and 83 WB-positives were detected (3.7% prevalence). Samples were first screened by GS HIV-1/HIV-2 Plus O EIA (Bio-Rad Laboratories, Redmond WA) and reactive samples were further tested by WB Genetic Systems HIV-1 Western Blot (Bio-Rad Laboratories, Redmond WA).

[0043] *HIV-1 nucleic acid amplification.* A total of 33 samples of frozen serum previously identified as positive for antibody to HIV-1 (Stevens et al, 1986, JAMA 255: 2167-2172; Szmuness et al, 1981, Hepatology 1: 377; Koblin et al., 1992, J Epidemiology 136:646-656) were assayed from New York City; a total of 20 frozen serum samples from San Francisco (Jaffe et al., 1985, Ann Intern Med 103: 210-214), identified as part of the present study as positive for antibody to HIV-1, were assayed. The New York City samples were from 1978 and 1979 though no complete

genomic sequences from 1978 were developed. The San Francisco samples were all from 1978. Additionally, a sample of PMBC and a sample of serum were both assayed; these had been collected from a single individual in 1983 (Patient 0), and the samples were stored at CDC Atlanta. Other than Patient 0, now deceased, the data recorded were unlinked to individual identifiers and the work was approved by the Human Subjects Protection Program at the University of Arizona.

[0044] Four panels of degenerate primers were designed using a suite of North American subtype B sequences. Primers were designed to be able to amplify both conserved regions and predictably variable sites. Primers within each panel were designed to generate sequence from the 5' end of *gag* to the 3' end of *nef* and were designed to amplify overlapping fragments. Two panels "HIVL" (N=25) and "HIVLb" (N=22) were designed to amplify fragments of ~500-650 bases in length. Two other panels "HIVm" (N=50) and "HIVr" (N=46) were designed to amplify fragments of ~200-320 bases in length. Nucleic acids from 100ul aliquots of serum (or PMBCs in the case of Patient 0) were isolated using the QIAamp Viral RNA Mini Kit (Qiagen, Gaithersburg, MD) with 5mcg added carrier RNA. Serum samples were then treated with DNase I (Invitrogen, Life Technologies, Carlsbad, CA) prior to reverse transcription. PMBC nucleic acids were left untreated. Proviral DNA from Patient 0's PMBCs was amplified with all four primer panels and from multiple separate isolations. Amplification was achieved using Invitrogen Platinum Taq DNA polymerase High Fidelity (Life Technologies, Carlsbad, CA) and run for 55 cycles at an annealing temperature of 52°C. Additionally, attempts were made to amplify longer fragments using PCR SuperMix High Fidelity (Life Technologies, Carlsbad, CA) and forward and reverse primers matched from the HIVLb primer panel for long fragment length followed by nesting with primers for slightly shorter fragment length. A single fragment of slightly more than 7000 bases was generated after multiple attempts with multiple primer combinations and cloned using the Invitrogen TOPO XL PCR Cloning Kit (Life Technologies, Carlsbad, CA). Fragments of individual clones were then amplified using HIVLb forward and reverse primers matched to give approximately 1000-base overlapping fragments and then sequenced.

[0045] Methods of the present invention using the serum samples proceeded as follows: Aliquots of isolated RNA were reverse transcribed using the GoScript Reverse Transcription System (Promega, Madison, WI) using a program of 4 cycles

of 50°C for 30' followed by 55°C for 30' and an 85°C final incubation. Primers used were pools of reverse primers from widely spaced amplicons, abrogating the possibility of incorporation of an internal primer into any given amplicon. RT products were then briefly amplified in multiplex reactions (denaturation for 3' at 94°C followed by 30 cycles of 94°C for 30", 52°C for 30", 68°C for 30", and a final extension of 68°C for 5') with matching forward primer pools and then amplified via single primer pairs (denaturation for 3' at 94°C followed by 40 cycles of 94°C for 30", 52°C for 30", 68°C for 30", and a final extension of 68°C for 5'). Two separate isolates were amplified from each sample in this manner with a minimum of one amplification with each primer panel per isolate. Sequencing was performed at the University of Arizona Genetics Core using an ABI 3730XL. The Patient 0 sample contained considerable heterogeneity (mixed bases) both in proviral assembly and in viral RNA amplifications. Heterogeneity in the NY and SF samples (all sequences derived from viral RNA) was low. In all cases consensus sequences were used in the phylogenetic analyses.

EXAMPLE 3 – Viral Enrichment and Multiplex RT-PCR Viral Genetic Screening Assays

[0046] Example 3 describes the development of viral enrichment and multiplex RT-PCR viral genetic screening assays that are more sensitive than existing RT-PCR protocols for detection of viral RNA. The methods of the present invention could allow recovery of viral genomic information from challenging source material (e.g., specimens with low concentration of template viral RNA, samples containing no viable (culturable) virus, etc.). The methods of the present invention may be useful in conventional samples as well, e.g., for conventional samples in resource-limited settings where culturing of virus may be precluded but RT-PCR may be possible. Without wishing to limit the present invention to any theory or mechanism, it is believed that the methods of the present invention may allow for detection of viral RNA with very high sensitivity (e.g., up to 100% specificity after sequencing). Thus, this may allow for the use of the methods of the present invention in resource-limited settings (e.g. without the need for culturing viral isolates), for detecting viral RNA in low-concentration, damaged or otherwise challenging samples (e.g., pooled mosquito specimens, archival human specimens, or perhaps even municipal influent (e.g., sewage) samples), etc.

[0047] In some embodiments, the methods of the present invention feature RNA (or DNA capturing, e.g., for concentration of viral nucleic acids from large pooled samples. This may increase the chances of finding the target sequence and may be effective at sequestering target nucleic acids away from potential inhibitors.

[0048] Referring to FIG. 3, the methods further comprise using non-degenerate and/or degenerate primers directed to a wide spectrum of viral strains, which are designed to amplify 70-100nt fragments in overlapping fashion (e.g., for FFPE samples) or 200-300nt fragments (e.g., for serum/plasma). Primers are then pooled in non-overlapping fashion for reverse transcription, and then a 'preliminary-amplification' (pre-amplification) step is employed to amplify with corresponding primer mates. The pre-amplified mix is then broken up for amplification with single primer pairs. The pre-amplification step greatly increases the effective titer of the target molecules, thus increasing both sensitivity and coverage.

[0049] This technique allows for HIV detection that is not otherwise detectable using previous techniques (see FIG. 2) and for development of long sequences by alignment of overlapping short sequences in samples that would otherwise be recalcitrant to viral genome sequencing. This technique provides a greater degree of assurance that a virus can be identified in samples with very low copy number and/or damaged RNA. Without wishing to limit the present invention to any theory or mechanism, it is believed that sequencing short reads in samples that have damaged RNA increases the likelihood of detecting polymorphisms.

[0050] Bell et al., 1971. *Arch. Gesamte Virusforsch.* 35:183–193; Calisher et al., 1989. *J. Gen. Virol.* 70:37–43; Crill et al., 2004. *Cell* 78:13975–13986; Dick et al., 1952. *Trans. R. Soc. Trop. Med. Hyg.* 46:509–20; Duffy et al., 2009, N Engl J Med. 360: 2536–2543; Faye et al., 2014. *PLOS, Neglected Tropical Diseases.* DOI: 10.1371/journal.pntd.0002636; Fischer and Hayes, 2009. *N. Engl. J. Med.* 360:2536–2543; Hayes, 2009. *Emerg. Infect. Dis.* 15:1347–1350; Kuno et al., 1998. *J. Virol.* 72:73–83. Gilbert et al., 2007. *PLoS ONE* 2: e537. Lanciotti et al., 2008; *Emerg. Infect. Dis.* 14:1232–1239; Messina et al., 2014. *Trends Microbiol.* 22:138–146. Elsevier Ltd; Mlakar et al., 2016. *N. Engl. J. Med.* 160210140106006; Rolland et al., 2012, Nature 490: 417–420; Smith et al., 2014, J Virol. Am Soc Microbiol 88: 9976–9990; Tofanelli et al., 1999. *Ancient Biomolecules*, V2, pp. 307-320; Wagner et

al., 2014, Science 345(6196): 570--573; Worobey et al., 2008. *Nature* 455, 661-664l; Worobey et al., 2014. *Proc Natl Acad Sci USA*. 104: 18566-18570; Worobey et al.,2016. *Nature* 539, 98-101; Xu et al., 2015. *Science* 348(6239).

[0051] Various modifications of the invention, in addition to those described herein, will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. Each reference cited in the present application is incorporated herein by reference in its entirety.

[0052] Although there has been shown and described the preferred embodiment of the present invention, it will be readily apparent to those skilled in the art that modifications may be made thereto which do not exceed the scope of the appended claims. Therefore, the scope of the invention is only to be limited by the following claims. Reference numbers recited in the claims are exemplary and for ease of review by the patent office only, and are not limiting in any way. In some embodiments, the figures presented in this patent application are drawn to scale, including the angles, ratios of dimensions, etc. In some embodiments, the figures are representative only and the claims are not limited by the dimensions of the figures. In some embodiments, descriptions of the inventions described herein using the phrase "comprising" includes embodiments that could be described as "consisting of", and as such the written description requirement for claiming one or more embodiments of the present invention using the phrase "consisting of" is met.

[0053] The reference numbers recited in the below claims are solely for ease of examination of this patent application, and are exemplary, and are not intended in any way to limit the scope of the claims to the particular features having the corresponding reference numbers in the drawings.

WHAT IS CLAIMED IS:

1. A method of detecting a target RNA in a sample, said method comprising:
    a. subjecting the sample to reverse transcription using reverse transcriptase and one primer from each of one or more pairs of primers, the pairs of primers are each adapted to amplify a fragment of the target RNA from 60 nt to 300 nt in length;
    b. subjecting the sample of (a) to polymerase chain reaction (PCR) amplification using Taq polymerase and the other primer of each of the one or more pairs of primers;
    c. subjecting a portion of the sample of (b) to PCR amplification using Taq polymerase and both of the primers from each of the one or more pairs of primers; and
    d. making detectable amplified product of (c), wherein detectable amplified product of (c) is indicative of the presence of the target RNA in the sample.
2. The method of claim 1, wherein the sample has no quantifiable RNA.
3. The method of claim 1 or claim 2, wherein the primers have the same annealing temperature or are within 5 degrees of an average annealing temperature.
4. The method of any of claims 1-3, wherein the fragment of the target RNA from 60 nt to 300 nt in length includes the primer.
5. The method of any of claims 1-4, wherein the Taq polymerase is proofreading Taq polymerase.
6. The method of any of claims 1-5, wherein the primers used in (a) are non-overlapping.
7. The method of any of claims 1-6, wherein the sample has low viral density.
8. The method of any of claims 1-7, wherein the sample comprises degraded nucleic acid.
9. The method of any of claims 1-8, wherein the sample comprises a low concentration of nucleic acid.
10. The method of any of claims 1-9, wherein the sample comprises a formalin-fixed paraffin-embedded (FFPE) sample.
11. The method of any of claims 1-9, wherein the sample comprises serum or plasma.

12. The method of any of claims 1-11, wherein the target RNA is a human immunodeficiency virus (HIV) RNA.
13. The method of any of claims 1-11, wherein the target RNA is a hepatitis C virus (HCV) RNA.
14. The method of any of claims 1-11, wherein the target RNA is a hepatitis B virus (HBV) RNA.
15. The method of any of claims 1-11, wherein the target RNA is an influenza virus RNA.
16. The method of any of claims 1-11, wherein the target RNA is an ebolavirus RNA.
17. The method of any of claims 1-16 further comprising subjecting the sample to DNAse prior to step (a).
18. A method of sequencing a target RNA in a sample, said method comprising:
    a. subjecting the sample to reverse transcription using reverse transcriptase and a first primer from each of two or more pairs of primers comprising the first primer and a second primer, the pairs of primers are each adapted to amplify a fragment of the target RNA from 60 nt to 600 nt in length, the first primers are non-overlapping with respect to each other, the second primers are non-overlapping with respect to each other;
    b. subjecting the sample of (a) to polymerase chain reaction (PCR) amplification using Taq polymerase and the second primers of each of the pairs of primers;
    c. subjecting a portion of the sample of (b) to PCR amplification using Taq polymerase and both of the primers from each of the pairs of primers; and
    d. sequencing amplified products of (c).
19. The method of claim 18, wherein the sample has no quantifiable RNA.
20. The method of claim 18 or 19, wherein the primers have the same annealing temperature or are within 5 degrees of an average annealing temperature.
21. The method of any of claims 18-20, wherein the fragment of the target RNA from 60 nt to 300 nt in length includes the primer.
22. The method of any of claims 18-21, wherein the Taq polymerase is proofreading Taq polymerase.

23. The method of any of claims 18-22, wherein the primers used in (a) are non-overlapping.
24. The method of any of claims 18-23, wherein the sample has low viral density.
25. The method of any of claims 18-24, wherein the sample comprises degraded nucleic acid.
26. The method of any of claims 18-25, wherein the sample comprises a low concentration of nucleic acid.
27. The method of any of claims 18-26, wherein the sample comprises a formalin-fixed paraffin-embedded (FFPE) sample.
28. The method of any of claims 18-26, wherein the sample comprises serum or plasma.
29. The method of any of claims 18-28, wherein the target RNA is a human immunodeficiency virus (HIV) RNA.
30. The method of any of claims 18-28, wherein the target RNA is a hepatitis C virus (HCV) RNA.
31. The method of any of claims 18-28, wherein the target RNA is a hepatitis B virus (HBV) RNA.
32. The method of any of claims 18-28, wherein the target RNA is an influenza virus RNA.
33. The method of any of claims 18-28, wherein the target RNA is an ebolavirus RNA.
34. A kit for detecting or sequencing a target RNA in a sample, said kit comprising two or more pairs of primers, the pairs comprising a first primer and a second primer, wherein the pairs of primers are each adapted to amplify a fragment of the target RNA from 60 nt to 600 nt, the first primers are non-overlapping with respect to each other, the second primers are non-overlapping with respect to each other.
35. The kit of claim 34 further comprising reverse transcriptase.
36. The kit of claim 34 or 35 further comprising Taq polymerase.
37. The kit of any of claims 34-36 further comprising 5 or more pairs of primers.
38. The kit of any of claims 34-36 further comprising 10 or more pairs of primers.
39. The kit of any of claims 34-36 further comprising 20 or more pairs of primers.

FIG. 1

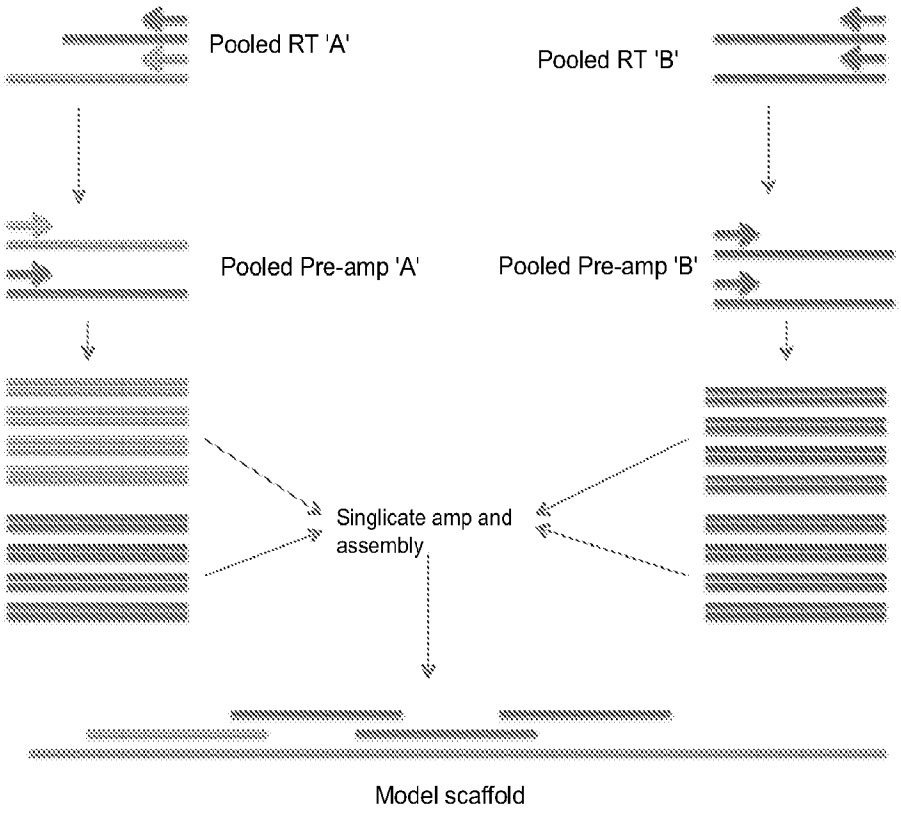
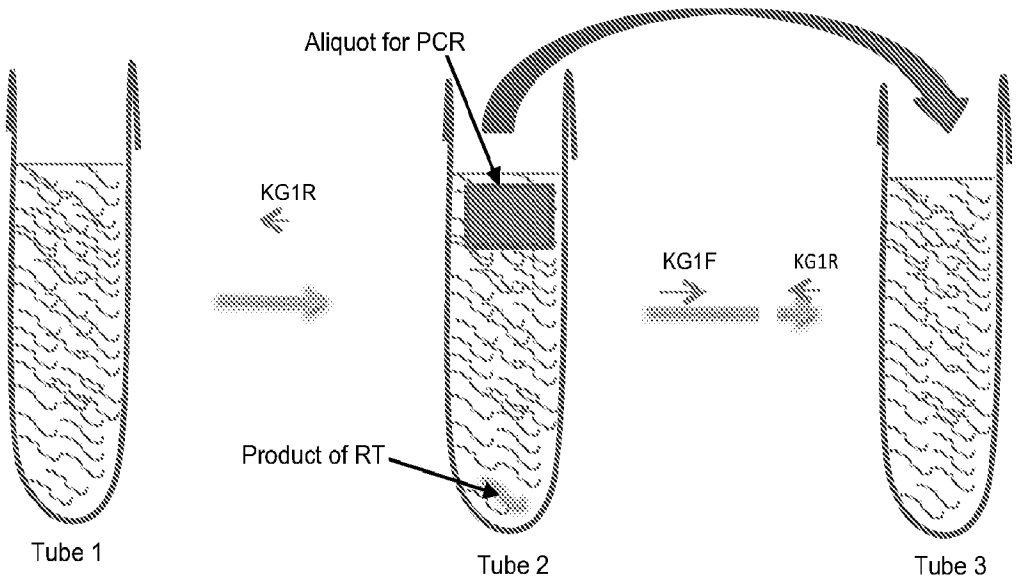
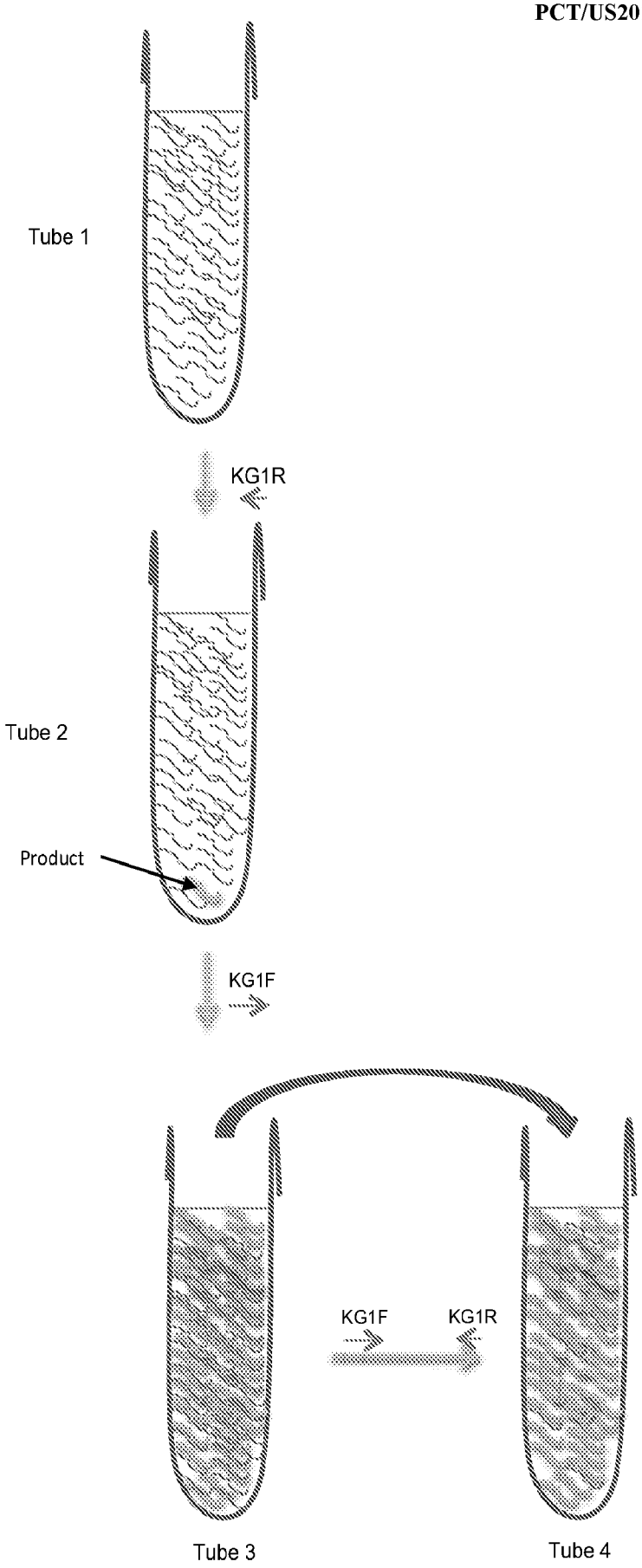


FIG. 2



Prior Art

FIG. 3



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/28591

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68; C12Q 1/70; C07H 21/04 (2017.01)
CPC - C12Q 1/6844; C12Q 1/686; C12Q 1/68; G01N 33/56983; C12Q 2533/101

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2014/0234830 A1 (EXNER et al.) 21 August 2014 (21.08.2014), Abstract, para [0002], [0004], [0005], [0006], [0015], [0017], [0042], [0045], [0049], [0050], [0052], [0053], [0088], [0091], [0103], and [0105]	1-3, 18-20
Y	US 2012/0141989 A1 (CHEN et al.) 07 June 2012 (07.06.2012), Abstract, para [0006], [0008], [0012], [0033], [0034], [0041], [0042], [0061], [0066], and Table 1	1-3, 18-20
Y	US 2011/0311971 A1 (HARTSHORN et al.) 22 December 2011 (22.12.2011), Abstract, para [0002], [0008], [0025], [0027], [0033], [0037], [0039], [0050], [0056], [0057], and [0073]	18-20
A	TANG et al., Quantitation of Taura syndrome virus by real-time RT-PCR with a TaqMan assay. J Virol Methods. 2004, Vol. 115(1), p. 109-14. Entire documentation, especially Abstract	1-3, 18-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 July 2017	21 SEP 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/28591

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

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

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

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

3. ☒ Claims Nos.: 4-17, 21-33, 37-39
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

Group I, Claims 1-3, 18-20, directed to a method of detecting a target RNA in a sample; or a method of sequencing a target RNA in a sample.

Group II, claims 34-36, directed to a kit.

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

**********Continued in the extra sheet**********

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3, 18-20

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/28591

Continuation of:
Box No III (unity of invention is lacking)

Special Technical Feature
Groups I-II are related as a product (Group II) and methods of potentially using the product (Group I).

Group I includes the special technical feature of a. subjecting the sample to reverse transcription using reverse transcriptase, b. subjecting the sample of (a) to polymerase chain reaction (PCR) amplification using Taq polymerase, not required by Group II.

Group II includes the special technical feature of a kit, not required by Group I.

Common Technical Features
The inventions of Groups I-II share the technical feature of two or more pairs of primers, the pairs comprising a first primer and a second primer, wherein the pairs of primers are each adapted to amplify a fragment of the target RNA from 60 nt to 600 nt, the first primers are non-overlapping with respect to each other, the second primers are non-overlapping with respect to each other, for detecting a target RNA in a sample (part of claim 34).

However, these shared technical features do not represent a contribution over prior art as being anticipated by US 2012/0141989 A1 to CHEN et al. (hereinafter 'Chen') as follows:

Chen discloses two or more pairs of primers (Abstract - 'detecting a target nucleic acid fragment comprising a magnetic bead; an inner primer pair and an outer primer pair'; para [0008] - 'Specific nucleic acid sequences in the target samples can be amplified by using four designated primers'),
--the pairs comprising a first primer and a second primer (para [0006] - 'molecular diagnosis based on polymerase chain reaction (PCR), RT-PCR... incorporated with specific primer sets for nucleic acid amplification', wherein each of 'specific primer sets' is 'the pairs comprising a first primer and a second primer'),
-- wherein the first primers are non-overlapping with respect to each other, the second primers are non-overlapping with respect to each other (para [0041] - 'the inner primer pair and an outer primer pair being specific to the amplification specific fragment', indicating 'wherein the first primers are non-overlapping with respect to each other, the second primers are non-overlapping with respect to each other'; para [0066]- 'performing a conventional RT-PCR process with the purified RNA samples and two sets of primer pairs including the NNV RNA1 and NNV RNA2 primer sets, which were designed from accession nos. AY721616 and AY721615, respectively, ...two sets of primers including the outer primer pair (NNV-F3/NNV-B3) and the inner primer pair (NNV-FIP/NNV-BIP) were also developed ... Details of the primer sets and the probe sequences can be found in Table 1'; Table 1, wherein non of the primers is overlaping with others),
--for detecting a target RNA in a sample (Abstract - 'detecting a target nucleic acid fragment'; para [0042] - 'target nucleic acid is a RNA fragment....carrying on a reverse transcription polymerase chain reaction before the purification and amplification'; para [0008] - 'Specific nucleic acid sequences in the target samples can be amplified by using four designated primers').
Although Chen does not expressively teach wherein the pairs of primers are each adapted to amplify a fragment of the target RNA from 60 nt to 600 nt, this limitation is encompassed by the teaching of Chen, because Chen discloses using the primer pairs suitable for nucleic acid amplification for detecting a target nucleic acid fragment about 200-500 bp, wherein the nucleic acid comprising RNA, which comprising the target RNA about 200-500 nt (Abstract - 'detecting a target nucleic acid fragment ... an inner primer pair and an outer primer pair suitable for ...amplification'; para [0042] - 'target nucleic acid is a RNA fragment'; para [0066]- 'performing a conventional RT-PCR process with the purified RNA samples and two sets of primer pairs'; para [0036] - 'specific fragment is from about 200 bp to about 500 bp', wherein '200 bp to about 500 bp' is for nucleic acid, which including double stranded, for RNA, it will be single stranded, and will be '200-500 nt'), which is under the condition in the scope of 'wherein the pairs of primers are each adapted to amplify a fragment of the target RNA from 60 nt to 600 nt'.

Without a shared special technical feature, the inventions lack unity with one another.

Groups I-II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Continuation of item 4: Claims 4-17, 21-33, 37-39 are not drafted in accordance with the second and third sentences of Rule 6.4 (a). These claims are improper multiple dependent claims.