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(54) Title: RESPIRATORY SYNCYTIAL VIRUS (RSV) VIRAL LOAD DETECTION ASSAY

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The invention concerns a method for the extraction of nucleic acids from biological samples e.g. tissue material or sputum derived from human or animal species and the quantitative detection thereafter of said nucleic acids e.g. in terms of viral load, more specifically RSV viral load detection.

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Respiratory Syncytial Virus (RSV) viral load detection assay

The invention relates to a method for the extraction of nucleic acids from biological samples e.g. tissue material or sputum derived from human or animal species and the quantitative detection thereafter of said nucleic acids e.g. in terms of viral load, more specifically RSV viral load detection.

Human respiratory syncytial virus (RSV) is a negative sense, single-stranded RNA virus of the family Paramyxoviridae, which includes common respiratory viruses such as those causing measles and mumps. RSV is a member of the paramyxovirus subfamily Pneumovirinae. The virion is variable in shape and size (average diameter of between 120 and 300 nm), is unstable in the environment (surviving only a few hours on environmental surfaces), and is readily inactivated with soap and water and disinfectants.

Clinical features: Respiratory syncytial virus (RSV) is the most common cause of bronchiolitis and pneumonia among infants and children under 1 year of age. Illness begins most frequently with fever, runny nose, cough, and sometimes wheezing. During their first RSV infection, between 25% and 40% of infants and young children have signs or symptoms of bronchiolitis or pneumonia, and 0.5% to 2% require hospitalization. Most children recover from illness in 8 to 15 days. The majority of children hospitalized for RSV infection are under 6 months of age. RSV also causes repeated infections throughout life, usually associated with moderate-to-severe cold-like symptoms; however, severe lower respiratory tract disease may occur at any age, especially among the elderly or among those with compromised cardiac, pulmonary, or immune systems.

Epidemiologic features: RSV is spread from respiratory secretions through close contact with infected persons or contact with contaminated surfaces or objects. Infection can occur when infectious material contacts mucous membranes of the eyes, mouth, or nose, and possibly through the inhalation of droplets generated by a sneeze or cough. In temperate climates, RSV infections usually occur during annual community outbreaks, often-lasting 4 to 6 months, during the late fall, winter, or early spring months. The timing and severity of outbreaks in a community vary from year to year. RSV spreads efficiently among children during the annual outbreaks, and most children will have serologic evidence of RSV infection by 2 years of age.

Diagnosis: Diagnosis of RSV infection can be made by virus isolation, detection of viral antigens, detection of viral RNA, demonstration of a rise in serum antibodies, or a combination of these approaches. Clinical laboratories use either antigen detection assays to diagnose infection or nucleic acid amplification techniques to detect viral nucleic acids.

To demonstrate antiviral activity of pharmaceutical compounds on the market or in development, a sensitive viral load test or assay is needed to measure any drop in RSV viral load upon treatment with the compound(s).

Viral load is a measure of the severity of a viral infection, and can be calculated by determining the amount of virus in an involved body fluid, for example, it can be given in nucleic acid copies per milliliter of blood. Determination of viral load is part of the therapy monitoring during chronic viral infections for instance in immunocompromised patients.

Thus, there is an ongoing need in the art for simple, fast and cost effective methods for detecting, identifying and quantifying RSV in biological samples in terms of viral load. Biological samples can be obtained from any organism but are prefereably from human or animal origin.

Preferably such a method must be able to process RNA derived from sputum and naso-pharyngeal wash (NPW). As a consequence thereof the detection assay, i.e. the RSV viral load detection assay, must include a sputum and NPW treatment method in preparation for RNA extraction from these samples. The assay must also be quantitative and able to distinguish between RSV-A and RSV-B in order not to miss a potential shift in RSV outbreak in a certain season.

The problem with sputum and NPW is that aliquots thereof are quite difficult to handle especially with regard to the extraction of nucleic acid material from said aliquots. Using the so-called EasyMAG® procedure (BioMérieux) for the extraction of nucleic acid material (such as RNA) from a sample, requires a more or less liquid solution for the extraction of nucleic acid from said solution. It is obvious that sputum, lung tissue but also NPW or cellular fractions containing RSV to be detected, do not qualify as a feasible liquid solution for further nucleic acid extraction thereof.

The solution is now found in a treatment method of samples, especially sputum and NPW, prior to the extraction of nucleic acid from said samples. After the extraction of nucleic acid from the samples, the nucleic acid is quantified e.g viral load detection either by an additional real-time Polymerase Chain Reaction (q-RT-PCR) or additional by the so-called multi component nucleic acid enzyme technology (MNAzyme).

The inventive method for treating samples, whereafter an extraction method is used for isolation and subsequent detection of nucleic acids (RNA and/or DNA), is not limited to RSV RNA containing samples but can be applied for the detection of any viral (RNA or DNA) or bacterial (DNA) respiratory pathogen such as but not limited to Influenza A and B, Para-influenza types 1, 2, 3 and/or 4, meta-pneumovirus, rhinovirus and adenovirus.

In accordance with the present invention a method for the extraction of nucleic acids from a biological sample has been found comprising the following steps:

a) obtaining a biological sample (e.g. tissue material from a human being or animal species,
b) adding phosphate buffered saline / 10% dithiothreitol to said tissue material of step a),
c) incubating under ultrasonic conditions,
d) adding lysis buffer,
e) obtaining lysed tissue material and
f) extracting said nucleic acid from said lysed tissue material.

Above mentioned step c) is preferably performed with a so-called Covaris S2 ultrasonic apparatus while the extraction step f) is preferably performed using the so-called EasyMAG procedure (BioMérieux).

Above mentioned step c) can alternatively be a mixing step using a Dyspomix apparatus specifically for (lung) tissue.

The nucleic acid can be either or both DNA or RNA from pathogens such as viruses or any other micro-organism while said tissue material can be either frozen sputum, naso-pharyngeal wash (NPW) or lung tissue wherein said virus is for instance human respiratory syncytial virus (RSV).

Said nucleic acid is further quantified by either the so-called quantitative real time Polymerase Chain Reaction technology (q-RT PCR) or by the so-called multi component nucleic acid enzyme technology (MNAzyme).

The combination of the above described extraction method of nucleic acid preferably from RSV containing patient samples with the further quantification by either q-RT-PCR or by the MNAzyme technology is also part of the present invention.

The invention further relates to a method for identifying, detecting or quantifying the presence of at least one target Respiratory Syncytial Virus (RSV) comprising the following steps:

(a) providing two or more oligonucleotide components wherein at least a first oligonucleotide component and at least a second oligonucleotide component are capable of self-assembly in the presence of said target to form a catalytically active multi-component nucleic acid enzyme (MNAzyme);
(b) contacting said oligonucleotide components with a sample putatively containing said at least one target under conditions:
(1) permitting the binding of said target to said oligonucleotide components and
(2) permitting the catalytic activity of the MNAzyme; and
(c) identifying, detecting or quantifying the presence of the catalytic activity of the MNAzyme, wherein the presence of the catalytic activity is indicative of the presence of said target.

Preferably the viral load of the target, RSV, is quantified by the above-mentioned method.

For a detailed explanation of q-RT-PCR technology specific reference is made to EP 543, 942 filed by F.Hoffmann-La Roche AG, Switzerland, while the MNAzyme technology is explained in great detail in patent application PCT/AU2006/001473 filed by Johnson & Johnson Research Pty Limited, Australia) and both documents are herewith incorporated by reference for sake of detailed explanation of both technologies.

Regarding the MNAzyme technology a general description of said technology is given hereafter.

MNAzymes (patent application PCT/AU2006/001473 by Johnson & Johnson Research Pty Limited, Australia) are novel catalytic nucleic acids based on DNAzymes. MNAzymes consist of two or more oligonucleotide sequences (e.g. partzymes) which, only in the presence of a MNAzyme assembly facilitator molecule (e.g. target which may be quantitatively amplified), form an active nucleic acid enzyme that is capable of catalytically modifying a substrate, for example a reporter substrate. An exemplary MNAzyme comprising partzyme A and partzyme B is depicted in Figure 2. With reference to Figure 2, DNA partzymes A and B each bind to a target, i.e. the MNAzyme assembly facilitator molecule (e.g., through Watson-Crick base pairing with a nucleic acid target). The MNAzyme only forms when the sensor arms of partzymes A and B hybridize adjacent to each other on the target. The substrate arms of the MNAzyme engage the reporter substrate, the cleavage of which is catalyzed by the catalytic core of the MNAzyme, formed by the interaction of the catalytic domains of partzymes A and B. The MNAzyme cleaves the substrate between a fluorophore and a quencher dye pair, thus generating signal. Cleavage of a DNA/RNA chimera (reporter substrate) is exemplified in the drawing. The term “MNAzyme” is also referred to as “multi-component nucleic acid enzyme”. An MNAzyme may also comprise a stabilizing oligonucleotide, which provides stability of the MNAzyme by interacting with an assembly facilitator or substrate. It is apparent that formation of an MNAzyme requires the assembly of at least the partzyme components with the target (or assembly facilitator), as well as the binding of a reporter substrate, for catalytic activity to be detectable, and that the absence of any of these components will result in a lack of catalytic activity.

The reporter substrate used with the MNAzymes can be labeled by any of a large variety of means including, for example, fluorophores (with or without one or more additional components, such as quenchers), radioactive labels, labeling with biotin (e.g. biotinylation) or chemiluminescent labels. Reporter substrates for catalytic nucleic acids may also include protein or nucleic acid enzymes, for example, covalently attached to their termini.

The reporter substrates used with the MNAzymes may be generic reporter substrate systems, which allow rapid assay development by allowing facile design changes to create new MNAzymes which recognize different targets.

The substrate arm portion and the catalytic core portion of the partzymes may remain unchanged, with changes only to the sensor arm portion of one or more partzymes required for new targets. Generic substrate sequences are provided and the same substrate can therefore be incorporated in assays for many different targets. Further, the same substrate can be incorporated into the methods in various embodiments herein, including assays where the substrate is free in solution or is tethered or attached to a support. A series of generic substrates can be used in a multiplex reaction allowing simultaneous detection of multiple targets. MNAzyme strategies using generic substrates offer a major advantage over technologies such as TaqMan® or Beacons which require the design and use of probes specific for each new target.

As described in more detail below, MNAzymes have an advantageous property in certain embodiments of being able to utilize a universal or generic reporter substrate. Such a substrate is shown in Figure 2 in a presently preferred configuration wherein the reporter substrate comprises both a detectable portion and a quencher portion. The quencher portion is adapted to diminish or eliminate a detectable signal from the detectable portion of the substrate until the MNAzyme cleaves the substrate. For example, the quencher portion may comprise “Black Hole Quencher 1” (BHQ1) or “Black Hole Quencher 2” (BHQ2). Thus, the MNAzyme cleaves the reporter substrate between the detectable portion and the quencher portion allowing the two portions to separate in solution, thereby allowing the detectable signal to appear or increase as the quencher portion is distanced from, or effectively removed from the local environment of the detectable portion.

The use of the generic or universal reporter substrate is enabled through the design of the MNAzyme’s component partzymes. By altering only the sensor arms of the partzymes, but by leaving the substrate arms unchanged, a large variety of MNAzymes specific for each of a plurality of targets can be designed all of which utilize a universal reporter substrate for detection. The skilled artisan will appreciate the advantages that this offers in terms of eliminating the need for customized or unique substrates for each target. Each new target requires only one or more changes in one or more of the sensor arm portions; the substrate arm portion and the catalytic core portion can remain constant. Thus, a single reporter substrate can be used for a single target using an MNAzyme, and multiple targets in a series of assays using altered MNAzymes. A plurality of reporter substrates allows multiplexing to detect multiple targets in

a single assay using multiple MNAzymes, one for each target. Such multiplexed methods of using MNAzymes are readily accomplished in solution or with attachment to a support system. It is contemplated herein that multiplexed assays can thus be accomplished in systems involving attaching one or more of the reporter substrate, or the MNAzyme partzymes or assembly facilitator, or additional enzyme activities, to a support as described herein.

Substrates can be modified by an MNAzyme thereby providing a detectable effect. In the detection process, the reporter substrate modification by an MNAzyme may involve, for example, cleavage, ligation, porphyrin metallation, and formation of carbon-carbon bonds, ester bonds or amide bonds. As a consequence of the reporter substrate modification by an MNAzyme, a detectable effect is generated and the magnitude of the effect may therefore be indicative of the quantity of the target sought to be measured. The detectable effect may be detected by a variety of methods, including fluorescence spectroscopy, surface plasmon resonance, mass spectroscopy, NMR, electron spin resonance, polarization fluorescence spectroscopy, circular dichroism, immunoassay, chromatography, radiometry, photometry, scintigraphy, electronic methods, UV, visible light or infra red spectroscopy, enzymatic methods or any combination thereof.

MNAzymes may contain one or more substitutions such as analogues, derivatives, modified or altered bases, ribonucleotides, alterations of the sugar or phosphate backbone, various deletions, insertions, substitutions, duplications or other modifications, or any combination of these, well known to those skilled in the art. Such modifications, substitutions, deletions, insertions, etc may be made in the sensor and/or substrate arms and/or in the catalytic core portions such that the molecule retains catalytic activity. Substitutions and modifications to arms that bind the substrate or assembly facilitator may be well tolerated and in fact are the basis of allowing tailoring of the molecules to different substrates/assembly facilitators. For example, modification of the sensor arms will allow tailoring to different assembly facilitators, while modification of the substrate arms will allow tailoring to different substrates.

The skilled artisan will appreciate that MNAzymes comprise either deoxyribonucleotides or ribonucleotides, or even both. Those MNAzymes comprising at least one and more preferably, all, deoxyribonucleotide component oligonucleotides are presently preferred. Also preferred are those

MNAzymes comprising at least one deoxyribonucleotide base, or its analogue, within the catalytic core of the MNAzyme. Even more preferred are those embodiments where such a base is required for catalytic activity.

A basic example of a MNAzyme structure is depicted in Figure 2. The structure shown comprises partzyme A and partzyme B which have base-paired with an MNAzyme assembly facilitator molecule, shown here simply as Target. Partzymes A and B by interacting with Target, have allowed the catalytic core to come into close proximity and thereby form. The substrate arms of the MNAzyme have interacted with and base-paired with a substrate, here Reporter Substrate. Thus the MNAzyme has self-assembled and this process is facilitated through the presence of the MNAzyme assembly facilitator molecule Target. In the absence of Target, no MNAzyme will form. Modification (in this case, cleavage) of the substrate is catalyzed by the catalytic core of the MNAzyme at the MNAzyme Cleavage Site within the substrate denoted by the vertical arrow. The substrate in this particular embodiment of the invention comprises a detectable portion having a detectable signal, for example fluorophore F, and a quencher portion having a quenching effect on the detectable signal F through the action of quencher Q. Upon cleavage at the MNAzyme Cleavage Site, there is a substantial increase in detectable signal, here fluorescence, which is readily detected or quantified.

More specifically, the partzyme A and partzyme B, shown in Figure 2, each comprise a substrate arm portion, a catalytic core portion, and a sensor arm portion. In the presence of a target, the sensor arm portions of partzyme A and partzyme B can begin to hybridize to, and base pair with complementary portions of the target, for example a DNA or RNA sequence. Upon contacting the target in this fashion, the MNAzyme self-assembles forming a catalytic core, which can modify a substrate which is bound by the substrate arms. Preferably the presence of the MNAzyme is detected through the detection or measurement of its catalytic activity. The substrate arms of the thus assembled MNAzyme can engage a substrate, for example the reporter substrate shown in Figure 2, through the interaction of the complementary sequences on the substrate arms and the substrate. Once the substrate is so engaged with the substrate arms, the catalytic core can promote the modification (eg. cleavage) of the substrate, which can in turn be measured or detected, directly or indirectly.

Multiple MNAzymes are useful in the present invention as they allow detection of related sequences differing by as little as a single nucleotide. Similarly, a unique reporter substrate is required to detect each of the several targets. In some cases, to multiplex the method requires the use of a distinct or unique detectable signal for each reporter substrate to facilitate the design of the method.

The target nucleic acid can be amplified in accordance with a procedure for amplifying that nucleic acid (i.e. DNA or RNA). Preferably, standard methods of *in vitro* amplification are used. The amplicons generated during the amplification serve as targets for an MNAzyme, thus MNAzyme activity is indicative of the presence of the target. The skilled artisan will appreciate that such monitoring can be conducted in a single vessel under conditions that permit both the amplification and the MNAzyme assembly and catalytic activity, or the MNAzyme assay can be conducted subsequent to, or at time points throughout the amplification, by removing samples at the end or during the course of the amplification reactions.

The method for detecting the presence of at least one target or assembly facilitator may further comprise providing at least a third and fourth oligonucleotide component, wherein said at least third and at least fourth oligonucleotide component are capable of self assembling in the presence of at least one additional target or assembly facilitator to form at least one additional catalytically active MNAzyme, and wherein at least one additional reporter substrate is present in the sample, said additional reporter substrate is capable of being modified only by the additional MNAzyme, wherein said modification provides said additional detectable effect.

Standard curves of threshold cycle (Ct) values over time for the target(s) are obtained and plotted onto the standard curves, generally obtained from a control or housekeeping gene, and the proportion of a given target in the viral population or in a given environment is visualized and calculated.

The protocols and products of the present invention may be used for diverse diagnostic, clinical, toxicological, research and forensic purposes including, drug discovery, designing patient therapy, drug efficacy testing, and patient management. The present methods may be used in combination with other assays. The results may be implemented in computer models and databases.

Additionally, the protocols and products of the present invention also allow monitoring of the effect of anti-RSV compounds on viral load.

Definitions

The terms “assembly facilitator molecule”, “assembly facilitator”, “MNAzyme assembly facilitator molecule”, “facilitator” and “MNAzyme assembly facilitator” as used herein refer to entities that can facilitate the self-assembly of component partzymes to form a catalytically active MNAzyme. In preferred embodiments an assembly facilitator is required for the self-assembly of an MNAzyme. An assembly facilitator in some embodiments comprises a target such as a nucleic acid or non-nucleic acid analyte. Assembly facilitator molecules may comprise one or more regions or molecules that may pair with, or bind to, one or more oligonucleotide “partzymes,” which constitute components or portions of an “MNAzyme”. It is not required that the assembly facilitator interact with, pair with, or bind to each component partzyme or oligonucleotide provided that it interacts with, pairs with, or binds to, at least one of the component partzymes of an MNAzyme. As used herein, MNAzyme assembly facilitator molecules are intended to encompass the broadest range of constituents, which can facilitate self-assembly of an MNAzyme. In some embodiments, an assembly facilitator may comprise a nucleic acid. In other embodiments, an assembly facilitator may comprise any cell or any portion thereof, for example, any eukaryotic or prokaryotic cell, a virus, prion, yeast or fungus, or any other molecule, for example, including but not limited to a protein, polypeptide, peptide or nucleic acid. In other embodiments, an assembly facilitator may comprise a virus, prion, yeast or fungus, or any other molecule, for example, including but not limited to glycoproteins, lipids, lipoproteins, entire organisms, cells, viruses, bacteria, archaea, fungi, antibodies, metabolites, pathogens, toxins, contaminants, poisons, small molecules, polymers, metal ions, metal salts, prions or any derivatives, portions or combinations thereof.

The term “target” as used herein includes any natural or synthetic entity, constituent or analyte, which is sought to be detected, identified or quantified by a particular MNAzyme(s). Targets therefore encompass the broadest range of detectable entities, constituents or analytes for which methods of sensitive detection, identification and/or quantification are desirable. In some embodiments, a target comprises an assembly facilitator. Some exemplary

targets include, but are not limited to, protein, polypeptide, peptide or nucleic acid, glycoproteins, lipids, lipoproteins, entire organisms, cells, viruses, bacteria, archaea, yeast, fungi, antibodies, metabolites, pathogens, toxins, contaminants, poisons, small molecules, polymers, metal ions, metal salts, prions or any derivatives, portions or combinations thereof. Other targets are also contemplated for use herein.

The terms “substrate”, “substrate molecule” and “chemical substrate” as used herein include any molecule which is capable of being recognized, and acted upon or chemically modified by a catalytic molecule. In particular embodiments, a substrate may be recognized and modified by an enzyme. In other embodiments, a substrate may be recognized and modified by a catalytic nucleic acid molecule. The chemical modification of a substrate can be measured by the appearance of, or increase in, a product of the modification reaction, or by the disappearance of, or decrease in, a substrate of the modification reaction(s). A particular catalytic molecule may recognize one or more different substrate molecules provided each substrate molecule has at least a minimum structure, which is recognizable for catalytic activity by the catalytic molecule.

A “reporter substrate”, “reporter probe” or “reporter probe substrate” as used herein is a substrate that is particularly adapted to facilitate measurement of either the disappearance of a substrate or the appearance of a product in connection with a catalysed reaction. Reporter substrates can be free in solution or bound (or “tethered”), for example, to a surface, or to another molecule. A reporter substrate can be labelled by any of a large variety of means including, for example, fluorophores (with or without one or more additional components, such as quenchers), radioactive labels, labelling with biotin (e.g. biotinylation) or chemiluminescent labels. Reporter substrates for catalytic nucleic acids may also include protein or nucleic acid enzymes, for example, covalently attached to their termini.

As used herein, the terms “partzyme”, “component partzyme” and “component oligonucleotide” refer to a DNA-containing or RNA-containing or DNA-RNA-containing oligonucleotide, two or more of which, only in the presence of a MNAzyme assembly facilitator molecule, can together form an “MNAzyme.” In certain preferred embodiments, one or more component partzymes, and preferably at least two, may comprise three regions or domains: a “catalytic”

domain, which forms part of the MNAzyme's catalytic core that catalyzes a chemical modification; a "sensor arm" domain, which may associate with and/or bind to an assembly facilitator (e.g. a target); and a "substrate arm" domain, which may associate with and/or bind to a substrate. A depiction of these regions or domains can be seen, for example, in Figure 2. A partzyme may comprise one or more molecules.

Abbreviations

MNAzyme : multi-component nucleic acid enzyme, or multipartite nucleic acid enzyme;

DNAzyme : deoxyribonucleic acid enzyme;

RNAzyme : ribonucleic acid enzyme, or ribozyme;

PCR : polymerase chain reaction;

dH_2O : deionized distilled water;

F : fluorophore;

Q : quencher;

JOE or 6-JOE : 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein;

FAM or 6-FAM : 6-Carboxyfluorescein;

BHQ1 : Black Hole Quencher 1;

BHQ2 : Black Hole Quencher 2.

Figures

Figure 1 is a diagnostic workflow for RSV viral load determination.

Figure 2 is a depiction of an exemplary design for an MNAzyme, wherein substrate arm portions (A) of partzymes A and B bind to a Reporter substrate, to which is attached a fluorescent tag (left) and a quencher (right). Catalytic core portions (C) are located between substrate arm portions (A) and sensor arm portions (B). Upon binding of sensor arm portions (B) to a Target, the Reporter substrate is cleaved at the MNAzyme Cleavage Site, thereby increasing fluorescence.

Figure 3: Schematic representation of an exemplary multiplex analysis of multiple targets: Two or more targets can be simultaneously detected using two or more substrates, each specific for one MNAzyme. Substrates are preferably labeled with different fluorophores. In this example, Target 1 can be detected by monitoring the increase in FAM fluorescence and Target 2 can be

detected by monitoring the increase in JOE fluorescence. Q: quencher; FAM, JOE: fluorophores.

Figure 4: Schematic representation of the External Quantification Control sequences cloned in the TOPO-TA vector (Invitrogen, Merelbeke): EQC-A (A) and EQC-B (B). The vectors indicate the T7 promotor and the flanking primers used to generate the amplicon that was cloned. Also shown are the two primers used during the q-RT-PCR assay in each vector as well as the location of the N*de*I restriction site used to linearize the vectors before transcription.

Figure 5a/b: Schematic representation of the internal extraction control sequences. The q-RT-PCR amplification uses an RSV-A and RSV-B conserved and specific sequence in the RSV genome. These sequences are located in the F-gene for RSV-A (1) and the N-gene for RSV-B (3). The primers to amplify the RSV RNA or the RSV internal extraction control are identical per sub-strain: The sequences in between the primers are probe-specific: RSV-A RNA = RSVA-Fgen-TP probe (1), RSV-A internal extraction control = RSVA-EC-Fgen-TP probe (2); RSV-B RNA = RSVB-Ngen-TP probe (3), RSV-B internal extraction control = RSVB-EC-Ngen-TP probe (4).

Figure 6: Schematic representation of the Internal Extraction Control (IEC) sequences cloned in the TOPO-TA vector (Invitrogen, Merelbeke): pIEC-A (A) and pIEC-B (B). The vectors indicate the T7 promotor, the two primers and the probe location for each vector as well as the location of the Apa*L*I restriction sites used to linearize the vectors before transcription.

The following non-limiting examples help to illustrate the principles of the invention.

Examples

Example 1

Sputum and NPW was received from various sources (REGA, Leuven, Belgium; O.L.V. Ziekenhuis, Aalst, Belgium). Sputa and NPW samples used in these experiments when not infected with RSV were spiked with an RSV virus stock (RSV-A: RSV-GFP 011828) whenever applicable.

Additionally a Dyspomix (Wilten Instruments, Cat nr 900.021.00, Berchem, Belgium), an AFA (Covaris, Hoddesdon, UK) and an ABI9700HT (Applied Biosystems, CA, USA) were used during the course of these experiments.

Sample processing in preparation of automated RNA extraction

A method was developed for the preparation of different RSV samples for automated RNA extraction (see Figure 1 for the schematic diagnostic workflow).

Different kinds of processing could occur with different kinds of samples (as described in Table 1).

Table 1. Overview of different sample types and different preparation methods. NPW: Nasopharyngeal Wash; BAL: Bronchoalveolar Lavage

	Sputum (human)	NPW (human)	Bal (mouse) fluid	Virus Culture (Supernatant)	Virus Culture (Cell fraction)	Lung tissue (rat)
Covaris S2	Yes	Yes	Yes	Yes	Yes	No
Dyspomix	Yes	Yes	Yes	Yes	Yes	Yes
EasyMAG	No	Yes	Yes	Yes	No	No

Samples were stored in-house at -80°C. The weight of the frozen samples was determined while still frozen, 1 volume of PBS/10% DTT was added to the samples with a minimal final volume of 2,2 ml (in Becton Dickinson 14 ml polypropylene round-bottom tube, ref number 352059 (25 per bag, sterile)). Samples with an estimated initial volume larger than 1,5 ml were first transferred to a 50ml tube before addition of one volume PBS/10% DTT by slightly warming the 15 ml tube until the frozen sample could be slid into the 50 ml Falcon tube (Red cap). The frozen sample/PBS-10% DTT was placed in the Covaris S2 instrument and processed with SonoLAB Single v2.4.3 with the following settings:

Mode = Power Tracking, Number of Cycles = 10, Bath Temperature Limit = 15°C.

Treatment 1: “Duty Cycle” = 20%, “Intensity” = 10 and “Cycles/Burst” = 100 for 30s.

Treatment 2: “Duty Cycle” = 0.1%, “Intensity” = 0.1 and “Cycles/Burst” = 50 for 10s.

Mostly one (1) cycle was sufficient to dissolve the sample, if not, extra cycles were added as needed.

The cooling of the Covaris was set at 1°C.

After Covaris treatment, 4 initial sample volumes of Lysis buffer (EasyMAG, BioMérieux) were added and incubated for 10 minutes.

The Covaris S2 instrument as described above can be found on the website of Covaris viz www.covarisinc.com . In summary the Covaris process produces a controlled acoustic field inside a sealed vessel. The process is based on a computer-controlled, focused acoustic energy technology. The Covaris Adaptive Focused Acoustics (AFA) process works by sending acoustic energy wave packets from a dish-shaped transducer that converges and focuses to a small-localized area (it is sometimes visualized as a vibrating loudspeaker cone). At this focal point, the energy density may be controllably focused into the sample of interest which has proven to be beneficial to numerous applications of sample preparation. Essentially, the process enables mechanical energy to be applied to a sample without directly contacting the sample.

An advanced Covaris instrument comprises a so-called intensifier which is attached to the transducer producing the waves. Said waves starting from the centre of the concave intensifier are reflected by the cone and the obtained vertical waves produces an extra heating at the bottom of the vessel.

Automated RNA extraction

The RNA was extracted using the EasyMAG platform (BioMérieux) according to the instructions provided by the supplier with the following adaptation: 3ml of the lysed sample volume was used (No lysis dispensing on the EasyMAG), per 8 samples to be extracted: 110 µl IEC-B (internal extraction control for RSV-B, stored in -80°C) was mixed with 440µl EasyMAG Buffer 3 and 550µl magnetic beads. A total of 125µl of this mix was dispensed in separate wells using the pre-programmed settings number 2 of the EasyMAG dispensing pipet. Using the pre-programmed settings number 3 of the EasyMAG dispensing pipet 100µl of this mix was added to each sample. Elution occurred in 110µl (Buffer 3, EasyMAG). As soon as the extraction was completed, the q-RT-PCR technology or MNAzyme technology was performed as described in Example 2 (A) and (B) respectively. The remainder of the RNA was stored at –80 °C.

Two possibilities were used for quantification, either q-RT PCR using Taqman technology (q-RT PCR technology and Taqman probes) or the MNAzyme technology.

For a detailed explanation of q-RT-PCR technology specific reference is made to EP 543, 942 filed by F.Hoffmann-La Roche AG, Switzerland, while the MNAzyme technology is explained in great detail in patent application PCT/AU2006/001473 filed by Johnson & Johnson Research Pty Limited, Australia) and both are herewith incorporated by reference for detailed explanation of both technologies.

Example 2

(A) Use of q-RT PCR technology and Taqman probes for the quantification of RSV-A and RSV-B viral strains and a control nucleic acid sequences via monoplex real time PCR

Probes and Primers:

The primers for the RSV-A q-RT-PCR amplification (and hence also pIEC-A) were:

Oligonucleotide	Sequence	
RSV-A-Fgen-FW	5'-CTGTGATAGA RTTCCAACAA AAGAACA-3'	SeqID No. 1
RSV-A-Fgen-RV	5'-AGTTACACCT GCATTAACAC TAAATTCC-3'	SeqID No. 2

The primers for the RSV-B q-RT-PCR amplification (and hence also pIEC-B) were:

Oligonucleotide	Sequence	
RSV-B-Ngen-FW	5'-GGCTCCAGAA TATAGGCATG ATTC-3'	SeqID No. 3
RSV-B-Ngen-RV	5'-TGGTTATTAC AAGAGCAGCT ATACACAGT-3'	SeqID No. 4

The probe for RSV-A was:

Probe	Sequence	
RSV-A-Fgen-TP	FAM 5'-CAGACTACTAGAGATTACC-3' NFQ-MGB*	SeqID No. 5

*NFQ-MGB: Non-Fluorescent Quencher – Minor Groove Binding Sequence

The probe for RSV-B was:

Probe	Sequence	
RSV-B-Ngen-TP	FAM 5'-TATCATCCCACAGTCTG-3' NFQ-MGB	SeqID No. 6

The probe for pIEC-A was:

Probe	Sequence	
RSV-A-EC-Fgen-TP	FAM 5'-AATGACCAATCCATACGCA-3' NFQ-MGB	SeqID No. 7

The probe for pIEC-B was:

Probe	Sequence	
RSV-B-EC-Ngen-TP	FAM 5'-TACCGTACTCTAGCCTA-3' NFQ-MGB	SeqID No. 8

For each sample, three q-RT-PCR mixes were prepared in duplicate:

RSV-A, RSV-B and IEC

Samples	**1**				
Reaction Vol. (µl)	**30**				
Mix item	**Concentration**			**Volume for (µl)**	
	Unit	**Stock**	**Final**	**1 sample**	**X samples**
Rnase free water				0.77	2.31
Yeast tRNA	ng/ml	10000	120	0.360	1.08
2x Reaction buffer	X	2.00	1.000	15.000	45.00
Euroscript RT	kU/ml	50.00	0.250	0.150	0.45
Probe RSV	µM	25.00	0.100	0.120	0.36
RSV primer FW	µM	20.00	0.900	1.350	4.05
RSV primer RV	µM	20.00	0.900	1.350	4.05
$MgCl_2$	mM	50.00	1.500	0.900	2.70
	Total Volume Mix (µl)				60.00

Volume Mix / Tube (µl)	20.00
Total RNA RSV-A	10.00

When all components were added to the plate, the plate was sealed with an Optical Adhesive Cover (ABI) and centrifuged for 1 minute at 1500rpm. Before

processing on the ABI7900HT, the plate was covered with a MicroAmp™ Snap-On Optical Film Compression Pad (ABI).

Thermal Profile was:

"Stage 1" reverse transcriptase reaction: 48°C, 30 minutes

"Stage 2" activation of polymerase: 95°C, 10 minutes

"Stage 3": 45 repeats:

95°C denaturation, 15 seconds

60°C elongation, 1 minute

Construction of the external quantification control (EQC) for the RSV q-RT PCR assay

In order to define the dynamic range of the q-RT-PCR assay, external quantification controls were constructed. This included:

- Design of the following plasmid constructs containing:
  - The sequence situated between the forward (EQC-RSV-A-FW) and reverse primer (EQC-RSV-A-RV) covering a region of RSV-A of 1004 base pairs including the region where the RSV-A q-RT-PCR assay primers and probes anneal. This construct is labeled as external quantification control RSV-A (pEQC-A).
  - The sequence situated between the forward (EQC-RSV-B-FW) and reverse primer (EQC-RSV-B-RV) covering a region of RSV-A of 1399 base pairs including the region where the RSV-B q-RT-PCR assay primers and probes anneal. This construct is labeled as external quantification control RSV-B (pEQC-B).

Primers and probes

The primers ordered the pEQC-A (EQC-RSV-A-FW, EQC-RSV-A-RV and EQC-RSV-A-RVret) and pEQC-B (EQC-RSV-B-FW, EQC-RSV-B-RV and EQC-RSV-B-RVret) constructs are described hereunder.

Oligonucleotide			
EQC-RSV-A-FW	5'-AATCAAAATAAACTCTGGGGC-3'	bp 5631-5651*	SeqID No. 9
EQC-RSV-A-RV	5'-GTTGGTTGTACATAGAGGGG-3'	bp 6634-6615*	SeqID No. 10
EQC-RSV-A-RVret	5'-TACATGTTTCAGCTTGTGGG-3'	bp 6734-6715*	SeqID No. 11
EQC-RSV-B-FW	5'-ATATTTATCAATCATGGCGGG-3'	bp 908-928**	SeqID No. 12

Oligonucleotide			
EQC-RSV-B-RV	5'-CTACATCATCTTCTTTGGGG-3'	bp 2306-2286**	SeqID No. 13
EQC-RSV-B-RVret	5'-GTGCCAGATGTTATCGGGC-3'	bp 2524-2507**	SeqID No. 14

* Positions are base on the Long strain (AY911262, RSV-A)

** Positions are based on AY353550 (RSV-B)

RSV samples

For this experiment an aliquot of RSV-A-GFP (GST011828) and RSV-B-P3-Hep2 (REGA) virus stock were used.

RNA extraction and amplification of desired RSV genome fragments

An RSV-A-GFP (GST011828) and RSV-B-P3-Hep2 (REGA) virus stock was extracted on the EasyMAG: 1000 µl input, 55 µl output. The RNA was subsequently submitted to an RT-hemi-nested PCR amplification. The PCR mixes and conditions are specified hereunder.

Overview of the different (RT) PCR amplification mixes and conditions to generate the hemi-nested amplification products for RSV-A (1004 bp) and RSV-B (1399 bp). The exact primer sequences are mentioned above.

Mix preparation PCR

RT-PCR

# Samples	1

Reaction Vol. (µl)	35				
Mix Item	Concentration			Volume for (µl)	
	Unit	Stock	Final	1 sample	x samples
Rnase Free water				6.10	12.20
2x Reaction buffer HiFi	X	2.00	1.000	17.50	35.00
Primer FW	µM	20.00	0.200	0.35	0.70
Primer RVret	µM	20.00	0.200	0.35	0.70
SuperscriptTM III HiFi	U/µl	100.00	2.000	0.70	1.40
	Total Volume Mix (µl)			25.00	50.00

Total Volume RNA (µl)	10.00

thermal cycling

step	temp. (°C)	time	cycles
1	53	30'	
2	94	2'	
3	92	15 "	40
4	55	30 "	
5	68	1'30 "	
6	68	7'	
7	4	hold	

Heminested

# Samples	1

Reaction Vol. (µl)	50				
Mix Item	Concentration			Volume for (µl)	
	Unit	Stock	Final	1 sample	x samples
Rnase Free water				39.17	78.34
PCR Buffer (15mM $MgCl_2$)	X	10.00	1.000	5.00	10.00
dNTPs	mM	25.00	0.200	0.40	0.80
Primer FW	µM	20.00	0.200	0.50	1.00
Primer RV	µM	20.00	0.200	0.50	1.00
Expand™HF PCR	U/µl	3.50	0.030	0.43	0.86
	Total Volume Mix (µl)			46.00	92.00

Total Volume RT-PCR mix (µl)	4.00

thermal cycling

step	temp. (°C)	time	cycles
1	94	2 min	
2	94	15 "	35
3	60	30 "	
4	68	1'	
5	68	7'	
6	4	hold	

TOPO-TA cloning

The obtained PCR fragments were cloned using the TOPO TA cloning kit (Invitrogen, Merelbeke, Belgium) following the manufacturer's instructions. Briefly, 4µl PCR product (unpurified) was mixed with 1 µl salt solution and 1 µl vector.

The ligation reaction was transformed into TOP10 chemically competent E.*coli* cells following the manufacturer's instructions.
After growing the E.*coli* cultures overnight, 10 colonies per construct (pEQC-A and pEQC-B) were transferred to liquid LB/Ampicillin medium, grown overnight and a miniprep (Qiagen, Hilden, Germany) was performed. Final volume = 50 µl, concentration not measured.

Sequencing

A total of 0,75µl of the generated DNA was submitted to sequencing. Sequencing was done using the Big Dye Terminator (BDT) Cycle Sequencing Kit v3.1 (Cat n°. 4337457, Applied Biosystems, CA, USA).
The mix composition of each well is indicated in below table. The sequencing reactions were performed using the primer set
"T3" (5'-ATTAACCCTCACTAAAGGGA-3') (SeqID No. 15) and
"T7" (5'-TAATACGACTCACTATAGGG-3') (SeqID No. 16) which are located on the TOPO-TA vector.
The sequencing PCR was done using the 9800 Fast Thermal Cycler (Applied Biosystems (ABI), Cat. nr. 4356204, CA, USA). The PCR conditions were 96°C for 5 seconds, 50°C for 5 seconds, 60°C for 1 minute and 15 seconds and this for 25 cycles in total followed by a final hold at 12°C.

Table :The mix composition of a single sequencing reaction, with a final volume of 5.75µl. (* T7 or T3 sequencing primer was used). "BDT" = Big Dye Terminator, "DB" = Dilution Buffer

Sequencing Mix preparation (µl):

	1 well	x wells **20**	
BDT	0.25	5.25	µl
DB	1.25	26.25	µl
Water	1.50	31.50	µl
Primer*	2.00	42.00	µl
tot mix	**5.00**	105.00	µl
amplicon	0.75		

Speed PCR program:

96°C	5"	**# cycli**
50°C	5"	**25**
60°C	1'15"	
12°C	hold	

In Vitro RNA transcription

One correct clone of each EQC construct (Clone 6 for EQC-A and Clone 3 for EQC-B) was linearized using the restriction enzyme N*de*I (Fig. 4 A & B).

Restriction enzyme digestion proceeded at 37°C for 3 hours using restriction enzyme buffer 4. The reactions were stopped by incubation at 65°C (20 minutes) and purified. To the restriction digest mix, 1/20 volumes of 0.5M EDTA (1µl), 1/10 volumes of NaOAC (2µl) and 2 volumes of EtOH (40µl) were added. The mixture was placed at -20°C for minimum 15 minutes, and then spun for 15 minutes at maximum speed. The supernatant was discarded and the pellet was dissolved in 50µl TE buffer. The linearized constructs were then transcribed using the MEGAshortscript™ T7 Kit (Ambion, Cat. nr. 1354, Texas, USA). The reaction mixtures were incubated at 37°C for 4 hours. The total length of the transcribed RNA was predicted to be 1250 (EQC-A) and 1600 (EQC-B) nucleotides. Multiple parallel reactions were set up for each EQC.

DNA removal

The transcription mixtures were treated with TURBO DNase™ (2 µl added to transcription mix, Ambion, Cat. Nr. 2238, Texas, USA). The DNase treatment lasted for 1 hour at 37°C in a final volume of 100µl. The RNA was purified by means of an RNA extraction on the EasyMAG (BioMérieux) eluting in 100µl. The purified eluates were pooled resulting in a final volume of about 1 ml per EQC.

Real-time EQC quantification

The purified transcripts were submitted to a real-time PCR quantification either in the presence or the absence of the RT enzyme. The mix compositions of the various mixes can be found hereafter:

EQC-A

# Samples	1				

Reaction Vol. (µl)	30				
Mix item	**Concentration**			**Volume for (µl)**	
	Unit	**Stock**	**Final**	**1 sample**	**X samples**
Rnase free water				1.670	5.01
Yeast tRNA	ng/ml	10000	120	0.360	1.08
2x Reaction buffer	X	2.00	1.000	15.000	45.00
Euroscript RT	kU/ml	50.00	0.250	0.150	0.45
Probe RSV-A	µM	25.00	0.100	0.120	0.36
RSV-A primer FW	µM	20.00	0.900	1.350	4.05
RSV-A primer RV	µM	20.00	0.900	1.350	4.05
	Total Volume Mix (µl)				60.00

Volume Mix / Tube (µl)	20.00
Total RNA	10.00

EQC-B

# Samples	1				

Reaction Vol. (µl)	30				
Mix item	**Concentration**			**Volume for (µl)**	
	Unit	**Stock**	**Final**	**1 sample**	**X samples**
Rnase free water				1.670	5.01
Yeast tRNA	ng/ml	10000	120	0.360	1.08
2x Reaction buffer	X	2.00	1.000	15.000	45.00
Euroscript RT	kU/ml	50.00	0.250	0.150	0.45
Probe RSV-B	µM	25.00	0.100	0.120	0.36
RSV-B primer FW	µM	20.00	0.900	1.350	4.05
RSV-B primer RV	µM	20.00	0.900	1.350	4.05
	Total Volume Mix (µl)				60.00

Volume Mix / Tube (µl)	20.00
Total RNA	10.00

The program used on the ABI9700 HT real-time PCR equipment was 48°C for 30 minutes, 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds

and 60°C for 1 minute. For all experiments only FAM-labeled probes were used (Monoplex assays).

Reproducibility of EQC (frozen aliquots)

From the EQC RNA pools (evaluated for residual DNA content), dilution series were made over 8 logs (8 aliquots, diluted 1/10) with a total volume of 2500µl. From these stocks aliquots were taken of 23µl each and stored at -80°C until processing occurred.

Sample processing, an example

In total 5 clinical samples were processed (Flow chart,see Figure 1). All samples were transferred to a 15ml falcon tube and PBS/1% DTT was added to a final volume of 2,2 ml. This mix was processed on the AFA (Covaris) for 60 seconds prior to the addition of 4 ml lysis buffer (EasyMAG, BioMérieux). From these 6.2 ml per sample, 3 ml was processed on the EasyMAG (BioMérieux) and the RNA was eluted in 110µl of which 10µl was processed in the q-RT-PCR in duplicate. The experiment was repeated once starting from extraction with the remaining 3 ml.

<u>Results</u>

PCR

The RT-Heminested PCR reactions were set up in duplo and both generated good results for each amplicon. The expected size for the RSV-A amplicon was 1004bp and for RSV-B 1399bp. These PCR amplified fragments were ligated in a TOPO-TA vector and transformed into competent E.*coli*. The transformants were grown overnight on solid LB/ampicillin plates. A total of 10 colonies were transferred to liquid LB/Ampicillin medium and again grown overnight. A miniprep (Qiagen, Hilden, Germany) was prepared from these cultures and the resulting DNA was submitted to sequencing.

Sequencing

Not all PCR fragments ligated in the same direction in the TOPO-TA vector. A total of 2 clones for pEQC-A and 6 clones for pEQC-B were cloned in the correct direction into the TopoTA vector. Of these clones, Clone 6 (pEQC-A) and Clone 3 (pEQC-B) were chosen for the in vitro transcription.

In vitro transcription

Clone 6 (pEQC-A) and Clone 3 (pEQC-B) were submitted to a restriction digest using N*de*I and purified as described above. The linearized and purified constructs were submitted to an *in vitro* transcription (using the MEGAshortscript™ T7 Kit (Ambion)). The residual DNA was removed using the TURBO DNase™ (Ambion). The transcripts were purified by means of an RNA extraction on the EasyMAG (BioMérieux) as described above.

Real-time PCR results

The pure transcripts were diluted 1/1000 and submitted to a real-time PCR quantification reaction with (dark blue signal) and without (light blue signal) RT enzyme to determine the residual DNA content of the transcripts. The 4-hour transcript of pEQC-A showed a difference of 12 Cts between the reaction performed without RT and the reaction performed in the presence of RT.

The residual DNA in the RSV-B transcripts was comparable to that of pEQC-A with about 10 Cts difference between the reactions performed in the presence or the absence of the RT enzyme.

Both controls contain less than 1 DNA molecule in 1000 RNA molecules. This is acceptable to use as an RNA control.

Stability (reproducibility) of Ct value determination on dilution series of EQC

Over several days and by different operators frozen EQC (A and B) dilution series were analyzed by means of q-RT-PCR quantification. The results of these tests (n = 6) show a dynamic range of 6-7 logs. The repeated experiments indicated a good reproducibility with an average slope of –3.39 (EQC-A) and –3.33 (EQC-B), a Y-axis intercept of 42.56 (EQC-A) and 41.51 (EQC-B) and a correlation of 0.999 of the data points for both EQC-A and EQC-B. For EQC-A, 5/6 runs had a linear range of 7 logs. The mean Ct value of the lowest dilution (n = 6) was 11.98511 (stdev 0.484211) and of the highest dilution was 35.90961 (stdev 0.988846) (n = 5, for run 5 the highest dilution was not measured). For EQC-B 4/6 only had a linear range of 6 logs . The mean Ct value of the lowest dilution (n = 6) was 10.98107 (stdev 0.569094) and of the highest common dilution (n = 6) was 30.8342 (stdev 0.657034).

Interpretation of the Standard curve, an example

A total of 4 NPW samples and one sputum sample were processed. For both experiments also the external quantification controls EQC-A and EQC-B were

taken along. Two settings were tested to investigate what the best interpretation mode was for the standard curves (i.e. what would allow to calculate "Ct value 1" of a sample in one experiment according to standard curve 1, and "Ct value 2" of that same sample in a second experiment according to standard curve 2 in such a way that both Ct values would be closest to each other): setting the threshold identical for all experiments or setting the Y-axis intercept identical for all experiments?

In a first setting, the threshold of the standard curves in both experiments was set to 0.1. As can be observed, none of the measurements deviated by more than 1 Ct. The mean of the differences was 0.56 Ct with a standard deviation of 0.39.

In another setting, it was tried to minimize the difference of the standard curves concerning the Y-axis intercept. As such, the threshold of the repeat experiment was modified to make the Y-axis intercept of the second EQC-A standard curve move closer to 42.43 (42.428). With this setting, one measurement differed more than 1 Ct (NPW 5: 1.04). The average of the differences however was only 0.47 with a standard deviation of 0.43.

CONCLUSION

Two vectors were constructed and sequenced. They both contained a part of the RSV genome (RSV-A 1004bp and RSV-B 1399bp). The purified DNase-treated transcripts were analyzed in the presence or the absence of the RT enzyme in a real-time PCR quantification assay. This indicated that for both EQCs, a suited RNA control transcript was obtained that contained less than 1 DNA molecule in 1000 RNA molecules.

Repeated experiments indicated that the EQC dilution series delivered stable, reproducible results over different days and different operators with a dynamic range of 6 to 7 logs.

The sequence of pEQC-A (clone 6) and pEQC-B (clone 3) respectively are depicted below.

<u>Sequence of pEQC-A:</u> (SeqID No. 17)

(**Bold** = primer sequence; <u>underlined</u> = probe sequence)

TGCACTGGCCAGGGGGATCACCATCCGTCGCCCCGGCGTGTCAATAATATCACTCTGTACATCCACAAAC
AGACGATAACGGCTCTCTCTTTTATAGGTGTAAACCTTAAACTGCCGTACGTATAGGCTGCGCAACTGTT
GGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCG
ATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATAC

GACTCACTATAGGGCGAATTGAATTTAGCGGCCGCGAATTCGCCCTTAATCAAAATAAACTCTGGGGCAA
ATAACAATGGAGTTGCCAATCCTCAAAGCAAATGCAATTACCACAATCCTCGCTGCAGTCACATTTTGCT
TTGCTTCTAGTCAAAACATCACTGAAGAATTTTATCAATCAACATGCAGTGCAGTTAGCAAAGGCTATCT
TAGTGCTCTAAGAACTGGTTGGTATACTAGTGTTATAACTATAGAATTAAGTAATATCAAGGAAAATAAG
TGTAATGGAACAGATGCTAAGGTAAAATTGATAAACCAAGAATTAGATAAATATAAAAATGCTGTAACAG
AATTGCAGTTGCTCATGCAAAGCACAACAGCAGCAAACAATCGAGCCAGAAGAGAACTACCAAGGTTTAT
GAATTATACACTCAACAATACCAAAAAAACCAATGTAACATTAAGCAAGAAAAGGAAAAGAAGATTTCTT
GGTTTTTTGTTAGGTGTTGGATCTGCAATCGCCAGTGGCATTGCTGTATCTAAGGTCCTGCACTTAGAAG
GAGAAGTGAACAAGATCAAAAGTGCTCTACTATCCACAAACAAGGCCGTAGTCAGCTTATCAAATGGAGT
TAGTGTCTTAACCAGCAAAGTGTTAGACCTCAAAAACTATATAGATAAACAATTGTTACCTATTGTGAAT
AAGCAAAGCTGCAGAATATCAAATATAGAAA**CTGTGATAGAGTTCCAACAAAAGAACA**ACAGACTACTAG
AGATTACCAG**GGAATTTAGTGTTAATGCAGGTGTAACT**ACACCTGTAAGCACTTACATGTTAACTAATAG
TGAATTATTGTCATTAATCAATGATATGCCTATAACAAATGATCAGAAAAAGTTAATGTCCAACAATGTT
CAAATAGTTAGACAGCAAAGTTACTCTATCATGTCCATAATAAAAGAGGAAGTCTTAGCATATGTAGTAC
AATTACCACTATATGGTGTGATAGATACACCTTGTTGGAAATTACACACATCCCCTCTATGTACAACCAA
CAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAAT
CATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAG
CATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCC
GCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTT
TGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGC
GGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATG
TGAGCAAAAGGCCAGCAAAAGCCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCC
GCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAG
ATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATAC
CTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGG
TGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATC
CGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAAC
AGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACA
CTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTC
TTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGA
AAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCAC
GTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAG
TTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCA
CCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGA
TACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGA
TTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCC
ATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTG
TTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCA
ACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATC
GTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTG

TCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTAT
GCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAA
GTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTT
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AAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTC
TTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTA
TTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGTATGCGGTGTGAA
ATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGAAATTGTAAGCGTTAATAATTCAGAAGAACT
CGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCG
GTCAGCCCATTCGCCGCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCC
GCCACACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGC
AGGCATCGCCATGGGTCACGACGAGATCCTCGCCGTCGGGCATGCTCGCCTTGAGCCTGGCGAACAGTTC
GGCTGGCGCGAGCCCCTGATGCTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCGAGTA
CGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATCAAGCGTATGCAGCC
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CGGCACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGA
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CGGTCTTGACAAAAAGAACCGGGCGCCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCAGCCGAT
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TCTTGTTCAATCATGCGAAACGATCCTCATCCTGTCTCTTGATCAGAGCTTGATCCCCTGCGCCATCAGA
TCCTTGGCGGCGAGAAAGCCATCCAGTTTACTTTGCAGGGCTTCCCAACCTTACCAGAGGGCGCCCCAGC
TGGCAATTCCGGTTCGCTTGCTGTCCATAAAACCGCCCAGTCTAGCTATCGCCATGTAAGCCCACTGCAA
GCTACCTGCTTTCTCTTTGCGCTTGCGTTTTCCCTTGTCCAGATAGCCCAGTAGCTGACATTCATCCGGG
GTCAGCACCGTTTCTGCGGACTGGCTTTCTACGTGAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCT
CATGCCTGACATTTATATTCCCCAGAACATCAGGTTAATGGCGTTTTTGATGTCATTTTCGCGGTGGCTG
AGATCAGCCACTTCTTCCCCGATAACGGAGACCGGCACACTGGCCATATCGGTGGTCATCATGCGCCAGC
TTTCATCCCCGATATGCACCACCGGGTAAAGTTCACGGGAGACTTTATCTGACAGCAGACG

Sequence of pEQC-B: (SeqID No. 18)

(**Bold** = primer sequence; underlined = probe sequence)

TGCACTGGCCAGGGGGATCACCATCCGTCGCCCCGGCGTGTCAATAATATCACTCTGTACATCCACAAAC
AGACGATAACGGCTCTCTCTTTTATAGGTGTAAACCTTAAACTGCCGTACGTATAGGCTGCGCAACTGTT
GGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCG
ATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATAC
GACTCACTATAGGGCGAATTGAATTTAGCGGCCGCGAATTCGCCCTTATATTTATCAATCATGGCGGGTT
TCTAGAATGTATTGGCATTAAGCCTACAAAACACACTCCTATAATATACAAATATGACCTCAACCCGTAA
ATTCCAACAAAAAACTAACCCATCCAAACTAAGCTATTCCTTAAATAACAGTGCTCAACAGTTAAGAAGG
GGCTAATCCATTTTAGTAATTAAAAATAAAGGTAAAGCCAATAACATAAATTGGGGCAAATACAAAGATG
GCTCTTAGCAAAGTCAAGTTAAATGATACATTAAATAAGGATCAGCTGCTGTCATCTAGCAAATACACTA
TTCAACGTAGTACAGGAGATAATATTGACACTCCCAATTATGATGTGCAAAAACACTTAAACAAACTATG

TGGTATGCTATTAATCACTGAAGATGCAAATCATAAATTCACAGGATTAATAGGTATGTTATATGCTATG

TCCAGGTTAGGAAGGGAAGACACTATAAAGATACTTAAAGATGCTGGATATCATGTTAAAGCTAATGGAG

TAGATATAACAACATATCGTCAAGATATAAATGGAAAGGAAATGAAATTCGAAGTATTAACATTATCAAG

CTTGACATCAGAAATACAAGTCAATATTGAGATAGAATCTAGAAAGTCCTACAAAAAAATGCTAAAAGAG

ATGGGAGAAGT**GGCTCCAGAATATAGGCATGATTC**TCCAGACTGTGGGATGATAAT**ACTGTGTATAGCTG**

CACTTGTAATAACCAAATTAGCAGCAGGAGATAGATCAGGTCTTACAGCAGTAATTAGGAGGGCAAACAA

TGTCTTAAAAAACGAAATAAAACGCTACAAGGGCCTCATACCAAAGGATATAGCTAACAGTTTTTATGAA

GTGTTTGAAAAACACCCTCATCTTATAGATGTTTTTGTGCACTTTGGCATTGCACAATCATCCACAAGAG

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AAGATGGGGAGTTTTAGCCAAATCTGTAAAAAATATCATGCTAGGACATGCTAGTGTCCAGGCAGAAATG

GAGCAAGTTGTGGAAGTCTATGAGTATGCACAGAAGTTGGGAGGAGAAGCTGGATTCTACCATATATTGA

ACAATCCAAAAGCATCATTGCTGTCATTAACTCAATTTCCTAACTTCTCAAGTGTGGTCCTAGGCAATGC

AGCAGGTCTAGGCATAATGGGAGAGTATAGAGGTACACCAAGAAACCAGGATCTTTATGATGCAGCCAAA

GCATATGCAGAGCAACTCAAAGAAAATGGAGTAATAAACTACAGTGTATTAGACTTAACAGCAGAAGAAT

TGGAGGCCATAAAGCATCAACTCAACCCCAAAGAAGATGATGTAGAAAGGGCGAATTCGTTTAAACCTGC

AGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTG

AAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCC

TAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGT

GCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTC

CTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTA

ATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGCCCA

GGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAA

TCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGC

TCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAA

GCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGG

CTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAAC

CCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAG

GCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTG

CGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCT

GGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTT

TGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATT

ATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATAT

GAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTC

GTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCC

CAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCC

GGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGG

AAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGT

GTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCC

CCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAG

TGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTC

TGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCG
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CTTGGTGGTCGAATGGGCAGGTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGA
TACTTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCAGCCAG
TCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATA
GCCGCGCTGCCTCGTCTTGCAGTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAAAAAGAACCGGGCG
CCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCAGCCGATTGTCTGTTGTGCCCAGTCATAGCCG
AATAGCCTCTCCACCCAAGCGGCCGGAGAACCTGCGTGCAATCCATCTTGTTCAATCATGCGAAACGATC
CTCATCCTGTCTCTTGATCAGAGCTTGATCCCCTGCGCCATCAGATCCTTGGCGGCGAGAAAGCCATCCA
GTTTACTTTGCAGGGCTTCCCAACCTTACCAGAGGGCGCCCCAGCTGGCAATTCCGGTTCGCTTGCTGTC
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AACATCAGGTTAATGGCGTTTTTGATGTCATTTTCGCGGTGGCTGAGATCAGCCACTTCTTCCCCGATAA
CGGAGACCGGCACACTGGCCATATCGGTGGTCATCATGCGCCAGCTTTCATCCCCGATATGCACCACCGG
GTAAAGTTCACGGGAGACTTTATCTGACAGCAGACG

A similar procedure as for above described EQC was worked out for the construction of the internal extraction control (IEC) for said RSV q-RT PCR assay.

<u>Construction of the internal extraction control (IEC) for the RSV q-RT PCR assay</u>

In order to monitor the RNA extraction efficiency of the EasyMAG (BioMérieux, Boxtel, The Netherlands) internal extraction controls (IEC) were constructed
This included design of the following plasmid constructs containing:

- Primer sequence identical to the RSV-A selected primers with a random 'in- between' sequence. A Taqman probe containing the fluorescent dye

TET or FAM will detect this sequence. This construct is labeled as internal extraction control RSV-A (pIEC-A)

- Primer sequence identical to the RSV-B selected primers with a random 'in- between' sequence. A Taqman probe containing the fluorescent dye NED or FAM will detect this sequence. This construct is labeled as internal extraction control RSV-B (pIEC-B)

Primers and probes

The primers ordered to generate the sequence to be cloned in pIEC-A (RSV-A-Fgen-EC-FW and RSV-A-Fgen-EC-RV) and pIEC-B (RSV-B-Ngen-EC-FW and RSV-B-Ngen-EC-RV) are described below and cover roughly one primer sequence and half of the specific probe sequence. A schematic representation of the RSV sequences and the corresponding primers and probes can be found in Fig. 5. The primers overlap partly, which enables them to anneal during a PCR amplification (see below).

Oligonucleotide	Sequence	
RSV-A-Fgen-EC-FW	5'-CTGTGATAGA GTTCCAACAA AAGAACAATG CGTATGGATT GGT –3'	SeqID No. 19
RSV-A-Fgen-EC-RV	5'-AGTTACACCT GCATTAACAC TAAATTCCCT AATGACCAAT CCA-3'	SeqID No. 20
RSV-B-Ngen-EC-FW	5'-GGCTCCAGAA TATAGGCATG ATTCTCTACC GTACTCTAGC C-3'	SeqID No. 21
RSV-B-Ngen-EC-RV	5'-TGGTTATTAC AAGTGCTGCT ATACACAGTA TTAGGCTAGA G-3'	SeqID No. 22

Amplification and cloning

The complementary primer sequences (pIEC-A (RSV-A-Fgen-EC-FW and RSV-A-Fgen-EC-RV) and pIEC-B (RSV-B-Ngen-EC-FW and RSV-B-Ngen-EC-RV) were submitted to a PCR reaction forming a short piece of double stranded DNA. The PCR mixes and conditions are specified below:

RSV-A

Component	Vol (µl)	# Samples **8**	
Rnase free water	39.17	352.53	µl
PCR Buffer (15 mM $MgCl_2$)	5	45	µl
dNTPs	0.4	3.6	µl
RSV-A Fgen-EC-FW	0.5	4.5	µl
RSV-A Fgen-EC-RV	0.5	4.5	µl
Expand™HF PCR	0.43	3.87	µl
Total volume	**46**	**414**	**µl**

RSV-B

Component	Vol (µl)	# Samples **8**	
Rnase free water	39.17	352.53	µl
PCT Buffer (15 mM $MgCl_2$)	5	45	µl
dNTPs	0.4	3.6	µl
RSV-B Ngen-EC-FW	0.5	4.5	µl
RSV-B Ngen-EC-RV	0.5	4.5	µl
Expand™HF PCR	0.43	3.87	µl
Total volume	**46**	**414**	**µl**

The PCR program used was 94°C for 2 minutes followed by 35 cycles of 94°C for 15 seconds, 54°C-62°C (gradient, performed on cycler 857) for 30 seconds and 68°C for 30 seconds. A final step was performed at 68°C for 10 minutes concluded with a final hold at 4°C.

TOPO-TA cloning

The obtained PCR fragments were cloned using the TOPO TA cloning kit (Invitrogen, Merelbeke, Belgium) following the manufacturer's instructions. Briefly, 4µl PCR product (without purification) was mixed with 1 µl salt solution and 1 µl vector.

The ligation reaction was transformed into TOP10 chemically competent E.*coli* cells following the manufacturer's instructions.

After growing the E.*coli* cultures overnight, 10 colonies per construct (pIEC-A and pIEC-B) were transferred to liquid LB medium, grown overnight and a

miniprep (Qiagen, Hilden, Germany) was performed (total volume of 50 µl plasmid DNA (concentration not measured).

Sequencing

A total of 0,75µl of the generated miniprep DNA was submitted to sequencing. Sequencing was done using the Big Dye Terminator (BDT) Cycle Sequencing Kit v3.1 (Cat n°. 4337457, Applied Biosystems, CA, USA). The mix composition with a final volume of 5.75 micro liter in each well was as follows:

Component	Volume (µl)
BDT	0.25
Water	1.50
Total volume	**5.00**
amplicon	0.75

BDT = Big Dye Terminator

The sequencing reactions were performed using the primer set "T3" (5'-ATTAACCCTCACTAAAGGGA-3') (SeqID No. 15) and "T7" (5'-TAATACGACTCACTATAGGG-3') (SeqID No. 16) which are located on the TOPO-TA vector.

The sequencing PCR was done using the 9800 Fast Thermal Cycler (Applied Biosystems (ABI), Cat. nr. 4356204, CA, USA). The PCR conditions were 96°C for 5 seconds, 50°C for 5 seconds, 60°C for 1 minute and 15 seconds and this for 25 cycles in total followed by a final hold at 12°C.

In Vitro RNA transcription

One correct clone of each IEC construct was linearized using the restriction enzyme Apa*L*I (Figure. 6). Restriction enzyme digestion (Apa*L*I) proceeded at 37°C for 3 hours using restriction enzyme buffer 4 and BSA. After restriction, the reactions were purified. To the restriction digest mix, 1/20 volumes of 0.5M EDTA (1µl), 1/10 volumes of NaOAC (2µl) and 2 volumes of EtOH (40µl) were added. The mixture was placed at -20°C for minimum 15 minutes, and then spun for 15 minutes at maximum speed. The supernatant was discarded and the pellet was dissolved in 50µl TE buffer. These purified templates were then

transcribed using the MEGAshortscript™ T7 Kit (Ambion, Cat. nr. 1354, Texas, USA. The reaction mixtures were incubated at 37°C for 4 hours. After incubation at 37°C the reaction mixtures were put at 4°C. The total length of the transcribed RNA is predicted to be 1100 nucleotides.

DNA removal

The transcription mixtures were treated with TURBO DNase™ (2 µl added to transcription mix, Ambion, Cat. Nr. 2238, Texas, USA). The DNase treatment lasted for 1 hour at 37°C in a final volume of 100µl. The RNA was purified by means of an RNA extraction on the EasyMAG (BioMérieux) eluting in 100µl.

Real-time IEC quantification

The purified transcripts were diluted 1/1000 and submitted to a real-time PCR quantification either in the presence or the absence of the RT enzyme. The compositions of the various mixes are depicted below.

The amplification program used on the ABI9700 HT real-time PCR equipment was 48°C for 30 minutes, 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Reaction Vol. (µl)	**25**				
Mix item	**Concentration**			**Volume for (µl)**	
	Unit	**Stock**	**Final**	**1 sample**	**X samples**
Rnase free water				0.325	7.15
Yeast tRNA	ng/ml	10000	120	0.300	6.60
2x Master Mix w/o UNGr	X	2.00	1.000	12.500	275.00
40X MultiScribe and Rnase Inhibitor Mix	X	40.00	0.013	0.625	13.75
Probe IEC-B	µM	5.00	0.100	0.500	11.00
primer 1	µM	20.00	0.300	0.375	8.25
primer 2	µM	20.00	0.300	0.375	8.25
	Total Volume Mix (µl)				330.00

Volume Mix / Tube (µl)	15.00
Total RNA	10.00

Sample processing, an example

In total 5 clinical samples were processed Each clinical sample was diluted in PBS/1% DTT to a final volume of 2,2 ml. This mix was processed on the AFA (Covaris) for 60 seconds prior to the addition of 4 ml lysis buffer (EasyMAG, BioMérieux). From these 6.2 ml per sample, 3 ml was processed on the EasyMAG (BioMérieux) and the RNA was eluted in 110µl of which 10µl was processed in the q-RT-PCR in duplicate. The experiment was repeated once starting from extraction with the remaining 3 ml.

Aliquoting and storage of IEC

A total of 24 negative samples (PBS, 10%DTT, 1ml final volume) were lysed with 2 ml Lysis buffer (BioMérieux) and incubated for 10 minutes. Three aliquots of IEC-B (110µl) were defrosted and diluted with buffer 3 (440µl, BioMérieux). A total of 550µl magnetic silica beads (BioMérieux) were added to this IEC dilution and all three mixes were pooled to obtain a final volume of 3300µl IEC/magnetic silica beads mix. This mix was diluted as described for the IEC addition of the EasyQ (BioMérieux), i.e., 125µl mix was dispensed in 24 wells (using the pre-programmed protocol “2” on the automatic dispensing pipet of BioMérieux) and of this total mix, 100µl was added to each sample, using the pre-programmed protocol “3” on the automatic dispensing pipet of BioMérieux. The IEC were further extracted on the EasyMAG and eluted in 110µl. From this mix 10µl was submitted to a q-RT-PCR amplification.

Results

PCR

The amplicon for the RSV-A internal extraction control construct had a size of 77 base pairs while the amplicon for the RSV-B internal extraction control construct had a size of 74 base pairs. These PCR amplified fragments were ligated in a TOPO-TA vector and transformed into competent E.*coli*. The transformants were grown overnight on solid LB/ampicillin plates. A total of 10 colonies were transferred to liquid LB/Ampicillin medium and again grown overnight. A miniprep (Qiagen, Hilden, Germany) was prepared from these cultures and the resulting DNA was submitted to sequencing.

Sequencing

As expected, not all PCR fragments were ligated in the same direction in the TOPO-TA vector. The RSV-A internal extraction control PCR fragments in

clones 5, 8 and 10 were ligated in the correct orientation. This was also the case for clones 1, 3, 7 and 8 for the RSV-B internal extraction control.

In vitro transcription

Clone 5 (pIEC-A) and clone 1 (pIEC-B) were submitted to a restriction digest using Apa*L*I and purified as described above. The linearized and purified constructs were submitted to an *in vitro* transcription (using the MEGAshortscript™ T7 Kit (Ambion)). The residual DNA was removed using the TURBO DNase™ (Ambion). The transcripts were purified by means of an RNA extraction on the EasyMAG (BioMérieux) as described above.

Real-time PCR results

The pure transcripts were diluted 1/1000 and submitted to a real-time PCR quantification reaction with and without RT enzyme to determine the residual DNA content of the transcripts. For IEC-A, the signal generated in the presence of reverse transcriptase (RT) emerged at Ct 8 while in the absence of the RT enzyme the signal only emerged at Ct 30-31. For IEC-B this was respectively Ct 8 (+ RT) and Ct 30 (-RT). Hence both IECs had a difference of 22 cycles between the signal with and without RT. This corresponds to a difference of more than 6 logs (22/3,3 = 6.6), indicating 1 residual DNA molecule for 10^6 RNA molecules. This is acceptable to use as an RNA control. The IEC RNA was diluted 1000 000 times (estimated Ct value 27,4 for IEC-A and 27.6 for IEC-B), aliquoted in 90µl aliquots and stored at -80°C.

Analysis of Clinical Samples, an example

In this experiment, five clinical samples were processed Since all assays were run in monoplex, only one IEC was needed.

<u>Standard Curves</u>

In the standard curves for EQC-A the slope was –3.34, with a Y-axis intercept of 42.43 and a linear range of 7 logs.

For EQC-B the slope was –3.34, with a Y-axis intercept of 41.23 and a linear range of 7 logs.

<u>IEC</u>

When looking at the IEC signals it could be observed that all signals emerged above the noise at the same moment, around Ct 28. This experiment was

repeated once starting from extraction (Hence an independent addition of IEC-B to the samples during this extraction), resulting in comparable values. All values were combined and plotted. The average of all measurements of the IEC-B was 28.09, with a standard deviation of 0.36.

Sample Analysis

3/5 samples produced a signal for RSV-A: sputum 17.38 and 17.68, “NPW 4” 21.74 and 22.15 and “NPW 6” 24.61 and 25.60. No signal was detected for NPW 3 and NPW 5 on the RSV-A analysis plate. These samples did produce an RSV-B signal: “NPW 3” 21.63 and 22.15 and “NPW 5” 25.49 and 25.58.

CONCLUSION

Two vectors were constructed and sequenced. They both contained a unique sequence covering either the RSV-A or RSV-B specific primers and a unique sequence in between the two primer sequences that can anneal with a unique fluorescent probe. The purified DNase-treated transcripts were analyzed in the presence or the absence of the RT enzyme in a real-time PCR quantification assay. This indicated that for both IECs a suited RNA control transcript was obtained that contained less than 1 DNA molecule in 1000 000 RNA molecules, covering the 2 log difference specified as an acceptance criteria.

The IEC-B was diluted 1000 000 times and added to clinical samples during their extraction on the EasyMAG. This process was repeated during a second, independent analysis of the samples. This experiment revealed comparable signals emerging around Ct 28.09, with a standard deviation of 0.36, regardless of the RSV RNA present in the sample (subtype A or B).

The IEC-B was aliquoted and stored in 110µl aliquots in -80°C. The repeated testing of these aliquots revealed a Ct value of 26.32 when the threshold was set at 0.02.

The sequence of pIEC-A (clone 5) and pIEC-B (clone 1) respectively are depicted below:

Sequence of pIEC-A (SeqID No. 23):

(**Bold** = primer sequence; underlined = probe sequence)

TGCACTGGCCAGGGGGATCACCATCCGTCGCCCCGGCGTGTCAATAATATCACTCTGTACATCCACAAAC
AGACGATAACGGCTCTCTCTTTTATAGGTGTAAACCTTAAACTGCCGTACGTATAGGCTGCGCAACTGTT
GGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCG
ATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATAC

GACTCACTATAGGGCGAATTGAATTTAGCGGCCGCGAATTCGCCCTT**CTGTGATAGAGTTCCAACAAAAG**

AACAATGCGTATGGATTGGTCATTAG**GGAATTTAGTGTTAATGCAGGTGTAACT**AAGGGCGAATTCGTTT

AAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTT

CCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCT

GGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAA

CCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCT

TCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAA

AGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCA

AAAGCCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCAT

CACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCC

CTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCC

TTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCC

AAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTG

AGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAG

GTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTT

GGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAA

CCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGA

AGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTC

ATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAA

GTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTG

TCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCA

TCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACC

AGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTG

TTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGC

ATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTA

CATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTT

GGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGA

TGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCT

CTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAA

ACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGT

GCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAA

ATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTA

TTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAA

ATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGTATGCGGTGTGAAATACCGCACAGATGCGT

AAGGAGAAAATACCGCATCAGGAAATTGTAAGCGTTAATAATTCAGAAGAACTCGTCAAGAAGGCGATAG

AAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGC

CAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGGCC

ACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGCATCGCCATGGGTC

ACGACGAGATCCTCGCCGTCGGGCATGCTCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCT

GATGCTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCG

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CATAGCCGAATAGCCTCTCCACCCAAGCGGCCGGAGAACCTGCGTGCAATCCATCTTGTTCAATCATGCG
AAACGATCCTCATCCTGTCTCTTGATCAGAGCTTGATCCCCTGCGCCATCAGATCCTTGGCGGCGAGAAA
GCCATCCAGTTTACTTTGCAGGGCTTCCCAACCTTACCAGAGGGCGCCCCAGCTGGCAATTCCGGTTCGC
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TGCGCTTGCGTTTTCCCTTGTCCAGATAGCCCAGTAGCTGACATTCATCCGGGGTCAGCACCGTTTCTGC
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TTCCCCAGAACATCAGGTTAATGGCGTTTTTGATGTCATTTTCGCGGTGGCTGAGATCAGCCACTTCTTC
CCCGATAACGGAGACCGGCACACTGGCCATATCGGTGGTCATCATGCGCCAGCTTTCATCCCCGATATGC
ACCACCGGGTAAAGTTCACGGGAGACTTTATCTGACAGCAGACG

Sequence of pIEC-B (SeqID No. 24):

(**Bold** = primer sequence; Underlined = probe sequence)

TGCACTGGCCAGGGGGATCACCATCCGTCGCCCCGGCGTGTCAATAATATCACTCTGTACATCCACAAAC
AGACGATAACGGCTCTCTCTTTTATAGGTGTAAACCTTAAACTGCCGTACGTATAGGCTGCGCAACTGTT
GGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCG
ATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATAC
GACTCACTATAGGGCGAATTGAATTTAGCGGCCGCGAATTCGCCCTT**GGCTCCAGAATATAGGCATGATT**
CTCTACCGTACTCTAGCCTAAT**ACTGTGTATAGCAGCACTTGTAATAACCA**AAGGGCGAATTCGTTTAAA
CCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCT
GTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGG
GTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCT
GTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCC
GCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGG
CGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAA
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GAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTC
GGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAG
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CAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTG
CCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATC
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CGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGC
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GCTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATG
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CCCAGAACATCAGGTTAATGGCGTTTTTGATGTCATTTTCGCGGTGGCTGAGATCAGCCACTTCTTCCCC
GATAACGGAGACCGGCACACTGGCCATATCGGTGGTCATCATGCGCCAGCTTTCATCCCCGATATGCACC
ACCGGGTAAAGTTCACGGGAGACTTTATCTGACAGCAGACG

(B) Use of MNAzymes for the quantification of RSV-A and RSV-B viral strains and a control nucleic acid sequences via triplex real time PCR

A triplex PCR assay, which used three MNAzymes to facilitate real time monitoring, was developed for the simultaneous detection and quantification of (i) RSV-A (F gene sequences), (ii) RSV-B (N gene sequences) and (iii) an internal extraction control (IEC B) sequence. The RSV-A primers and partzymes, which targeted a region of the F gene, were designed to be fully complementary to the RSV-A sequence but to contain several base mismatches in the corresponding region of the RSV-B genome. Similarly, the RSV-B primers and partzymes, which targeted a region in N gene, were designed to be fully complementary to the RSV-B sequence but to contain several base mismatches in the corresponding region of the RSV-A genome.

#.1 Partzyme Oligonucleotides for a triplex RT-PCR assay

Multiple targets can be simultaneously detected in one multiplexed reaction that comprises multiple unique MNAzymes. Each MNAzyme has sensor arms specific for one target and substrate arms specific for a unique member of a series of generic reporter substrates, each one of which is labeled with a different fluorophore.

In the following example, two MNAzymes were designed to detect two strains of RSV from clinical samples with a third MNAzyme designed to detect a synthetic control sequence, which was applied to the sample prior to nucleic acid extraction.

Table 2

SEQ ID #	Target	Partzyme Name	Partzyme sequence listed in 5' to 3' direction (P = phosphate group)
25	RSVA	RSVAA5/2-P	CAGACTACTAGAGATTACCATACAACGAGAG GAAACCTT-P
26	RSVA	RSVAB6/2-P	TGCCCAGGGAGGCTAGCGGGAATTTAGTGTT AATGCA-P
27	RSVB	RSVBA5/3-P	AGACTGTGGGATGATAATACTACAACGAGGT TGTGCTG-P
28	RSVB	RSVBB6/3-P	CGGTTGGTGAGGCTAGCTGTGTATAGCTGCA CTTGTA-P
29	IEC B	IEC3A5/6-P	ACCTGCAGGACTAGTCCCTTTACAACGAGAG GCGTGAT-P
30	IEC B	IEC3B6/6-P	CTGGGAGGAAGGCTAGCTAGTGAGGGTTAAT TCTGAG-P

#.2 Reporter Substrates

In this example, three different reporter substrates, each labeled with a different fluorophore, were used. The sequences of the substrates are written 5' to 3' below. In the current example, a first substrate SubBi-2 was end-labeled with a 6-FAM moiety at the 5' end and a BHQ1 moiety at the 3' end and was designated SubBi-2-FB. The substrate SubBi-2-FB was used to monitor the accumulation of RSVA amplicons. The cleavage of SubBi-2-FB was monitored at 516 nm with excitation at 492 nm. A second substrate SubBi-3 was end-labeled with a 6-JOE moiety at the 5' end and a BHQ1 moiety at the 3' end and was designated SubBi-3-JB. The substrate SubBi-3-JB was used to follow the amplification of the region of the RSVB and SubBi-3-JB cleavage was monitored at 555 nm with excitation at 535 nm. The third substrate SubBi-6 was end-labeled with a Quasar 670 moiety at the 5' end and a BHQ2 moiety at the 3' end and was designated SubBi-6-Q6B2. The substrate SubBi-6-Q6B2 was used to monitor the amplification of the internal extraction control (IEC B) and cleavage of SubBi-3-Q6B2 was monitored at 665 nm with excitation at 635 nm. The sequences of the three substrates are listed below. The lower case bases represent RNA and the upper case bases represent DNA. The moieties at the 5' and 3' ends are indicated.

Table 3

SEQ. ID No.	Substrate sequence listed in 5' to 3' direction	Name
31	(FAM)AAGGTTTCCTCguCCCTGGGCA(BHQ1)	SubBi-2-FB
32	(JOE)CAGCACAACCguCACCAACCG(BHQ1)	SubBi-3-JB
33	(Q670)ATCACGCCTCguTCCTCCCAG(BHQ2)	SubBi-6-Q6B2

#.3. PCR primers for amplification of the target sequences

The primers 5RSVA/3 and 3RSVA/3 were used for the amplification of RSVA F gene sequence. The primers 5RSVB/3 and 3RSVB/3 were used to amplify the RSVB N gene sequence. The primers 5IECB/1 and 3IECA3/3 were used to amplify the Internal extraction control (IECB) sequence. The sequences of the oligonucleotide PCR primers are listed below.

Table 4

SEQ. ID No.	Primer sequence listed in 5' to 3' direction	Name
34	GTGATAGAGTTCCAACAAAAGA	5RSVA/3
35	AAGTGCTTACAGGTGTAGTTA	3RSVA/3
36	GCTCCAGAATATAGGCATGAT	5RSVB/3
37	GATCTATCTCCTGCTGCTAAT	3RSVB/3
38	CTTGTAATAACCAAAGGGCGA	5IECB/1
39	GGAAACAGCTATGACCATGATT	3IECA3/3

#.4. Reaction Components: Amplification and quantification of target sequences

Reverse transcription, real time amplification and quantification of the target sequences were performed in a total reaction volume of 25 µL. All reactions were conducted on an Mx3005P™ QPCR System (Stratagene). The cycling parameters were 50°C for 30 minutes (reverse transcription step), followed by 95°C for 7 minutes, then 10 cycles of 95°C for 15 seconds and initially 65°C for 30 seconds with a 1°C decrease in temperature per cycle to 55°C, and finally 50 cycles of 95°C for 15 seconds and 50°C for 120 seconds. The reactions contained 40 nM of 5RSVA/3, 40 nM of 5RSVB/3, 40 nM of 5IECB/1, 200nM of 3RSVA/3, 200nM of 3RSVB/3, 200nM of 3IECA3/3, 200 nM of each substrate (SubBi-2-FB, SubBi-3-JB and SubBi-6-Q6B2), 8 mM $MgCl_2$, 200 µM of each dNTP, 10 units Rnasin (Promega), 1 x Immobuffer (Bioline) 1 unit of Immolase (Bioline) and 40U of M-MLV(-H) (Promega). Each duplicate reaction contained a dilution of both RSVA and RSVB genomic RNA along with an RNA preparation of a T7 Transcription of the IECB sequence, or no template nucleic acid.

Two standard curves were produced, each performed in duplicate. The first standard curve was generated by performing four fold dilutions of both RSVA viral genomic RNA and the T7 transcript of the IECB through a constant concentration of background of RSVB viral genomic RNA. The second standard curve was generated by performing four fold dilutions of both RSVB viral RNA and the T7 transcript of the IECB through a constant concentration of background RSVA genomic RNA.

Table 5 : RSVA /IECB Standard curves

	Threshold (Ct)		
	RSVA (FAM)	**RSVB (JOE)**	IECB (Quasar 670)
Standard 1	22.9	19.6	11.6
Standard 2	25.3	19.9	13.8
Standard 3	28.1	20.4	15.9
Standard 4	30.6	20.4	19.0
Standard 5	32.9	20.3	20.8
Standard 6	36.4	20.6	23.4
Water only (no template control)	No Ct	No Ct	No Ct
Standard Curve	R^2 = 0.995 Slope = -4.102 Efficiency = 75.2%	N/A	R^2 = 0.997 Slope = -3.954 Efficiency = 79%

Table 6 : RSVB /IECB Standard curves

	Threshold (Ct)		
	RSVA (FAM)	**RSVB (JOE)**	IECB (Quasar 670)
Standard 1	26.7	15.4	11.2
Standard 2	27.1	17.6	13.7
Standard 3	27.3	20.0	16.3
Standard 4	27.4	22.4	18.6
Standard 5	27.4	25.3	21.1
Standard 6	27.3	28.3	22.2
Water only (no template control)	No Ct	No Ct	No Ct
Standard Curve	N/A	R^2 =0.996 Slope = - 4.276 Efficiency = 71.3%	R^2 =0.989 Slope = - 3.771 Efficiency = 84.2%

Each calibration dilution series produced a standard curve that had a high correlation coefficient (≥ 0.989), and high specificity indicated by the lack of background in no template controls. Further, the fact that the threshold cycle (Ct) for RSVB remained relatively constant (varying by ≤ 1 Ct value) in

reactions, where there was a constant input amount of RSVB but varying amounts of RSVA, indicates the specificity of the primer and substrate systems for the specific viral strains being quantified.

The suitability of the real time MNAzyme assay for the detection and quantification of RSVA and RSVB was further tested by analysing human specimens. RNA extracted from sputum and/or nasopharyngeal washes was analysed and RSVA and/or RSVB was detected in several samples.

Results of the comparison between method A (= q-RT PCR technology) and method B (= MNAzyme technology)

Table 7: Analysis of sputum and nasopharyngeal washes using Method A and Method B respectively.

Ct = threshold cycle/positive signal; ND = not detected; **+** = positive result; **-** = negative result.

Sample	Data (Cts) Method B			Data (Cts) Method A		Method B Results		Method A Results	
	RSVA	RSVB	IECB	RSVA	RSVB	RSVA	RSVB	RSV A	RSV B
Sputum 36	15.6/17.1	ND x 2r	30/27.3	21.2	ND	+	-	+	-
NPW 10	19.7/20.2	ND x 2r	28. 2	25.0	ND	+	-	+	-
NPW 11	34.4/36.0	23.2/24.0	29.1	39.8	22.4	+	+	+	+
NPW 12	37.1/39.3	25.1/26.3	28.3	42.5	24.0	+	+	+	+
NPW 13	ND x 2r	27.2/39	28.8	ND	25.1	-	+	-	+
NPW 14	18.9/19.5	ND x 2r	ND	24.3	ND	+	-	+	-
NPW 15	18.4/18.9	ND x 2r	28.5	23.5	ND	+	-	+	-
NPW 16	ND/41.7	26.1/26.8	27.8	43.6	24.7	+ (1/4)	+	+	+
NPW 17	38/37	ND x 2r	28.2	ND	40.7	+	-	-	+
NPW 18	ND x 2r	14.2/15.3	26.4	ND	15.1	-	+	-	+
NPW 19	37.1/ND	21.1/21.7	28.7	ND	20.8	+ (1/4)	+	-	+
NPW 20	ND/41.4	19.7/20.0	27.5	ND	19.6	+ (1/4)	+	-	+
NPW 21	19.2 /20.1	ND x 2r	29.1	23.7	ND	+	-	+	-
NPW 22	41.8 /ND	ND x 2r	28.6	ND	30.3	+ (1/4)	-	-	+
NPW 23	38.3/36.4	23.5/23.8	28.3	ND	22.7	+ (3/4)	+	-	+

Sample	Data (Cts) Method B RSVA	RSVB	IECB	Data (Cts) Method A RSVA	RSVB	Method B Results RSVA	RSVB	Method A Results RSV A	RSV B
NPW 24	40.6/ND	34.5/ND	27.9	ND	30.7	+ (1/4)	+ (1/4)	-	+
NPW 25	30.7/34.3	ND x 2r	28.5	34.8	42.5	+	-	+	+
NPW 26	18.6/19.3	ND x 2r	28.2	22.9	ND	+	-	+	-
NPW 27	ND x 2r	19.0/20.1	27.7	ND	19.6	-	+	-	+
NPW 30	18.1/19.2	ND x 2r	28.2	23.4	ND	+	-	+	-
NPW 32	ND x 2r	25.1/26.3	29.1	ND	24.3	-	+	-	+
NPW 33	15.2	ND	28.4	20.2	ND	+	-	+	-
NPW 34	21.4	ND	27.8	25.7	ND	+	-	+	-
NPW 36	19.4	ND	27.7	24.3	ND	+	-	+	-
NPW 37	ND	19.5	28.6	ND	20.7	-	+	-	+
NPW 38	17.1	ND	ND	21.8	42.0	+	-	+	+
NPW 39	36.7	21.5	27.3	40.6	21.2	+	+	+	+
Sputum 1	27.2/29.6	15.0/14.2	ND	35.4	15.8	+	+	+	+
Sputum 2	22.8/25.1	15.2/14.0	ND	28.0	15.5	+	+	+	+

Sample	Method B RSVA	RSVB	Method A RSVA		RSVB		Method B Results RSVA	RSVB	Method A Results RSVA	RSVB
Sputum 3	27.5/29.2	ND x 2r	NA	17.4	NA	-	+	-	+	-
NPW 6	25.4/26.5	ND x 2r	38.0	-	22.9	21.6	+	-	+	+
NPW 7	23.8/25.1	ND x 2r	25.9	21.7	-	-	+	-	+	-
NPW 8	23.7/25.6	ND x 2r	NA	-	NA	25.5	+	-	-	+
NPW 9	26.8/28.7	ND x 2r	26.1	24.6	-	-	+	-	+	-

Up to four (4) replicates of each sample were analyzed and when only some were detectable the number of positive per total number of replicates analyzed is indicated (e.g. 1 in 4 = 1/4).

When sufficient material was present each sample was analyzed in duplicate twice (i.e. duplicates in 2 separate runs).

The results in the Table show that there is high concordance between the two methods (A and B) used in terms of Ct value (threshold cycle value).

Claims

1. Method for the extraction of nucleic acids from a biological sample comprising the following steps:

a) obtaining a biological sample,
b) adding phosphate buffered saline / 10% dithiothreitol to said tissue material of step a),
c) incubating under ultrasonic conditions,
d) adding lysis buffer,
e) obtaining lysed tissue material and
f) extracting said nucleic acid from said lysed tissue material.

2. Method according to claim 1 wherein step c) is performed with an ultrasonic Covaris S2 apparatus and the extraction step f) is performed using EasyMAG procedure.

3. Method according to claim 1 or 2 wherein the nucleic acid is DNA or RNA from pathogens such as viruses.

4. Method according to claim 3 wherein said tissue material is either frozen sputum, naso-pharyngeal wash (NPW) or lung tissue and wherein said virus is human respiratory syncytial virus (RSV).

5. Method according to any of claims 1-4 wherein said nucleic acid is further quantified by quantitative real time polymerase chain reaction. (q-RT PCR).

6. Method according to any of claims 1-4 wherein said nucleic acid is further quantified by multi component nucleic acid enzyme technology (MNAzyme).

7. A method for identifying, detecting or quantifying the presence of at least one target Respiratory Syncytial Virus (RSV) comprising the following steps:

(a) providing two or more oligonucleotide components wherein at least a first oligonucleotide component and at least a second oligonucleotide

component are capable of self-assembly in the presence of said target to form a catalytically active multi-component nucleic acid enzyme (MNAzyme);

(b) contacting said oligonucleotide components with a sample putatively containing said at least one target under conditions:
(1) permitting the binding of said target to said oligonucleotide components and
(2) permitting the catalytic activity of the MNAzyme; and

(c) identifying, detecting or quantifying the presence of the catalytic activity of the MNAzyme, wherein the presence of the catalytic activity is indicative of the presence of said target.

8. A method according to any of claims 5 - 7 wherein the viral load of the target is quantified.

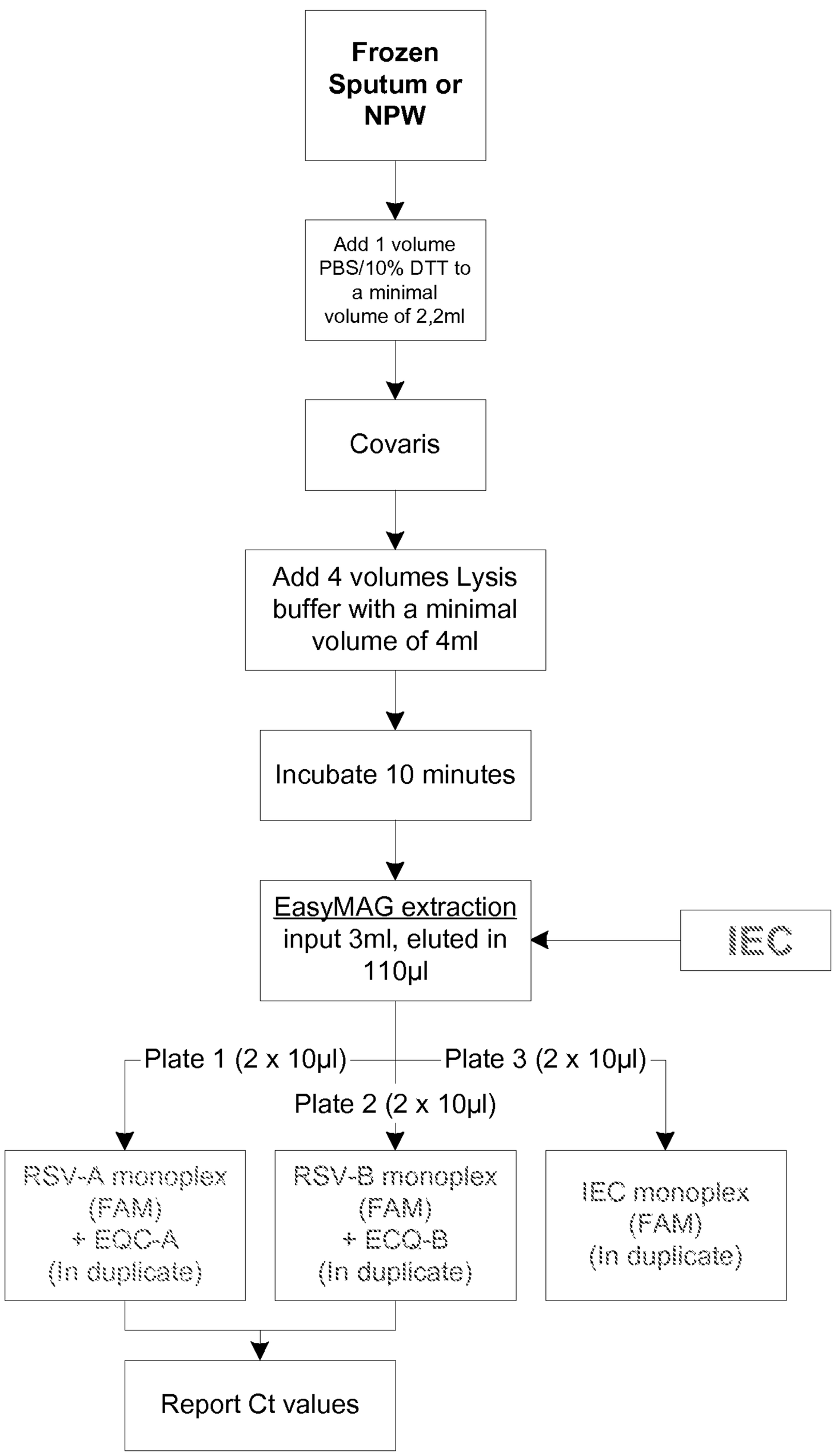


Figure 1. Diagnostic workflow for RSV viral load determination.

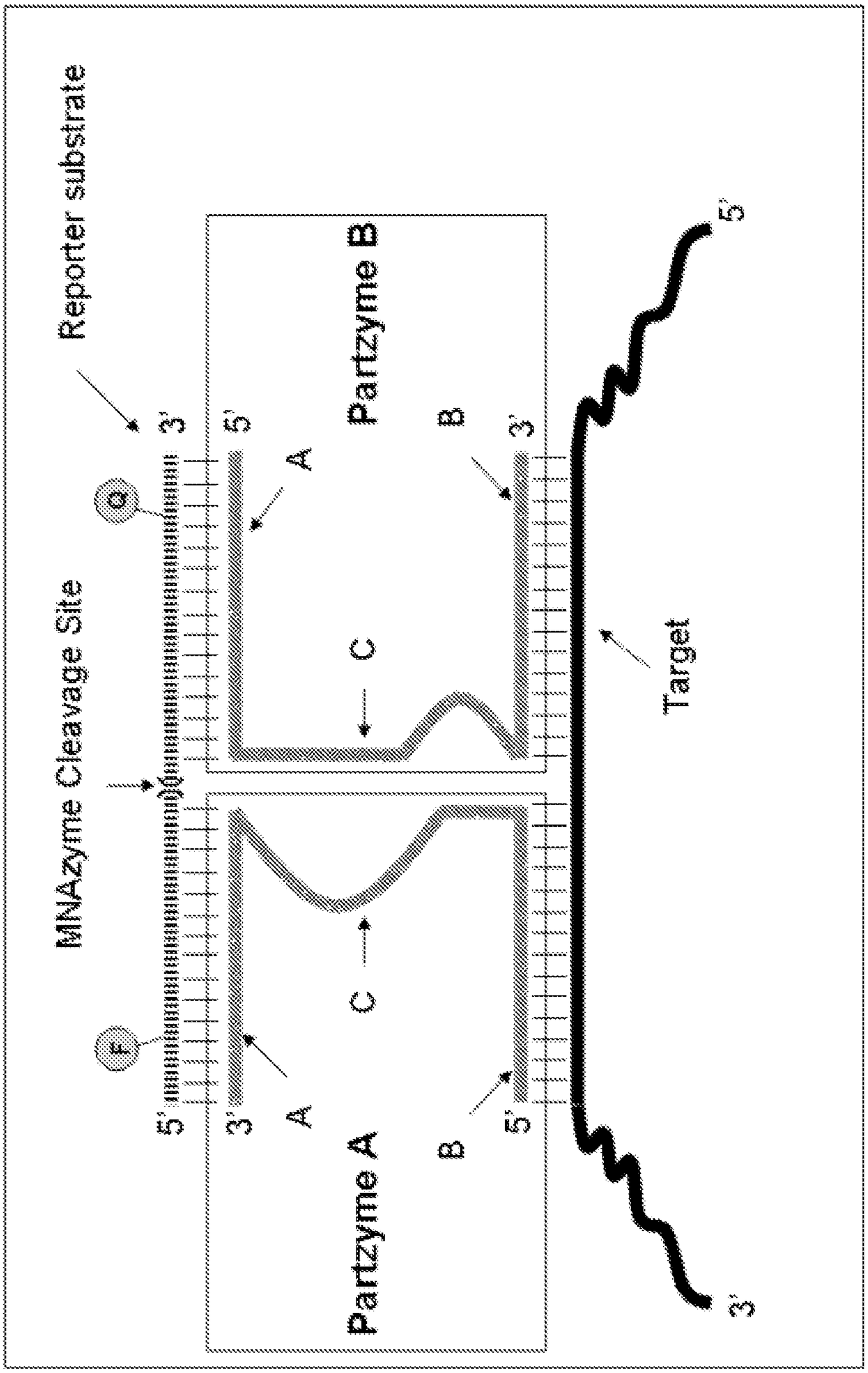

Reporter substrate
Reporter
3'
5'
A
Partzyme B
B
3'
Q
C
Cleavage Site
MNAzyme Cleavage Site
Target
5'
F
5'
3'
A
Partzyme A
C
B
5'
3'

Figure 2

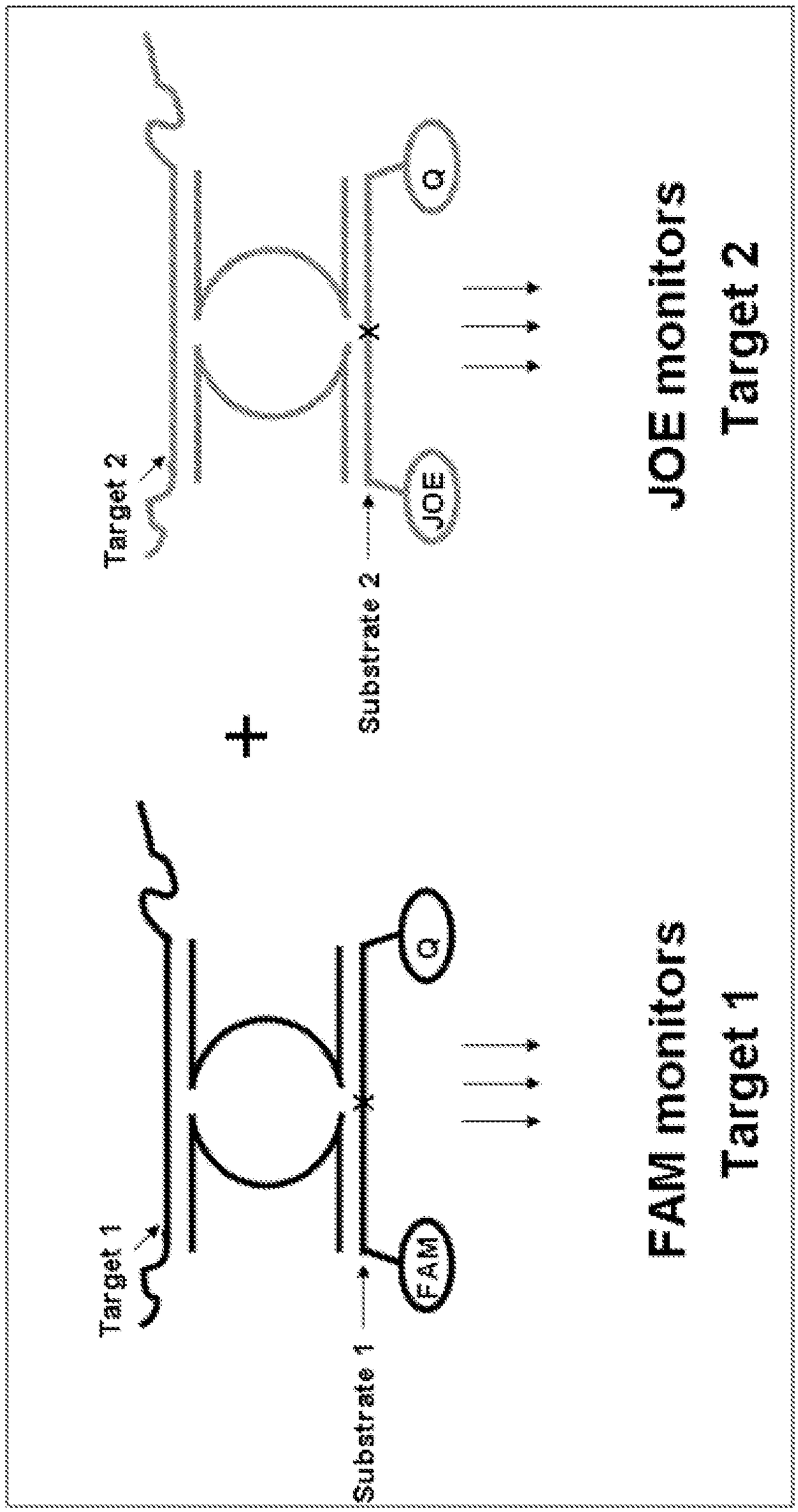
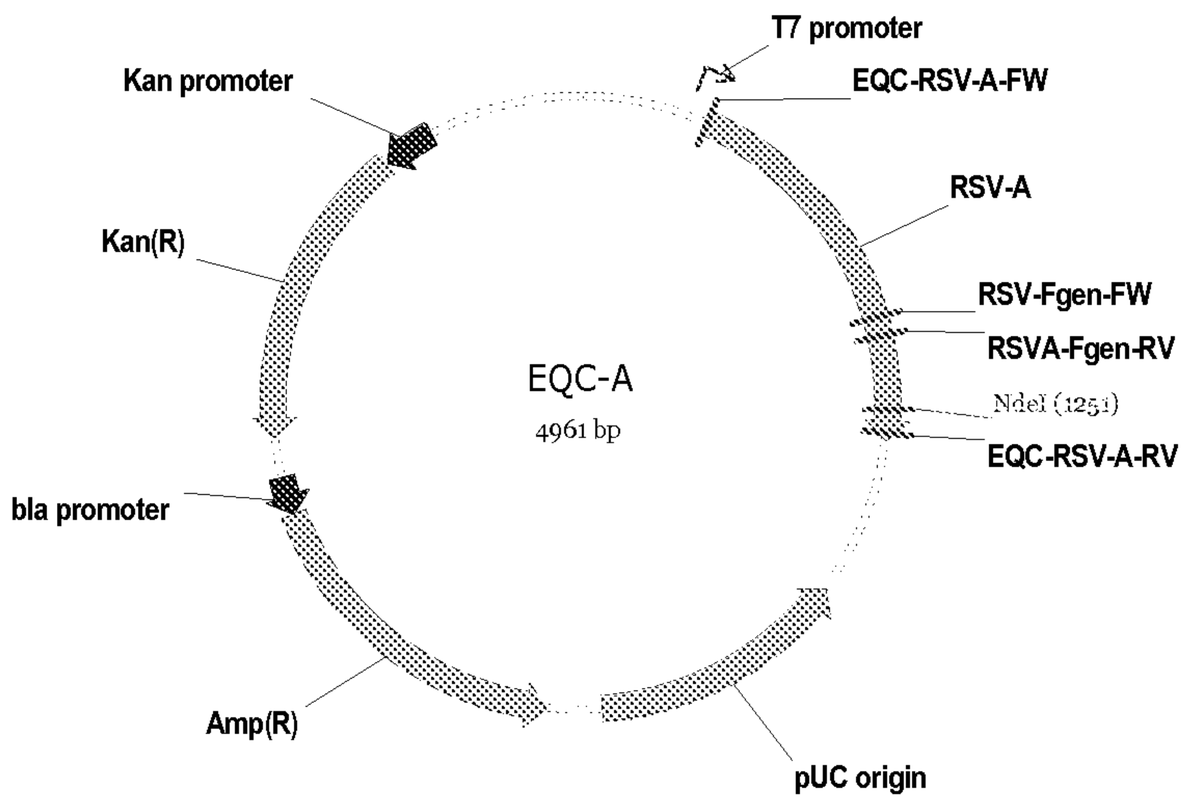

Target 2
Substrate 2
JOE
Q
JOE monitors
Target 2
Target 1
Substrate 1
FAM
Q
FAM monitors
Target 1

Figure 3

A.


T7 promoter
Kan promoter
EQC-RSV-A-FW
RSV-A
Kan(R)
RSV-Fgen-FW
RSVA-Fgen-RV
EQC-A
4961 bp
NdeI (1251)
EQC-RSV-A-RV
bla promoter
Amp(R)
pUC origin

B.

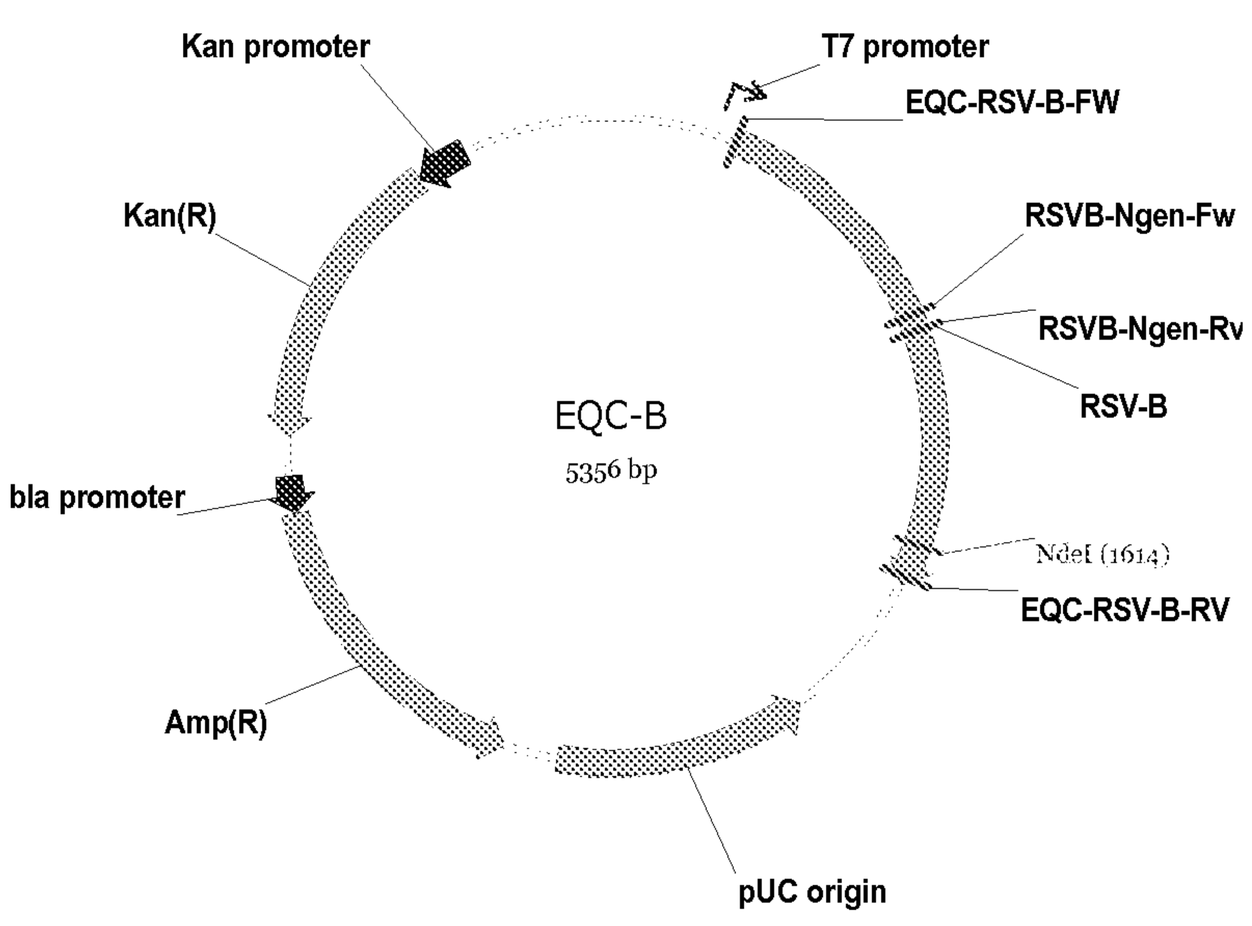

Kan promoter
T7 promoter
EQC-RSV-B-FW
Kan(R)
RSVB-Ngen-Fw
RSVB-Ngen-Rv
RSV-B
EQC-B
5356 bp
NdeI (1614)
EQC-RSV-B-RV
bla promoter
Amp(R)
pUC origin

Figure. 4.

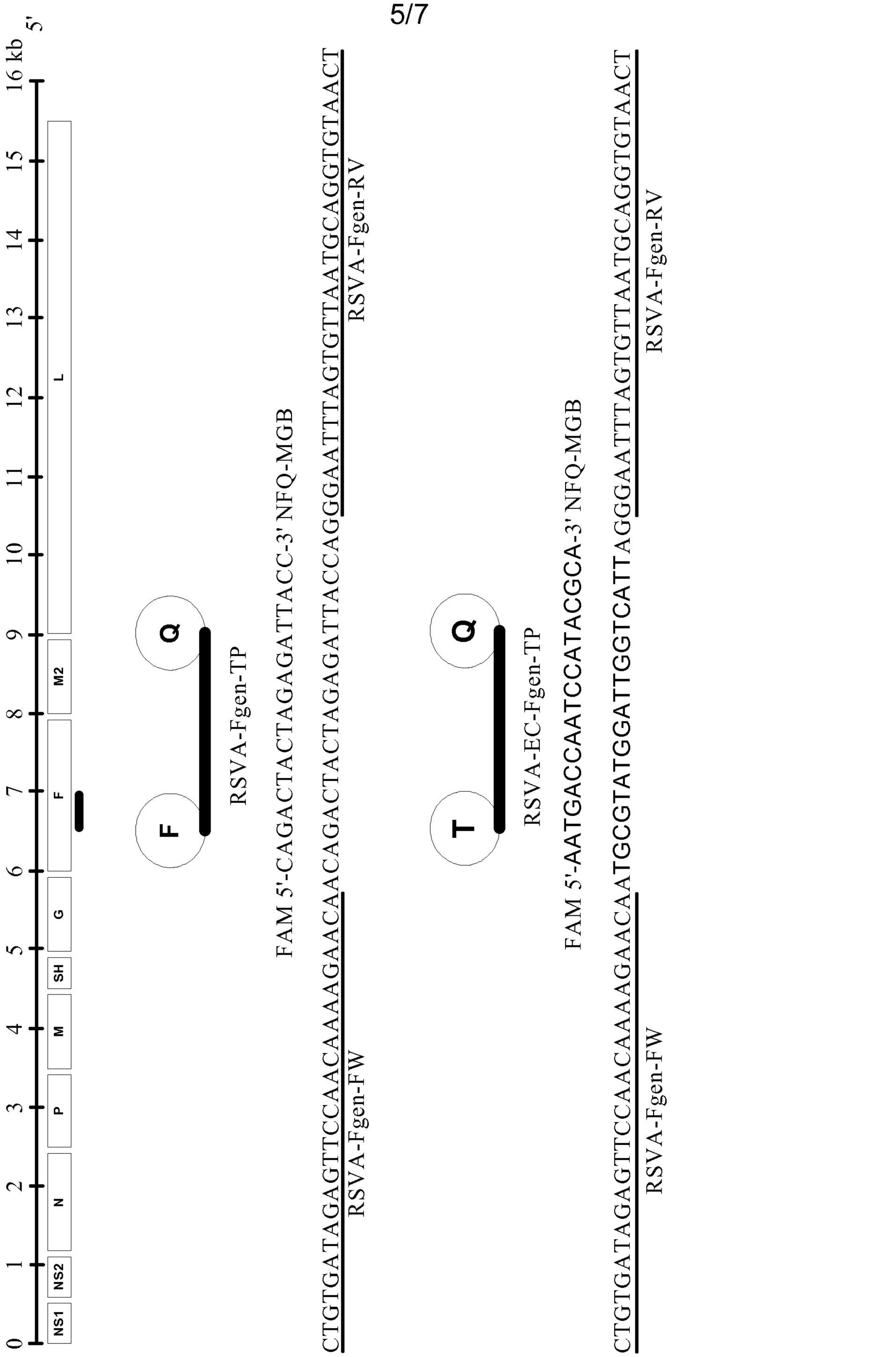

hRSV A/B
3'
5'
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16 kb
NS1
NS2
N
P
M
SH
G
F
M2
L
F
Q
T
Q
RSVA-Fgen-TP
FAM 5'-CAGACTACTCTAGAGATTACC-3' NFQ-MGB
RSVA-Fgen-FW
CTGTGATAGAGTTCCAACAAAAGAACAACAGACTACTCTAGAGATTACCAGGGAATTTAGTGTTAATGCAGGTGTAACT
RSVA-ECC-Fgen-TP
FAM 5'-AATGACCAACAATCCATACGGCA-3' NFQ-MGB
RSVA-Fgen-FW
CTGTGATAGAGTTCCAACAAAAGAACAATGCGTATGGATTGGTCATTAGGGAATTTAGTGTTAATGCAGGTGTAACT
RSVA-Fgen-RV

Figure 5a

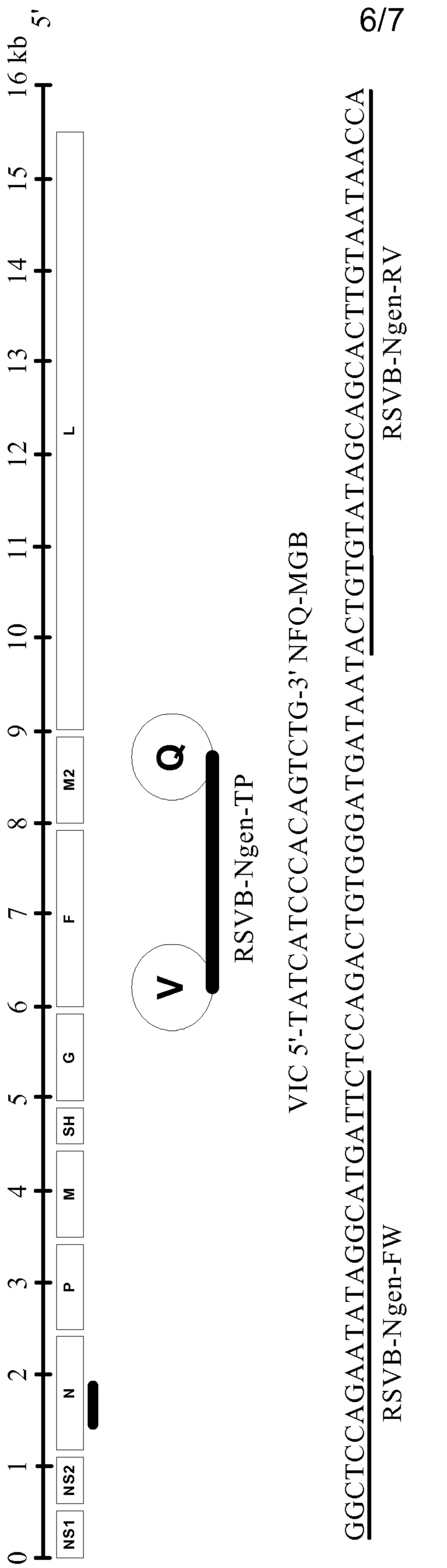
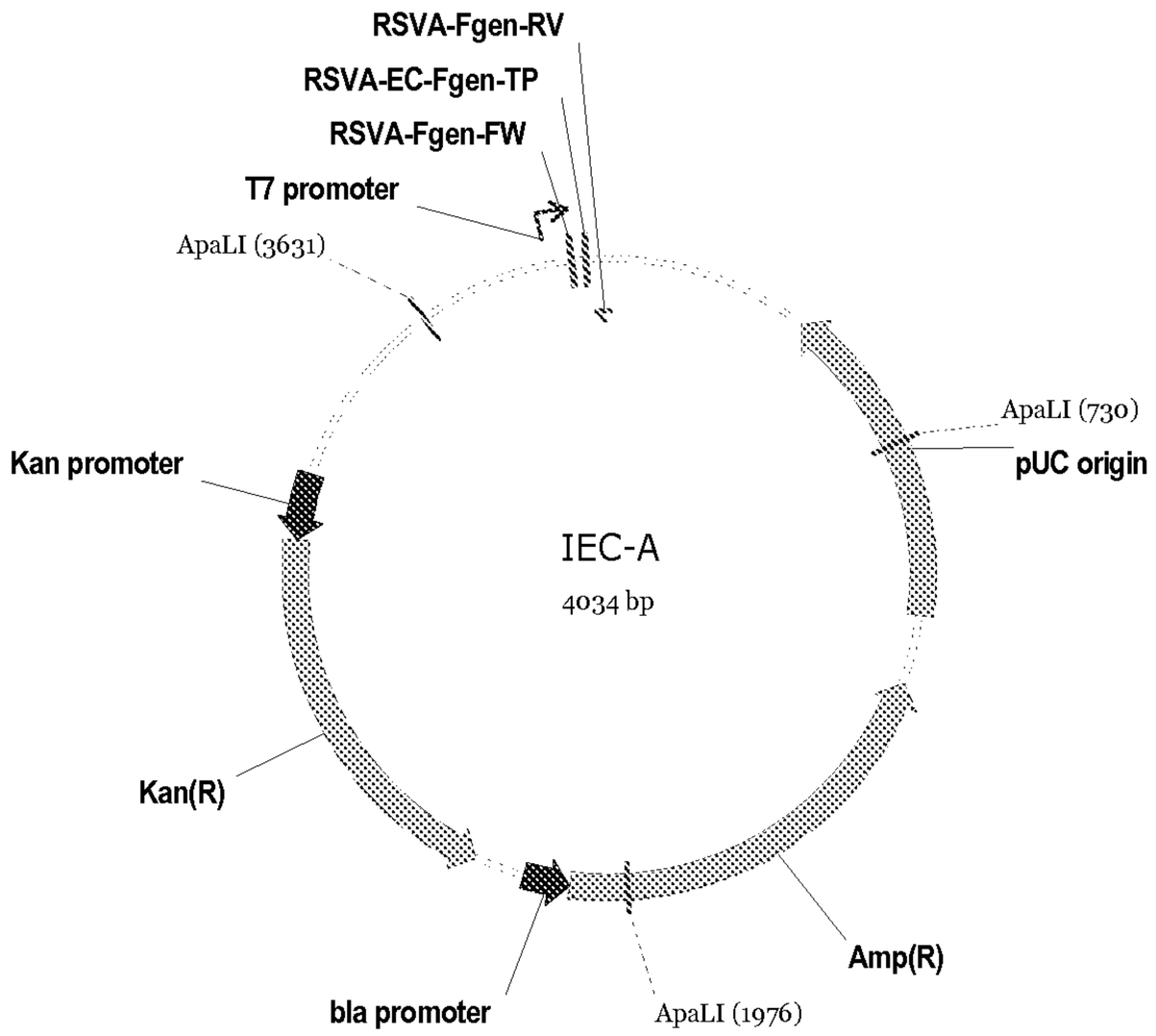

hRSV A/B
3'
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16 kb
5'
NS1
NS2
N
P
M
SH
G
F
M2
L
V
Q
RSVB-Ngen-TP
VIC 5'-TATCATCCACAGTCTG-3' NFQ-MGB
RSVB-Ngen-FW
GGCTCCAGAATATAGGCATGATTCTCCAGACTTGTAATAACCA
RSVB-Ngen-RV
N
Q
RSVB-EC-Ngen-TP
FAM 5'-TACCGTACTCTAGCCTA-3' NFQ-MGB
RSVB-Ngen-FW
GGCTCCAGAATATAGGCATGATTCTCTACCGTACTCTAGCCTAATACTGTGTATAGCAGCACTTGTAATAACCA
RSVB-Ngen-RV

Figure 5b

(A)


RSVA-Fgen-RV
RSVA-EC-Fgen-TP
RSVA-Fgen-FW
T7 promoter
ApaLI (3631)
ApaLI (730)
pUC origin
Kan promoter
IEC-A
4034 bp
Kan(R)
Amp(R)
bla promoter
ApaLI (1976)

(B)

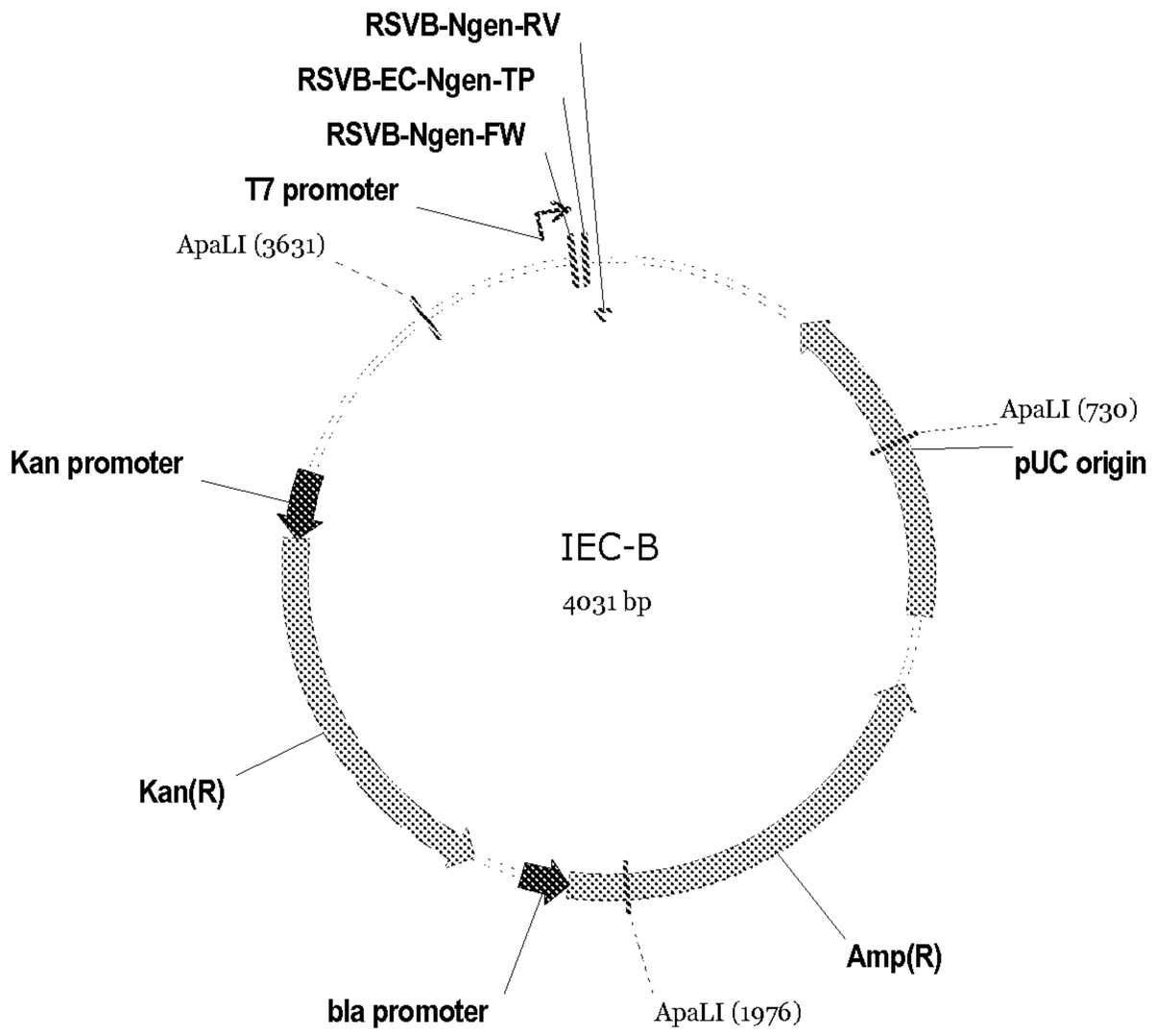

RSVB-Ngen-RV
RSVB-EC-Ngen-TP
RSVB-Ngen-FW
T7 promoter
ApaLI (3631)
ApaLI (730)
pUC origin
Kan promoter
IEC-B
4031 bp
Kan(R)
Amp(R)
bla promoter
ApaLI (1976)

Figure 6