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(54) Titre : DETECTION D'ACIDE NUCLEIQUE VIRAL ET METHODE POUR UNE TRANSCRIPTION INVERSE D'ARN
(54) Title: DETECTION OF VIRAL NUCLEIC ACID AND METHOD FOR REVERSE TRANSCRIBING RNA

(57) Abrégé/Abstract:
Provided are means and methods for efficiently reverse transcribing RNA obtained from a variety of sources. The invention further provides means and methods for efficiently and specifically detecting viruses and particularly human viruses in a sample.

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(54) Title: DETECTION OF VIRAL NUCLEIC ACID AND METHOD FOR REVERSE TRANSCRIBING RNA

(57) Abstract: Provided are means and methods for efficiently reverse transcribing RNA obtained from a variety of sources. The invention further provides means and methods for efficiently and specifically detecting viruses and particularly human viruses in a sample.

Title: Detection of viral nucleic acid and method for reverse transcribing RNA.

The invention relates to the fields of diagnostics and the copying and/or amplification of nucleic acid. In particular, the invention relates to means and methods for detecting virus using molecular biological tools.

The present invention is exemplified by means of viruses, in particular human enteroviruses, adenoviruses and influenza. However, the invention is by no means limited to enterovirus, adenovirus and influenza detection. Means and methods for isolating and reverse transcribing RNA are applicable using any type of RNA.

Human enteroviruses (EV) are members of the family Picornaviridae, are ubiquitous, and are mainly enterically transmitted. Enteroviruses have traditionally been identified by serotype-specific antisera in a virus-neutralizing test and 66 EV types are known to infect humans (20). Sixty-six EV serotypes were initially recognized and divided into 5 major groups: poliovirus (PV's; 1 to 3), Coxsackieviruses A (CAV's; 1 to 22 and 24), Coxsackieviruses B (CBV's; 1 to 6), echoviruses (1 to 7, 9, 11 to 27, and 29 to 33), and enteroviruses 68 to 71 (18). Recent molecular analyses have proven that echovirus 22 and 23 were genetically distinct from the members of the Enterovirus genus and were reclassified in a separate genus of Parechoviruses within the family of Picornaviridea (14, 21, 25). Infections with EV cause a wide range of clinical outcomes like asymptomatic infections, aseptic meningitis (meningeal inflammation in the absence of a bacterial pathogen), encephalitis, paralytic poliomyelitis, and myocarditis. Although the majority of EV infections do not cause significant disease, infection can cause serious illness, especially in infants and immune-compromised patients. EV infections are the most common cause of aseptic meningitis and account for 80 – 90% of all cases of CNS infection for which a possible causative agent is identified (26). In the neonate, aseptic meningitis induced complications and poor outcome of EV infections generally occur within the first 2 days of life

(1, 3). Aseptic meningitis in immune-competent adults is characterized by sudden onset of fever but neurological abnormalities are rare and both short term and long term outcome are generally good. Encephalitis caused by EV infections is a less common but a more severe disease than aseptic meningitis (19, 31, 32). Immune-compromised children and adults who are infected with EV may develop chronic meningitis and encephalitis, which may last years before becoming fatal (17).

Early clinical symptoms of meningitis caused by viruses, bacteria, and fungi are quite similar and difficult to distinguish but diagnosis, therapy, and outcome of disease caused by these pathogens vary considerably. Reliable laboratory diagnosis for EV is needed since specific and rapid diagnosis of EV meningitis has a significant impact on patient's management (11). Although, RT-PCR may become the diagnostic method of choice for EV infections of CNS, in many laboratories diagnosis of EV still relies on cell culture techniques. Stool specimens or rectal swabs, throat swabs, and CSF are used in virus culture. However, EV can be detected by RT-PCR in all kinds of clinical specimens like whole blood, plasma, serum, CSF, Stool specimens, throat swabs, vesicle fluids, pleuratic fluids, broncheo-alveolar-lavages, amniotic fluids, urine, and brain biopsies. Early in the acute phase of the illness, EV is frequently isolated from the throat whereas isolation of virus from CSF provides the most direct link to disease but is usually less successful. Cytopathic effect (CPE) is not recognizable within a few days and some enteroviruses do not grow in cell culture and therefore do not cause CPE at all (16, 27).

In recent years, several EV RT-PCR assays have been developed which are sensitive and rapid (9, 10, 15, 27, 28). Although these methods improved the sensitivity and speed of the detection of enteroviruses, it was found that the sensitivity can be further improved by means and methods of the present invention by 40%, range 25-55% (Table 5).

The invention therefore provides a method for reverse transcribing an RNA target molecule comprising incubating said RNA target molecule with a reverse

transcriptase. Preferably, the reverse transcriptase is a Moloney Murine Leukemia Virus Reverse Transcriptase [M-MLV RT], more preferably a reverse transcriptase known as SuperScript II or III (RNase H- Reverse Transcriptase) described in Potter et al, Focus Vol 25.1; pp 19-24. SuperScript II contains a series of point mutations in the Rnase H domain of M-MLV, or a functional part, derivative and/or analogue of said SuperScript II or III, in a solution comprising between 2,5 and 7,5 mM of a suitable salt, preferably 5.0 mM, between 0.05 % and 0.2 % of a non-ionic detergent, preferably 0.1%, between 60 and 240 µM per nucleotide, preferably 120 µM and a suitable buffer that buffers said solution at a pH between about 8 or 10, said method further comprising incubating said solution at a temperature of between 25 and 50 ^{0}C.

A functional part, derivative and/or analogue of superscript II or III comprises the same activity in kind not necessarily in amount as superscript II or III themselves under the above mentioned conditions. Superscript and suitable parts, derivatives and analogues are described in Potter J. et al (2003): Focus, Vol 25.1, pp 19-24). Suitable salts are salts of group I of the periodic system, preferably a sodium or potassium salt. The counter-ion in the salt is preferably chloride. The non-ionic detergent is non-denaturing and significantly improves both the yield and quality of the reverse transcriptase (RT) product. The non-ionic detergent is preferably non-idet p40, or triton X100, or a combination or an equivalent thereof. Preferably the non-ionic detergent is Triton X100 or an equivalent thereof. The non-ionic detergent is preferably present in amount of between about 0.05 and 0.2 % (vol/vol). Preferably the non-ionic detergent is present in an amount of about 0.1% (vol/vol). Preferably, all four of different categories of nucleotides characterised by dATP, dCTP, dGTP and dTTP are added to the solution. One or more of the categories may be omitted, for instance when a specific typically short RT product is desired. However, at least one of the above mentioned categories is required in the solution. Currently, many different derivatives and analogues of the prototype nucleotides are available. Such derivatives and analogues, when they can be incorporated into the nascent strand, can also be used in the present invention. It is also possible to add one or more so-called stopper nucleotides in the solution for, for instance, sequencing

purposes. In general any nucleotide or derivative and/or analogue thereof can be used in the present invention. A particular category of nucleotide is typically present in an amount of between about 60 and 240 μM per nucleotide. Preferably, a particular category of nucleotide is present in an amount of between about 100 and 140 μM, more preferably in an amount of about 120 μM. The temperature range is typically a range wherein superscript is active. Preferably, the temperature range is between about 25 and 50 ^{0}C. Preferably, between about 40 and 50^{0}C. More preferably, the incubation temperature is about 42 ^{0}C for Superscript II or a functional part, derivative and/or analogue thereof and about 50 ^{0}C for Superscript III or a functional part, derivative and/or analogue thereof. The buffer can be any compound when it is suitable for use in enzymatic processes and capable of buffering a solution between about pH 8 and 10. Preferably, buffers common in enzymatic processes in molecular biology are used. Preferably the buffer comprises TRIS or HEPES or a combination or equivalent thereof. Preferably, the solution is buffered by said buffer at a pH of between about 8 and 10. Preferably between about pH 8 and 9. Preferably, at an pH between about 8.1 and 8.5. More preferably the solution is buffered by said buffer at an pH of about 8.3. Counter ions for adjusting the buffer to the desired pH are preferably sodium, potassium and chloride. For efficient reverse transcription to take place it is important that the reverse transcriptase primes efficiently. Efficient priming requires, as yet, the presence of a primer for the reverse transcriptase. The RNA itself can act as a primer as, for instance, is the case with genomic retroviral RNAs. However, typically at least one primer is added to the solution. The primer can be any oligonucleotide or equivalent thereof that is capable of hybridising to the RNA of interest and capable of being elongated by the reverse transcriptase. An equivalent of a primer oligonucleotide for RT priming comprises the same hybridisation and RT priming capability in kind not necessarily in amount. The primer oligonucleotide typically comprises between about 4 and 30 nucleotides or equivalent thereof. For random priming, the primer typically contains between about 4 to 10 nucleotides or equivalent thereof. For specific priming the primer typically contains between about 10 and 30 nucleotides or equivalents thereof, more preferably between about 15 and 25

nucleotides, preferably between about 18 and 22 nucleotides, more preferably about 20 nucleotides.

In a particularly preferred embodiment of random priming the primer is a hexamer.. For random priming it is preferred that the primer is a mixture of at least 5 and preferably at least 10 and more preferably at least 20 oligonucleotides or equivalent thereof, wherein said oligonucleotides or equivalents thereof differ from each other by at least one nucleotide. In a particularly preferred embodiment the primer is a hexamer mixture comprising at least 20 of the mentioned different oligonucleotides or equivalents thereof.

The RNA may be derived from any sample. However, it was found that the incubation conditions for reverse transcribing RNA are more robust than other conditions and allow for efficient reverse transcription of input RNA also when samples are obtained from notoriously difficult sources such as faeces. Thus in a preferred embodiment the invention provides a method of the invention further comprising preparing RNA target molecule from a sample comprising faeces, preferably human faeces.

In one aspect of the invention, the RNA target molecule comprises sequences derived from an enterovirus, preferably a human enterovirus (EV) or an influenza virus, preferably influenza A. Until recently the presence or absence of EV in faeces was analysed using cell lines to propagate any virus from the faeces. Of late, molecular diagnostics have been developed based on for instance amplification of enteroviral nucleic acid. Thus in one embodiment a method of the invention further comprises amplifying produced RT product using one or more primers. In previous studies, EV-RNA was purified without a control (IC-RNA) that monitored both RNA extraction efficiency and RT-PCR efficiency. In molecular diagnostics, the use of an IC-RNA is used for a reliable interpretation of results because it enables the verification of the sensitivity of the assay and avoids false negative results. Thus in one embodiment a method of the invention further comprises providing a sample containing the RNA target molecule with an internal control nucleic acid. Preferably this internal control nucleic acid comprises RNA. This may be done by adding the internal control to the sample or

by adding the internal control already at an earlier stage, for example to the faeces or the isolated RNA there from. Thus in a preferred embodiment said solution further comprises an internal control RNA molecule. *In vitro* transcribed RNA can be used as an IC-RNA but is prone to degradation by RNAses. Packaging of IC-RNA into a phage protects the RNA from enzymatic degradation (24). These armoured RNA's can be spiked into clinical specimens without degradation, thus enabling simultaneous monitoring of the complete nucleic acid extraction and amplification process for each specimen. Thus in a preferred embodiment the internal control nucleic acid comprises armoured RNA. In a preferred embodiment the armoured RNA is prepared using the means and methods disclosed herein or disclosed in (24). Alternatively, or additionally, another method for at least in part avoiding degradation of RNA by RNAses is applied. Preferably the internal control RNA comprises a sequence 5'-CCC TGA ATG CGG CTA ATC CTA ACC ACG GAA CAG GCG GTC GCG AAC CAG TGA CTG GCT TGT CGT AAC GCG CAA GTC TGT GCT TGA GAC GTG CGT GGT AAC CGT CCG TGT TTC CTG TTA TTT TTA TCA TGG CTG CTT ATG GTG ACA AT-3'.

Like the wild-type EV target, IC-RNA's should have about the same length, contain the same primer binding sites, and have about the same GC content for identical extraction and amplification efficiency but should contain a different probe binding site for the differential detection of IC-RNA. Thus in a preferred embodiment the internal control nucleic acid comprises about the same length, primer binding site(s) and GC content as the region in the EV RNA that is selected for reverse transcription and subsequent amplification. To discriminate between target and internal control said internal control comprises at least one location where the nucleic acid encodes a sequence that differs from at least all human enteroviruses. Detection of this region, for instance, by means of a probe thus can distinguish between internal control and EV-RNA. In a preferred embodiment the region that is selected for amplification comprises sequences that are conserved between human enteroviruses as this allows the use of one primer set for the detection of all (currently identified) enteroviruses. Preferably the selected region further comprises, a further location that is conserved

between human enteroviruses, wherein the location is internal to the amplified region and does essentially not exhibit overlap with the location of the primer(s). Such a further conserved region allows the use of one detection means such as a probe for the detection of essentially all (currently known) human enteroviruses. Thus in one particularly preferred aspect the invention provides a method wherein said internal control RNA molecule comprises a sequence 5'-CCC TGA ATG CGG CTA ATC CTA ACC ACG GAA CAG GCG GTC GCG AAC CAG TGA CTG GCT TGT CGT AAC GCG CAA GTC TGT GCT TGA GAC GTG CGT GGT AAC CGT CCG TGT TTC CTG TTA TTT TTA TCA TGG CTG CTT ATG GTG ACA AT-3'. The primer(s) for amplification of this sequence allows amplification of essentially all human enteroviruses, whereas it further span a region that further comprises a further conserved region for the easy detection of amplified material from all, currently known, human enteroviruses. This sequence, and in particular the choice of the primer binding sites has the additional advantage that, whereas assay in the art detect also human viruses related to the human enteroviruses, the assay of the invention is more selective as at least the closely related human rhinoviruses are not detected using an assay of the invention. This feature of course greatly improves the reliability of the assay of the invention.

Thus in a particularly preferred embodiment the invention further provides a method for testing a sample for the presence therein of a nucleic acid derived from an enterovirus, comprising providing a nucleic acid amplification solution with said sample, with a first primer comprising a nucleotide sequence ID#1: 5'-CCC TGA ATG CGG CTA AT-3' or a functional equivalent thereof and with a second primer comprising a nucleotide sequence (ID#2: 5'-ATT GTC ACC ATA AGC AGC C-3' or a functional equivalent thereof, and incubating said amplification solution to allow for amplification of said nucleic acid when present. A functional equivalent of a primer comprises the same 10 bases when measured from the 3'-end but contains a mismatch of preferably 2, and more preferably 1 nucleotide in the 10 bases when measured from the 5'-end. It has been found that such functional equivalents comprise essentially the same specificity and amplification efficiency as the original they are derived from. The 3' end of a

functional equivalent of a primer of the invention is preferably identical to said primer, while the 5' end of said functional equivalent may differ from said primer such that the properties essentially remain the same in kind, not necessarily in amount. According to the invention, the 5' end of a primer of the invention can be varied while retaining the properties of said primer in kind, not necessarily in amount. In one embodiment, a functional equivalent of a primer of the invention contains at most 30, preferably at most 25, more preferably at most 20, more preferably at most 15, most preferably at most 10 additional nucleotides at its 5' end as compared to said primer. Furthermore, at least one nucleotide of said functional equivalent may be changed as compared to said primer, preferably in its 5' end region.

Said sample tested with a method of the invention preferably comprises product of a method for reverse transcribing an RNA target molecule according to the invention. In a preferred embodiment a method of the invention further comprises analysing the presence or absence of said nucleic acid by means of a probe. Preferably the presence of nucleic acid derived from a (human) enterovirus is analysed with a probe comprising a sequence ID#3: EV-specific probe; 5'-GCG GAA CCG ACT ACT TTG GGT-3' or a functional equivalent thereof. In a preferred embodiment a method for testing a sample of the invention further comprises analysing the presence or absence of an internal control nucleic acid by means of a probe comprising a sequence ID#4: IC-specific probe; 5'-CTT GAG ACG TGC GTG GTA ACC-3 or a functional equivalent thereof. Preferred primers, probes and internal control nucleic acid suitable for detecting enterovirus are depicted in Table 13.

The invention furthermore provides primers, probes and internal control nucleic acids which are preferably used for testing a sample for the presence therein of a nucleic acid derived from viruses other than enteroviruses. Tables 6-12 depict primers, probes and internal control nucleic acids suitable for testing a sample for the presence therein of a nucleic acid derived from influenza A virus, influenza B virus, metapneumovirus, Respiratory Syncitial Virus, Rhino virus,

Adenovirus and Parainfluenza virus. In these tables, R means G or A; S means G or C and Y means T or C. When at least one primer of tables 6-13 is used for amplification/detection of viral nucleic acid, the sensitivity and/or selectivity of the detection method is improved as compared to conventional methods of the art. According to the present invention, a primer of the invention provides an improved result, even if a prior art primer is capable of annealing somewhere in the same kind of viral nucleic acid region as a primer of the present invention. This is for instance shown in example 4. Hence, a primer of the invention provides a more sensitive and/or more specific assay. The invention thus provides a method for testing a sample for the presence therein of a nucleic acid derived from a virus, comprising providing a nucleic acid amplification solution with at least part of said sample and with a first and a second primer, wherein said first primer comprises a nucleotide sequence of a forward primer depicted in any one of tables 6-13, or a functional equivalent thereof, and/or wherein said second primer comprises a nucleotide sequence of a reverse primer depicted in any one of tables 6-13, or a functional equivalent thereof, and incubating said amplification solution to allow for amplification of said nucleic acid when present. For instance, if the presence of Influenza A nucleic acid in a sample is investigated, a nucleic acid amplification reaction is preferably provided with said sample and with at least one oligonucleotide comprising a sequence of a primer as depicted in Table 6, or at least one equivalent thereof.

Preferably, both primers of any one of tables 6-13, or at least one functional equivalent of at least one of said primers, is used. Hence, if the presence of Influenza A nucleic acid in a sample is investigated, a nucleic acid amplification solution is most preferably provided with said sample, with an oligonucleotide comprising a sequence of the forward primer depicted in Table 6, and with an oligonucleotide comprising a sequence of the reverse primer depicted in Table 6, or at least one functional equivalent of at least one of said primers. If the presence of Influenza B nucleic acid in a sample is investigated, a nucleic acid amplification solution is preferably provided with said sample and with an oligonucleotide comprising a sequence of the forward primer depicted in Table 7, and with an oligonucleotide comprising a sequence of the reverse primer depicted

in Table 7, or at least one functional equivalent of at least one of said primers. If the presence of adenoviral nucleic acid in a sample is investigated, a nucleic acid amplification solution is preferably provided with said sample and with an oligonucleotide comprising a sequence of the forward primer depicted in Table 11, and with an oligonucleotide comprising a sequence of the reverse primer depicted in Table 11, or at least one functional equivalent of at least one of said primers, et cetera. In a preferred embodiment a method of the invention is provided wherein said virus comprises an enterovirus. In a further preferred embodiment said virus comprises an adenovirus or influenza virus, because these viruses are commonly found in individuals.

One embodiment therefore provides a method of the invention, wherein said first primer comprises the sequence 5'-CAGGACGCCTCGGRGTAYCTSAG-3' or a functional equivalent thereof and said second primer comprises the sequence 5'-GGAGCCACVGTGGGRTT-3' or a functional equivalent thereof. These primers are particularly suitable for testing a sample for the presence of adenoviral nucleic acid. One further preferred embodiment provides a method of the invention, wherein said first primer comprises the sequence 5'-GACAAGACCAATCCTGTCACYTCTG-3' or a functional equivalent thereof and said second primer comprises the sequence 5'-AAGCGTCTACGCTGCAGTCC-3' or a functional equivalent thereof. These primers are particularly suitable for testing a sample for the presence of influenza A nucleic acid.

In case a sample is tested for the presence of nucleic acid derived from an RNA virus, said RNA is preferably converted into DNA. This is most preferably performed with a method of the present invention. Said sample therefore preferably comprises a product of a method for reverse transcribing an RNA target molecule according to the invention.

In a preferred embodiment a method of the invention further comprises analysing the presence or absence of viral nucleic acid by means of a probe. Preferably the presence or absence of nucleic acid derived from a virus is analysed with a probe comprising a nucleotide sequence of the virus-specific

probe depicted in the same table as said forward primer and/or said reverse primer, or a functional equivalent thereof. Hence, if at least one oligonucleotide comprising a sequence of a primer of Table 6, or a functional equivalent of said primer, is used for amplifying influenza A nucleic acid, a virus-specific probe as depicted in Table 6, or a functional equivalent thereof, is preferably used. Likewise, if sample nucleic acid is amplified using at least one oligonucleotide comprising a sequence of a primer of another Table (or a functional equivalent thereof), a virus-specific probe of the same Table is preferably used.

A method of the invention comprising analysing the presence or absence of viral nucleic acid with a probe comprising a nucleotide sequence of the virus-specific probe depicted in the same table as said forward primer and/or said reverse primer, or a functional equivalent of said probe, is therefore preferably provided. In one preferred embodiment, the presence of nucleic acid derived from an enterovirus is analysed with a probe comprising a sequence ID#3: EV-specific probe; 5'-GCG GAA CCG ACT ACT TTG GGT-3'or a functional equivalent thereof. In a further preferred embodiment the presence of nucleic acid derived from an adenovirus is analysed with a probe comprising a sequence 5'-CCGGGTCTGGTGCAGTTTGCCCGC-3'or a functional equivalent thereof. In yet another preferred embodiment the presence of nucleic acid derived from an influenza A virus is analysed with a probe comprising a sequence 5'-TTCACGCTCACCGTGCCCAGTGAGC-3'or a functional equivalent thereof.

In order to be capable of verifying the sensitivity of an assay of the invention, and to avoid false negative results, an internal control nucleic acid is preferably used. If the presence of RNA derived from an RNA virus in a sample is investigated, said internal control nucleic acid preferably also comprises RNA. Likewise, if the presence of DNA derived from a DNA virus in a sample is investigated, said internal control nucleic acid preferably comprises DNA. This allows for accurate verification of assay sensitivity and for accurately avoiding false negative results. In one embodiment a method of the invention therefore further comprises providing said nucleic acid amplification solution with an internal control nucleic acid molecule. This is for instance performed by adding said internal control to

said amplification solution and/or by adding said internal control to at least part of said sample which is used in an amplification reaction. As already explained above, said internal control comprises at least one location where the nucleic acid comprises a sequence that differs from all viruses that are tested for. Preferably, an internal control depicted in any one of tables 6-13 is used. If an oligonucleotide comprising a primer sequence of one specific table (or a functional equivalent thereof) is used, it is preferred to use an oligonucleotide comprising the internal control nucleic acid sequence of the same table (or a functional equivalent thereof). A combination of oligonucleotides comprising at least one primer, probe and/or internal control nucleic acid sequence of any one of Tables 6-13, or at least a functional equivalent of said at least one primer, probe and/or internal control, enhances the sensitivity and/or specificity of a method of the invention. The invention therefore provides a method of the invention, wherein said internal control nucleic acid molecule comprises a nucleotide sequence of the internal control depicted in the same table as said primer, or a functional equivalent of said internal control. As explained above, an internal control nucleic acid of the invention preferably contains the same primer binding sites, although this is not necessary. Said internal control nucleic acid furthermore preferably has essentially the same length and preferably has about the same GC content for essentially the same extraction and/or amplification efficiency but should contain a different probe binding site for differential detection of internal control nucleic acid. Detection of said internal control nucleic acid by a different IC-specific probe enables distinguishing between internal control and viral nucleic acid. Preferably, an IC-specific probe comprising a sequence as depicted in Tables 6-13 is used. The invention thus provides a method of the invention, further comprising analysing the presence or absence of said internal control nucleic acid by means of a probe comprising a nucleotide sequence of the IC-specific probe depicted in the same table as said internal control, or a functional equivalent of said probe. Preferably, said probe comprises the sequence 5'-CTT GAG ACG TGC GTG GTA ACC-3' , 5'-GATGTGTCCGCCGTGGTCCCCTGG-3', and/or 5'-TTCACTGGGCCCGACTCGCACTGAC-3', or a functional equivalent thereof,

in order to test a sample for the presence of a nucleic acid derived from enterovirus, adenovirus and/or influenza A, respectively.

For detection probes may be labelled by any means suitable. Many different methods are known in the art. Amplified product can be measured directly or indirectly through measurement of label associated with the probe. Amplified product may be measured at the end of the amplification step or in real-time during amplification. Such methods are known in the art. Real time measurement of amplified product is preferred because results are obtained faster with less effort, while the sensitivity is increased, as compared to end-point PCR, and the method is less prone to contamination. When measured in real time, a probe preferably comprises a fluorophore. Other preferred labels include radio-active and/or digoxigenine labels. Label may be associated directly to the probe, or the probe may be detected indirectly, through a labelled binding member specific for said probe.

In a further preferred embodiment, a sample is tested with a method of the invention for the presence therein of at least two different viruses. For instance, if an individual shows clinical signs of infection of the respiratory tract, it is preferred to test a sample of said individual for the presence of a variety of viruses capable of infecting the respiratory tract. In that case, a sample is preferably tested for the presence of nucleic acid of at least two viruses depicted in tables 6-13. Said two viruses preferably comprise Adenovirus and Influenza. A method of the invention is preferably performed using at least two oligonucleotides comprising at least one primer, probe and/or internal control sequence depicted in a first table selected from the group consisting of tables 6-13, or at least one functional equivalent thereof, and at least two oligonucleotides comprising at least one primer, probe and/or internal control sequence depicted in a second table selected from the group consisting of tables 6-13, or at least one functional equivalent thereof. In one embodiment the invention provides a method of the invention, wherein a sample is tested for the presence of a first virus as depicted in any one of Tables 6-13 and for the presence of a second virus

as depicted in any one of Tables 6-13, using at least two primers depicted in the same Tables as said first and said second virus.

More preferably, a sample is tested for the presence of nucleic acid of at least three viruses depicted in tables 6-13, preferably using oligonucleotides comprising at least one primer, probe and/or internal control sequence depicted in a first table of the invention, comprising at least one primer, probe and/or internal control sequence depicted in a second table of the invention, and comprising at least one primer, probe and/or internal control sequence depicted in a third table of the invention or at least one functional equivalent thereof, et cetera.

It is desirable to combine as many assays as possible in a single assay format. This increases the chance of a patient being diagnosed correctly.

In a preferred embodiment, a sample is tested in the same assay for the presence of nucleic acid derived from at least two virus species. More preferably, a sample is tested in the same assay for the presence therein of nucleic acid derived from at least three virus species. Hence, a nucleic acid amplification solution is preferably provided with at least part of a sample and with primers of the invention capable of selectively amplifying at least two virus species. Said nucleic acid amplification solution is preferably furthermore provided with probes of the invention specific for said at least two virus species. More preferably, said nucleic acid amplification solution is furthermore provided with at least one internal control and a probe specific for said internal control.

In a particularly preferred embodiment oligonucleotides comprising primer, probe and/or internal control sequences of at least two, preferably at least three, more preferably at least four, most preferably all, tables selected from the group consisting of tables 6-13, or a functional equivalent of at least one of said primer/probe/internal control sequences, are used in a single assay. The target viral nucleic acid and the internal control are preferably detected in a multiplex format, e.g. a microarray or fluidic beads system. This way, it is possible to rapidly test a sample for a variety of viruses. In a most preferred embodiment, oligonucleotides comprising primer, probe and/or internal control sequences of at

least two, preferably at least three, most preferably at least four tables selected from the group consisting of tables 6-13, or a functional equivalent of at least one of said primer/probe/internal control sequences, are used for a real-time PCR in one assay. Usually, multiplex amplification is only possible for two or at most three different nucleic acid sequences because a plurality of primers, probes and internal controls is at risk of hybridizing with each other instead of their target sequences. However, the primers, probes and internal control sequences of the invention are designed such that oligonucleotides comprising primer, probe and/or internal control sequences of the invention are suitable for amplification and detection of more than two, preferably more than three, more preferably more than four viral nucleic acid sequences simultaneously in the same assay, preferably in a real time PCR. This embodiment therefore provides a rapid, sensitive and reliable test for a wide variety of viruses.

In another aspect the invention provides an aqueous solution for reverse transcribing an RNA target molecule comprising between 25 and 75 mM of a suitable salt, between 0.05 % and 0.2 % of a non-ionic detergent and a suitable buffer that buffers said solution at a pH between about 8 or 10. Preferably said solution further comprises between 60 and 240 µM per nucleotide or equivalent thereof and a reverse transcriptase mentioned above or a functional part, derivative and/or analogue thereof. The invention also provides a concentrate of an aqueous solution of the invention. Preferably the concentrate comprises between 5 and 10 times the concentration of the chemicals mentioned for the aqueous solution of the invention.

In another aspect the invention provides an isolated or recombinant oligonucleotide comprising a sequence as depicted in any one of tables 6-13. Said oligonucleotide is suitable for performing a method of the invention. Preferably, a combination of oligonucleotides comprising at least two sequences depicted in one table selected from tables 6-13 is used. Such combination is particularly suitable for testing a sample for the presence therein of viral nucleic acid. Preferably, a combination of two primers is provided, comprising a first primer comprising a

sequence of a forward primer depicted in any one of tables 6-13, or a functional equivalent thereof, and a second primer comprising a sequence of the reverse primer depicted in the same table as said forward primer. The invention therefore provides a primer pair comprising a primer comprising a sequence of a forward primer as depicted in any one of Tables 6-13, or a functional equivalent thereof, and a primer comprising a sequence of the reverse primer depicted in the same table as said forward primer, or a functional equivalent thereof. A preferred primer pair comprises a primer comprising a sequence of a forward primer as depicted in Table 6, or a functional equivalent thereof, and a primer comprising a sequence of the reverse primer depicted in Table 6, or a functional equivalent thereof. This primer pair is particularly suitable for testing a sample for the presence of Influenza A. Another preferred primer pair comprises a primer comprising a sequence of a forward primer as depicted in Table 11, or a functional equivalent thereof, and a primer comprising a sequence of the reverse primer depicted in Table 11, or a functional equivalent thereof. This primer pair is particularly suitable for testing a sample for the presence of adenovirus. Yet another preferred primer pair comprises a primer comprising a sequence of a forward primer as depicted in Table 13, or a functional equivalent thereof, and a primer comprising a sequence of the reverse primer depicted in Table 13, or a functional equivalent thereof. This primer pair is particularly suitable for testing a sample for the presence of enterovirus.

In another aspect the invention provides an oligonucleotide comprising a sequence ID#1: 5'-CCC TGA ATG CGG CTA AT-3'; ID#2: 5'-ATT GTC ACC ATA AGC AGC C-3' ; ID#3: EV-specific probe; 5'-GCG GAA CCG ACT ACT TTG GGT-3'; ID#4: IC-specific probe; 5'-CTT GAG ACG TGC GTG GTA ACC-3' or internal control 5'-CCC TGA ATG CGG CTA ATC CTA ACC ACG GAA CAG GCG GTC GCG AAC CAG TGA CTG GCT TGT CGT AAC GCG CAA GTC TGT GCT TGA GAC GTG CGT GGT AAC CGT CCG TGT TTC CTG TTA TTT TTA TCA TGG CTG CTT ATG GTG ACA AT-3'.

The invention further comprises a recombinant virus particle comprising a sequence as depicted in any one of Tables 6-13 or a functional equivalent thereof, or the complement thereof.

The invention further comprises a recombinant virus particle comprising a control sequence (control probe or the complement thereof). Preferably, said recombinant virus particle is a recombinant phage particle. Preferably a phage having, expect for the nucleotide sequence of the region to be amplified using the oligonucleotide of the invention, the characteristics and elements of a phage as described in (24).

A further embodiment provides a kit for detecting viral nucleic acid comprising at least one and preferably at least two, more preferably at least three oligonucleotides comprising a sequence as depicted in any one of tables 6-13, or a functional equivalent of said sequence. A kit of the invention is particularly suitable for amplifying and/or detecting a virus, in particular any virus depicted in tables 6-13.

One preferred embodiment of the invention provides a kit for detecting enteroviral RNA comprising at least one and preferably at least two, more preferably at least three oligonucleotides of the invention. Said kit most preferably comprises at least one and preferably at least two, more preferably at least three oligonucleotides comprising a sequence as depicted in Table 13, or a functional equivalent thereof. Preferably, the kit comprises an internal control nucleic acid of the invention. In one embodiment, said kit comprises an armoured RNA according to (24) or the example herein, comprising an internal control sequence of the invention, preferably comprising a sequence 5'-CCC TGA ATG CGG CTA ATC CTA ACC ACG GAA CAG GCG GTC GCG AAC CAG TGA CTG GCT TGT CGT AAC GCG CAA GTC TGT GCT TGA GAC GTG CGT GGT AAC CGT CCG TGT TTC CTG TTA TTT TTA TCA TGG CTG CTT ATG GTG ACA AT-3' . Preferably, a kit of the invention comprises ID#4: IC-specific probe; 5'-CTT GAG ACG TGC GTG GTA ACC-3' and/or the complement thereof.

Another preferred embodiment of the invention provides a kit for detecting adenoviral DNA comprising at least one and preferably at least two, more preferably at least three oligonucleotides comprising a sequence as depicted in table 11, or a functional equivalent thereof. Said kit preferably comprises an internal control nucleic acid, comprising a sequence as depicted in table 11, or a functional equivalent thereof.

Yet another preferred embodiment provides a kit for detecting influenza A RNA comprising at least one and preferably at least two, more preferably at least three oligonucleotides comprising a sequence as depicted in table 6, or a functional equivalent thereof. Said kit preferably comprises an internal control nucleic acid, comprising a sequence as depicted in table 6, or a functional equivalent thereof.

In a preferred embodiment a kit of the invention further comprises an aqueous solution of the invention and/or concentrate thereof. Preferably said kit further comprises SuperScript II or III.

The invention is further explained in the following examples. These examples do not limit the scope of the invention, but merely serve to clarify the invention. Many alternative embodiments can be carried out, which are within the scope of the present invention.

Examples

Example 1

Materials and Methods

Chemicals and enzymes. Lysis buffer, wash buffers, and silica suspension were prepared as described previously (4). SuperScript II (SS II) was obtained from Life Technologies (Gaithersburg, MD). RNAse Inhibitor (RNAsin) was obtained from Promega, Madison, WI). Bovine serum albumin (BSA) was obtained from Roche Diagnostics (Almere, The Netherlands). Deoxynucleotides (dATP, dCTP, dGTP, dUTP), *Taq* DNA polymerase (Amplitaq Gold), uracil-N-glycosylase (Amperase) were from Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands). Streptavidin-coated magnetic beads (Dynabeads M-280) were from Dynal (Hamburg, Germany). Tris, KCl, $MgCl_2$, Calf Thymus (CT)-DNA, were obtained from Sigma (Zwijndrecht, The Netherlands. Serum and plasma samples were obtained in Vacutainer tubes (Becton Dickinson Systems, Meylan, France).

Clinical specimens. In total 322 clinical specimens, including 281 CSF samples, 18 faeces samples, 10 throat swap samples, 3 vesicle fluids, 3 pleuratic fluids, 2 broncheo-alveolar-lavages, 2 amniotic fluids, 1 urine, and 2 brain biopsies, were obtained from patients suspected to be infected with EV and tested by the RT-PCR.

Cytopathologic effect (CPE) for EV infection. Viral culture was done by cocultivation of CSF with human diploid fibroblasts, tertiary monkey kidney cells, or Vero cells. These viral cultures were examined twice weekly for the appearance of EV-specific CPE. Preliminary identification of isolates was performed by either unstained CPE or by CPE incubated with specific Mab (DAKO-Enterovirus, 5-D8/1 [DAKO, Glostrup, Denmark]).

Serotyping of isolates. Typing of EV's was performed at the National Institute of Public Health and the Environment by neutralization tests with antiserum

pools. These pools are prepared according to a combination so designed that an isolate can be screened for identity using 8 pools of 42 anti-sera in a single test.

Viral strains. The EV serotypes CVA [1-6, 18-22, 24], CVB [1-6], Echovirus [1-9, 11-22, 24-27, 29-33], Enterovirus [68-71], PV vaccines [1-3]), and Parechovirus 1 and 2 were kindly provided by the National Institute of Public Health and the Environment (Bilthoven, The Netherlands). The rhinovirus serotypes (1A, 1B, 3, 8, 11, 13, 14, 15, 16, and 88) were kindly provided by the Department of Virology from the Utrecht Medical Center (Utrecht, The Netherlands). The HAV serotype (HM175) was kindly provided by the Municipal Health Service Amsterdam.

Primers. Reverse transcription used random hexamers (Roche Diagnostics, Almere, The Netherlands), which were diluted in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) to 1.5 µg/µL. PCR primers were designed using computer-assisted analysis (OMIGA, Oxford Molecular, England) of all available 5' non-coding regions and full genomes of EV serotypes. HPLC-purified PCR primers were from Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands) and were diluted in TE buffer to 100 ng/µL. Primers for amplification of both wild type EV RNA and IC RNA are located in the conserved 5' non-coding region of EV. The primer pair used for amplification consisted of entero-1 (ID#1: 5'-CCC TGA ATG CGG CTA AT-3'; nucleotide positions [nt] 452-468) and Bio-entero-2 (ID#2: 5'-ATT GTC ACC ATA AGC AGC C-3', 5' biotinylated; nt 579-597). Nucleotide numbering was according to the Sabin polio type 2 strain as described by Toyoda et al. (30).

Armoured EV-RNA control. The armoured EV-RNA control, containing part of the 5' non-coding region (nucleotide 428-691) was obtained from Ambion (Ambion, Inc., RNA diagnostics, Austin, Tx, USA) and was constructed according to the armoured RNA technique (24). The method is based on the packaging of recombinant RNA into MS2-like particles, which are produced in *Escherichia coli*. The particles are isolated through a series of conventional protein purification procedures. According to Ambion, the approximate conversion factor from 1 mg of armoured RNA to copies of RNA is about 2×10^{14}. The armoured EV-RNA stock solution used in this paper with lot no. 040D49012A contained approximately 6.5×10^{14} EV-RNA c/mL.

Construction of armoured IC-RNA control. For the construction of IC-RNA 2 oligonucleotides were designed by us and synthesized by Applied Biosystems (Ent-hyb-3; 5'- *CCC TGA ATG CGG CTA ATC* CTA ACC ACG GAA CAG GCG GTC GCG AAC CAG TGA CTG GTC TGT CGT AAC GCG CAA GTC TGT GCT **TGA GAC GTG**-3', and Ent-hyb-4; 5'-*ATT GTC ACC ATA AGC AGC* CAT GAT AAA AAT AAC AGG AAA CAC GGA C**GG TTA CCA CGC ACG TCT CAA GCA** CAG ACT T-3'). These 2 oligonucleotides, which together represent the same part of the 5' non-coding region (nucleotide 428-691) as present in the armoured EV-RNA control, were overlapping over a stretch of 20 nucleotides (underlined) and contained the same primer binding sites as the armoured EV-RNA control (in *Italics*) but with a different probe region (in bold). This IC-probe region allowed for discrimination between EV and IC RNA amplimers after hybridisation and detection. IC-RNA control was constructed by hybridisation and elongation of 1 ng Ent-hyb-3 and 1 ng Ent-hyb-4, 2.5 U of Amplitaq Gold, 5 µg of BSA, 1 x PCRII buffer (10 mM Tris-HCl; pH 8.3, 50 mM KCl), dATP, dCTP, dGTP, and dTTP at a concentration of 200 µM each, and 3 mM $MgCl_2$. The mixture was incubated for 10 min at 95°C, 5 min at 55°C, and 10 min at 72°C. The resulting hybrid was subsequently amplified with primer pair entero-1 and non-Bio-entero-2 in the same mixture as described above and was incubated 10 min at 95°C, followed by 35 cycles each consisting of 20 s at 95°C, 20 s at 55°C, and 1 min at 72°C, followed by 5 min at 72°C. The concentration of resulting amplimer was estimated by measuring the UV-absorption at 260 nm and subsequently 2 ng was cloned into a plasmid vector (PCRII, Promega, Ma) resulting in plasmid pEntIC 2. The IC-RNA sequence was confirmed by dideoxynucleotide sequencing (Visible Genetics Inc., Toronto, Canada). Packaging of IC-RNA control into MS2-like particles was performed with plasmid pEntIC 2 as the template and was custom made by Ambion. According to Ambion, the approximate conversion factor from 1 mg of armoured RNA to copies of RNA is about 2×10^{14}. The armoured IC-RNA stock solution used in this paper with lot no. 021D49013A contained approximately 5.4 $\times 10^{14}$ IC-RNA c/mL.

Dilution buffer for armoured RNA controls. The armoured RNA controls were diluted in TSM dilution buffer containing 20 ng/µL CT-DNA, 10 mM Tris

(pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, 0.1% gelatin (Sigma, catalogue no. G-9382) and stored at -20°C. Final solution of armoured EV-RNA contained 500 copies/µL and the final solution of armoured IC-RNA contained 100 copies/µL.

RNA purification. EV-RNA was purified from clinical specimens as described earlier (5), with the following modifications; 20 µL of size-fractionated silica particles was used in combination with 900 µL of lysis buffer L6. To this silica-lysis buffer mixture, the clinical specimen and 500 armoured IC-RNA copies were added. RNA was eluted in 100 µL TE buffer.

RT-PCR. Forty µL of the 100 µL RNA eluate (2/5) was used for reverse transcription. The final reverse transcription mixture (50 µL) contained 1500 ng hexamers, 1 x CMB1 buffer (10 mM Tris-HCl; pH 8.3, 50 mM KCl, 0.1% Triton), 0.4 U/µL of SSII, 120 µM of each dNTP, 0.08 U/µL RNAsin, and 5 mM $MgCl_2$. The mixture was incubated for 30 minutes at 42°C and 25 µL (corresponding to 40 µL [1/5] of CSF) was subsequently used as input in the PCR. The PCR was performed in a 50 µL volume containing 200 ng of entero-1 (5'-CCC TGA ATG CGG CTA AT-3'; nucleotide positions [nt] 452-468) and 200 ng of Bio-entero-2 (5'-ATT GTC ACC ATA AGC AGC C-3', 5' biotinylated; nt 579-597), 0.05 U/µL of Amplitaq Gold DNA polymerase, 0.01 U/µL Amperase, 0.1 µg/µL of BSA, 1 x PCRII buffer (10 mM Tris-HCl; pH 8.3, 50 mM KCl), dATP, dCTP, and dGTP at a concentration of 200 µM each, and 400 µM dUTP. The final $MgCl_2$ concentration in PCR reaction was 2.5 mM. PCR's were performed in an Applied Biosystems 9600 thermocycler: 2 min at 50°C, 10 min at 95°C, followed by 45 cycles each consisting of 20 s at 95°C, 20 s at 55°C, and 1 min at 72°C, followed by 5 min at 72°C.

Hybridisation and measurement by ECL. After RT-PCR, excess primers were removed as earlier described (6), and amplicons were hybridised with TBR (Tris [2,2'-bipyridine] ruthenium [II] chelate)-labelled probes specific for EV-RNA and IC-RNA as recently described (6). Probes (nt 531-551) were 5' TBR-labelled and were as follows, TBR-entero-1 (ID#3: EV-specific probe; 5'-GCG GAA CCG ACT ACT TTG GGT-3' and TBR-entero-2 (ID#4: IC-specific probe; 5'-CTT GAG ACG TGC GTG GTA ACC-3'). Hybrids were captured with streptavidin-coated magnetic (beads and the electrochemiluminescence (ECL) signal, expressed in

luminosity units (LU), was measured by the M8 system (IGEN, Oxford, England). In real time PCR this step can be omitted. With this device, a 96-well plate is used and unhybridised TBR-labelled probes are automatically removed by washing. The amount of labelled hybrids is determined after excitation by applying an electric field.

Other labels can also be used, such as fluorophores in real-time PCT, radioactive labels, digoxigenine etc. A signal of more than 500 LU (= 2.5-times mean background signal for either probe) was considered to be positive. A clinical specimen was considered positive for EV-RNA if more than 500 LU were measured with the EV probe, regardless of the result obtained for the IC probe. A clinical specimen was considered to be negative for EV-RNA if less than 500 LU were measured with the EV probe and IC- RNA was detected at more than 500 LU. In the diagnostic RT-PCR, 4 controls were included in RNA extraction; 2 positive controls and 2 negative controls. The high positive control (HPC) contained 12,500 armoured EV-RNA copies/extraction and the low positive control (LPC) contained 2500 armoured EV-RNA copies/extraction, both together with 500 armoured IC-RNA copies. The first negative control contained 500 armoured IC-RNA copies and served as a control for the entire procedure and should be negative for EV probe but positive for the IC probe. The second negative control contained no armoured EV-RNA copies or armoured IC-RNA copies and should be negative for both probes with a mean of 200 LU.

Parechovirus specific RT-PCR. The conditions of the RT-PCR reaction to detect Parechoviruses were similar as the above described protocol except for the primers used in PCR. For the specific detection of Parechoviruses we used the primer pair as described by Oberste et al. (22).

Results

Determination of the lower limit of detection of the EV RT-PCR assay. To determine the lower limit of detection of the EV RT-PCR assay, we spiked decreasing amounts of both armoured EV-RNA and armoured IC-RNA diluted in TSM buffer into 200 µL of EV-negative CSF before RNA extraction with the Boom method (5). RNA was diluted in 100 µL and 40 µL of extracted RNA (2/5) was used in RT and subsequently 50% of the cDNA was used in PCR (1/5 of the extracted RNA). Limiting dilutions of the armoured EV-RNA revealed a detection limit of 84 EV-RNA copies in extraction with a 50% hit rate (5/10 runs) resulting in a detection limit of 17 copies of EV-DNA in PCR (1/5). Limiting dilutions of the armoured IC-RNA revealed a detection limit of 28 IC-RNA copies in extraction with a 60% hit rate (6/10 runs) resulting in a detection limit of 5 copies of IC-DNA in PCR (1/5) (Table 1). Identical results were found after the direct release of EV-RNA and IC-RNA from its phage (without CSF as background) after incubation at 70°C for 5 minutes (results not shown).

Testing of EV serotypes. All known EV serotypes (n = 64) were tested with the described EV RT-PCR. The extraction of every single EV serotype and non-enterovirus serotype was done alternated with negative extraction controls that contained IC-RNA. All EV serotypes were positive whereas the non-enteroviruses, including echovirus 22 and 23, rhinoviruses, HAV, and all negative controls were negative. The negative results were truly negative since the ECL signals for co-extracted armoured IC-RNA were all positive (Table 2).

Testing of the 2001 and 2002 QCMD EV proficiency panels. These proficiency panels from 2001 and 2002 consisted of coded freeze-dried samples, including an Echo 11 and CVA 9 dilution series, other EV serotypes representing different genetic clusters of human EV's, and negative controls. The freeze-dried samples were reconstituted with 1 mL of sterile water according to the QCMD protocol and 200 µL thereof was used in extraction and amplified as described in the materials and methods section. All samples of the 2001 QCMD proficiency panel were positive regardless of the serotypes and all included negative controls were truly negative, since the ECL signals for co-extracted armoured IC-RNA were all positive (Table 3).

Testing of clinical specimens. A total of 322 clinical specimens were obtained from patients suspected to be infected with EV and were tested by the RT-PCR. Forty-five positive results (14%), 267 negative results (82.9%), and 10 invalid results (3.1%) were found. These invalid results were found in 7/281 CSF samples (2.5%), in 2/18 faeces samples (11.1%), and in 1/10 throat swab samples (10%). Repeating these 10 invalid samples with the optimised RT-PCR revealed that all invalid results apart from 1 faeces sample were valid, negative for EV, and could be reported to the clinician. (Table 4).

Comparison of virus culture with RT-PCR. Eighty-seven clinical specimens suspected to be infected with EV were available to be tested in virus culture and in RT-PCR. Agreement between virus culture and RT-PCR was found in 72/87 clinical specimens (82.8%). In 54 clinical specimens a negative result was found for both virus culture and RT-PCR, whereas 18 clinical specimens were positive in virus culture and in RT-PCR. Discordant results were found in 15 clinical specimens. In 3 of them virus culture was positive whereas RT-PCR was truly negative for EV-RNA in RT-PCR since the results for IC-RNA were positive. Two of these RT-PCR negative samples were subjected to a specific Parechovirus RT-PCR and were found to be positive (results not shown). In the other RT-PCR negative sample CPE was only found after 4 weeks. The remaining discordant results consisted of 12 clinical specimens that were RT-PCR positive and negative in virus culture (Table 5).

Discussion.

Isolation of EV in cell culture is still regarded as the diagnostic gold standard. There are however many disadvantages to culture such as its labour-intensity, the delay of days to weeks to obtain a positive result, and its false negativity rate of approximately 25-35% because of failures in antibody neutralization and the inability of certain CVA serotypes to grow in cell culture (16, 27).

Many of the disadvantages of cell culture can be overcome by RT-PCR. Most frequently used primer and probe sets for the detection of EV are those described by Chapman et al. (10) and Rotbart et al. (27). These primers and probes have been proven to be reactive with all known EV and fail to amplify echovirus 22 and 23, which have been reported to be a genetic distinct genus in the family of Picornaviridae (14, 21, 25). We designed a primer and probe set with minor modifications in comparison to those first described by Chapman et al. and Rotbart et al. and found that not only all known EV serotypes would be detected but also that cross-reactivity between EV serotypes and rhinoviruses was avoided. This cross-reactivity with rhinoviruses has an impact if nasal or pharyngeal swab specimens are used for testing.

We have described a RT-PCR assay in a non-nested format for the detection of EV-RNA in clinical specimens in which 2500 c/mL of armoured IC-RNA mimicking the EV target were included in the RNA extraction and all subsequent steps of the procedure. This armoured IC-RNA can be spiked directly into clinical specimens without degradation of the RNA and enables monitoring of the complete nucleic acid extraction and amplification process for each specimen.

The sensitivity of the RT-PCR was evaluated with limiting dilutions of both armoured EV-RNA and armoured IC-RNA. We found a detection limit of 5 copies of IC-DNA in 60% of cases in PCR. Poisson statistics predicts that 63% of the reactions will be positive with a single copy of DNA in PCR (13). Thus, if RNA was extracted and reversed transcribed with 100% efficiency, the 60% detection rate should represent 1 copy of IC-DNA in PCR. Since we found comparable results after the direct release of RNA from the armoured RNA through incubation at 70°C for 5 minutes and after extraction of the RNA from the armoured RNA using the Boom method, our results might suggest a less efficient

reverse transcription step or the presence of a certain percentage of empty armoured RNA phages. In addition, our observed approximately 3-fold difference in detection between EV-RNA and IC-RNA might be explained by inaccuracies in the determination of the concentration and dilution steps of the stock solutions. The lower limit of sensitivity of the RT-PCR was based on the lower detection rate of IC-RNA and was about 150 IC-RNA c/mL. The presence of 500 copies of IC-RNA during extraction from 200 µL of clinical specimens allowed us to draw the conclusion that a specimen found to be negative for EV-RNA but positive for IC-RNA would contain less than 2500 copies of EV-RNA/mL.

The use of the armoured IC-RNA was critical in the detection of false-negative reactions or invalid results. Currently, viral culture is still the gold standard to determine an EV infection. Validation of a RT-PCR assay for the diagnosis of EV infections is difficult because cases are defined clinically and viral culture has a poor sensitivity of approximately 70%, partly due to the inability of certain CVA serotypes to grow in cell culture (16, 27). We found an overall good agreement of 82.8% between virus culture and RT-PCR. CSF provides the most direct link to disease but is usually less successful in virus culture. We found high discordant results (17.2%) between virus culture and RT-PCR and mainly in CSF samples. Delayed processing and improper handling of the clinical specimens may have contributed to false negative results in virus culture and our results showed the added value of the described RT-PCR assay for the diagnosis of an EV-infection. In conclusion, viral EV culture with a good clinical diagnosis will still be of use for EV diagnosis. However, the described EV RT-PCR assay will be of benefit as a sensitive, highly specific, faster assay for the detection of EV in clinical specimens, and the use of an armoured IC RNA strongly reduces false negative results.

Example 2

Construction of IC-RNA control sequences depicted in Tables 6-12.

For the construction of IC-RNA for each virus as listed in Tables 6-12, two IC-oligonucleotides were designed and synthesized by Applied Biosystems (for sequences of IC-oligonucleotides, see Tables 6-12). Each set of two oligonucleotides as given in each of the Tables 6 to 12, contains a stretch of complementary nucleotides, varying in length between 25 and 50 nucleotides. IC-RNA control was constructed by hybridization and elongation of 1 ng of each of the two IC-oligonucleotides (as listed for each virus in Tables 6-12), 2.5 U of Amplitaq Gold, 5 µg of BSA, 1 x PCRII buffer (10 mM Tris-HCl; pH 8.3, 50 mM KCl), dATP, dCTP, dGTP, and dTTP at a concentration of 200 µM each, and 3 mM $MgCl_2$. The mixture was incubated for 10 min at 95°C, 5 min at 55°C, and 10 min at 72°C. The resulting hybrid was subsequently amplified with the virus-specific primer pair (listed as forward and reverse in each of the Tables 6-13) in the same mixture as described above and was incubated 10 min at 95°C, followed by 35 cycles each consisting of 20 s at 95°C, 20 s at 55°C, and 1 min at 72°C, followed by 5 min at 72°C. The concentration of resulting amplicon was estimated by measuring the UV-absorption at 260 nm and subsequently 2 ng was cloned into a plasmid vector (PCRII, Promega, USA). For each virus, the IC-RNA sequence was confirmed by dideoxynucleotide sequencing. IC-RNA was made for each virus by *in vitro* transcription of the IC-containing plasmid with T7 RNA polymerase (Invitrogen, USA). For each virus the IC-RNA differs from the wild type virus RNA only in the central region of the amplicon, i.e. in the probe region, which allows for discrimination between wild type and IC RNA amplicon after hybridization and detection.

In case of DNA viruses (e.g. adenovirus), IC-DNA was made by linearizing the IC-containing plasmid by a unique enzyme, which digests the plasmid outside the amplicon region.

Example 3

Adenovirus: Materials & methods

DNA-purification with MagNApure

200µl respiratory specimen (sputum, BAL, etc.) and 350µl lysisbuffer, or 50µl faeces and 500µl lysisbuffer in an eppendorf tube, vortex.

Pre-lysis 10 min. at room temperature.

Spin for 2 min at maximum speed (13000 rpm).

Transfer 490µl supernatant to a MagNApure sample cartridge.

Add 10.000 copies of Adeno internal control (10µl with a concentration of 10E6 cop./ml)

MagNApure total NA isolation-external lysis protocol, elution volume 100µl.

10µl of each eluate was used for Taqman PCR (10µl contains 1000 copies of internal control).

The final PCR mixture (25 µl) contained:

1x Taqman® Universal PCR MasterMix (ABI)
900nM forward primer
900nM reverse primer
200nM wild-type probe
200nM internal control probe
400ng/µl α-casein

PCR's were performed in an ABI Prism 7000 sequence detection system as follows:

2 min 50°C and 10 min at 95°C, followed by 45 cycles each consisting of 15 sec at 95°C and 1 min at 60°C.

<u>Real-time PCR with an internal control detecting all 51 known Adenovirus serotypes</u>

Objectives: The gold standard for the diagnosis of adenovirus (AV) infection is culture. However, results are available after days to weeks and not all AV types

grow in culture. Especially in immune compromised patients severe infections with AV are described and a rapid diagnosis would be important. Therefore, an AV real-time PCR was developed, detecting all 51 known AV serotypes.

Methods: Primers were chosen in the hexon region. In order to cover all 51 serotypes the forward primer is degenerated at 3 positions and the reverse primer at 2 positions. The IC DNA contains the same primer binding sites, but has a shuffled probe region compared to WT virus. Primers, probes and internal control are depicted in Table 11. The IC DNA was added to the clinical sample in order to monitor extraction and PCR efficiency. Twelve (2-fold) serial dilutions were made from AV DNA and IC DNA in a background of AV negative throat fluid in order to assess the detection limit of the AV PCR. To investigate the linearity, 8 (ten-fold) serial dilutions from AV DNA (with 1000 copies IC DNA in every dilution) in a background of AV negative sputum were tested. A panel of AV prototype strains of all 51 AV serotypes was tested. To establish the clinical utility of the assay, a comparison of AV PCR and culture was performed in a panel of 152 clinical samples.

Results: In the serial dilutions in throat fluid the 50% detection limit of AV DNA was 8 copies per PCR assay (400 copies/ml) and of IC DNA 16 copies per PCR assay (800 copies/ml) (Table 14). The real-time AV PCR was linear from 125 copies per PCR assay (6250 copies/ml) until 1.25 E8 copies per PCR assay (6.25 E9 copies/ml) (Table 15). All 51 AV serotypes were detected in the panel of AV prototype strains (Table 18). Concordant results between culture or Ag detection and PCR were found in 139/152 (91.4%). In 10 cases, PCR was positive while culture was negative (6.6%) and in 12 cases PCR was positive while both culture and ELISA were negative (7.7%). In 1 case, PCR was negative while culture was positive (0.6%)(Table 17).

Conclusion: A sensitive AV real-time PCR assay was developed, detecting all 51 AV serotypes. The assay can be applied on different body fluids, among which faeces and respiratory materials. The assay has proven to be more rapid and more sensitive than AV culture.

Example 4

Primers of the invention with degenerated sites (Table 11) were compared with primers without degenerated sites in an Adenovirus detection experiment. The results are shown in Table 18.

Left part of Table 18 shows results obtained with primers Adeno-F and Adeno-R without degenerated sites:

Primer Adeno-F: CAggACgCCTCggAgTACCTgAg

Primer Adeno-R: ggAgCCACCgTggggTT

Right part of Table 18 shows results obtained with primers of the present invention with degenerated sites:

Primer of the invention: CAggACgCCTCggRgTAYCTSAg

Primer of the invention: ggAgCCACVgTgggRTT

Positive results are defined by detectable adenovirus CT value regardless of IC values. Negative Adenovirus results are true negative results only if IC is detectable as is indicated by CT values.

Results:

The non-degenerate primers failed to detect 4 Adenovirus serotypes.

The improved, degenerate primers of the present invention detected all known 51 Adenoviruses.

Example 5

Primers of the invention for detecting enterovirus were used in the following experiment:

From a previous series five samples that were cultured and found to represent an enterovirus infection (based on CPE), were tested at the AMC, using the newly developed AMC primer and probe set. The followed method is the same as described in the Material and Methods section of Example 1. All five samples were found to be positive for enterovirus (subtypes Echo 9, Echo 18, COX B5, COX A9, and EV Stuttgart).

This shows that the optimized AMC primer and probe set is capable of detecting various enteroviral subtypes.

Example 6

Over the last twelve months a large series of cerebro spinal fluid samples from patients suspected to suffer from myocarditis, sepsis, diarrhea, meningitis and respiratory disorders was analyzed at the Dept. of Clinical Virology. The samples were analyzed by PCR (see Materials and Methods section of Example 1).

Table 20 shows that over the last year 220 cerebro spinal fluid samples were analyzed for the presence of enterovirus. In the column 'Test result', pos means positive for the presence of enterovirus.

It is concluded from Table 20 that of the 220 cerebro spinal fluid samples that were analyzed at the AMC over the last year, 32 were positive. Hence, in a significant amount of cases enterovirus is detected. The AMC primer and probe set is therefore a significant improvement in the diagnostics of viruses in patient samples.

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Table 1a. Sensitivity for armored EV-RNA control phages.

EV-RNA copies/200 µL			
CSF in extraction	in RT (2/5)	in PCR (1/5)*	proportion positive
900	360	180	10/10 (100%)
450	180	90	9/10 (90%)
225	90	45	8/10 (80%)
112	45	23	8/10 (80%)
84	34	17	5/10 (50%)
56	23	11	6/20 (30%)
28	11	6	5/16 (31%)
14	6	3	1/10 (10%)
7	3	1	1/10 (10%)
0	0	0	0/10 (0%)

*For the copy numbers presented in Table 1 the following assumptions were made; (i) The armoured RNA stocks contain the specified number of phage particles and all contain EV-RNA or IC-RNA, (ii) a 100% efficiency in extraction, in reverse transcription, and in PCR.

Table 1b. Sensitivity for armoured IC-RNA control phages.

IC-RNA copies/200 µL			
CSF in extraction	in RT (2/5)	in PCR (1/5)*	proportion positive
225	90	45	10/10 (100%)
112	45	23	9/10 (90%)
56	23	11	8/10 (80%)
28	11	6	6/10 (60%)
0	0	0	0/10 (0%)

*For the copy numbers presented in Table 1 the following assumptions were made; (i) The armoured RNA stocks contain the specified number of phage particles and all contain EV-RNA or IC-RNA, (ii) a 100% efficiency in extraction, in reverse transcription, and in PCR.

Table 2. Specificity of EV RT-PCR for prototype strains of enteroviruses and controls.

Virus serotype	Source	Result	Mean LU EV-RNA (range)
CVA (2-4, 6-18, 20, 21, 24[a])	Cell culture	Pos	87,000 (57,000-117,000)
CVA (1, 5, 19, 22)	Suckling mice	Pos	87,000 (73,000-100,000)
CVB (1- 6)	Cell culture	Pos	91,000 (75,000-107,000)
Echovirus (1-9, 11-16, 17-21, 24-27, 29-33 [b,c,d,e])	Cell culture	Pos	98,000 (86,000-111,000)
Echovirus (22, 23[f])	Cell culture	Neg	218 (209-227)
Enterovirus (68-71)	Cell culture	Pos	77,000 (68,000-86,000)
Poliovirus (1, 2, 3)	Cell culture	Pos	104,000 (79,000-129,000)
Rhinovirus (1A, 1B, 3, 8, 11, 13, 14, 15, 16, 88)	Cell culture	Neg	228 (205-277)
HAV	Cell culture	Neg	237
Negative control		Neg	226 (217-235)
HPC (25,000 EV-RNA c/mL)		Pos	85,000 (62,000-106,000)
LPC (5,000 EV-RNA c/mL)		Pos	58,000 (46,000-70,000)
IC (2,500 IC RNA c/mL)		Pos	51,000 (40,000-63,000)

[a] Echovirus 34 is a variant of CVA24.
[b] Echovirus 8 is a variant of echovirus 1.
[c] CVA23 is a variant of echovirus 9.
[d] Echovirus 10 was reclassified as reovirus 1.
[e] Echovirus 28 was reclassified as human rhinovirus 1A.
[f] Echovirus 22 and 23 were reclassified as parechovirus types 1 and 2.

Source: adapted from Melnick, Fields Virology, 3rd edition, 1996 (18).

Table 3. Composition and results of the QCMD proficiency panel 2001.

Code	Serotype	Virus titer*	LU EV-RNA	LU IC-RNA	Result
EV-C01	CXVA 9	0.036	31,104	65,482	Positive
EV-C02	CXVA 9	0.36	87,325	81,793	Positive
EV-C03	CXVA 9	3.6	90,570	71,435	Positive
EV-C04	No virus		214	89,860	Negative
EV-C06	Echo 11	25.2	96,460	85,396	Positive
EV-C11	Echo 11	252	99,930	43,751	Positive
EV-C09	Echo 11	25,200	87,380	860	Positive
EV-C07	CXVB 5	317	83,867	32,092	Positive
EV-C08	EV 71	56.4	98,508	31,411	Positive
EV-C10	No virus		226	74,234	Negative
EV-C05	Echo 6	20,000	82,529	27,549	Positive

*Titer of original virus stocks before inactivation and freeze-drying ($TCID_{50}$/mL): CVA 9; 3.6×10^6, Echo 6; 2.0×10^8, Echo 11; 2.5×10^7, CVB 5; 3.2×10^7, EV 71; 5.6×10^6.

Table 4. Summary of results after testing clinical specimens.

Specimen	N	Positive	Negative
CSF	281	36	238
Faeces	18	6	10
Throat swabs	10	0	9
Other	13	3	10
Total no.	322	45 (14%)	267 (82.9%)

Table 5. Comparison of RT-PCR and virus culture on different clinical specimens obtained from patients with a clinically suspected EV infection.

Clinical specimen	No tested	RT-PCR + Culture +	RT-PCR + Culture -	RT-PCR - Culture -	RT-PCR - Culture +
CSF	67	15	10	40	2
Faeces	13	3	1	9	0
Throat swap	4	0	0	4	0
Urine	1	0	0	1	0
Biopsy	1	0	1	0	0
Total no.	86	18 (20.9%)	12 (14.0%)	54 (62.8%)	2* (2.3%)

* These culture isolates were identified as Parechovirus and not as Enterovirus.

Table 6: Influenza A virus

Oligo Name	Sequence (5' to 3')
Forward Primer INFa-165s	gACAAgACCAATCCTgTCACYTCTg
Reverse Primer INFa-261r	AAgCgTCTACgCTgCAgTCC
INFa-IC-linker-1	gACAAgACCAATCCTgTCACCTCTgACTAAggggATTTTAggATTTgTgTTCACTgggCCCgACTCgCACTgAC
INFa-IC-linker-2	AAgCgTCTACgCTgCAgTCCTCgTCAgTgCgAgTCgggCCCAgTgAACACAAATCCT
Virus-Specific Probe INFa-WT-TM	TTCACgCTCACCgTgCCCAgTgAgC
IC-Specific probe INFa- IC-TM	TTCACTgggCCCgACTCgCACTgAC
Internal Control	*gACAAgACCAATCCTgTCACCTCTg*ACTAAggggATTTTAggATTTgTg**TTCACTgggCCCgACTCgCACTgA CgA*ggACTgCAgCgTAgACgCTT***

Table 7: Influenza B virus

Oligo Name	Sequence (5' to 3')
Forward Primer INFb-matrix-F	TCgCTgTTTggAgACACAAT
Reverse Primer INFb-matrix-R	TTCTTTCCCACCgAACCA
INFb-IC-linker-1	TCgCTgTTTggAgACACAATTgCCTACCTgCTTTCATTgACAgAgAgAACAAgATAgCTCgAgAgAgA
INFb-IC-linker-2	TTCTTTCCCACCgAACCAACAgTgTAATTTTTCTgCTTCTCTCTCgAgCTATCTTgTTCTCTCTg
Virus-Specific Probe INFb-WT-TM	AgAAgATggAgAAggCAAAgCAgAACT
IC-Specific probe INFb-IC-TM	AgAgAgAACAAgATAgCTCgAgAgAgA
Internal Control	*TCgCTgTTTggAgACACAAT*TgCCTACCTgCTTTCATTgAC**AgAgAgAACAAgATAgCTCgAgAgAgAA** gCAgAAAAATTACACTgT*TggTTCggTgggAAAgAA*

Table 8: Metapneumovirus

Oligo Name	Sequence (5' to 3')
Foward Primer HMPV fusion 575-F	AgCTTCAgTCAATTCAACAgAAg
Reverse Primer HMPV fusion 717-R	CCTgCAGATGTYGGCATGT
HMPV-linker-1	AgCTTCAgTCAATTCAACAgAAggTTTCTAAATgTTgTgCggCAATTTTCAgACAATgCTggAATAACACCAgCAATATCCTTggA
HMPV-linker-2	CCTgCAgATgTTggCATgTTggAAACAgCTCTggCTAgTTCAgCATCTgTCATTAAgTCCAAggATATTgCTggTgTTATTCC
Virus-Specific HMPV-WT-probe-TM	TgTTgTgCggCAgTTTTCAgACAATgC
IC-Specific HMPV-IC-probe-TM	ACgTTTgTggTATACACgCgTgTATAC
Internal Control	*AgCTTCAgTCAATTCAACAgAAgg*TTTCTAAAA**CgTTTgTggTATACACgCgTgTATAC**TggAATAACACCAgCAATATCCTTggACTTAATgACAgATgCTgAACTAgCCAgAgCTgTTTCCA*AACATgCCAACATCTgCAgg*

Table 9: Respiratory Syncitial Virus

Oligo Name	Sequence (5' to 3')
Forward Primer RSV-fusion 1120-F	ATgAACAgTTTAACATTACCAAgT
Reverse Primer RSV-fusion 1272-R	gTTTTgCCATAgCATgACAC
RSV-IC linker-1	ATgAACAgTTTAACATTACCAAgTgAAgTAAATCTCTgCAATgTTgACATATTCAATCCCAAATATgATTgTAAAATTAgTTTgCAgTAC
RSV-IC linker-2	gTTTTgCCATAgCATgACACAATggCTCCTAgAgATgTgATAACTgAgTTTCTTTgACgTgTgTACTgCAAACTAATTTTACAA
Virus-Specific RSV-WT probe-TM	TgACTTCAAAAACAgATgTAAgCAgCTCC
IC-Specific RSV-IC probe-TM	gTTTgCAgTACACACgTCAAAgAAACTCA
Internal Control	*ATgAACAgTTTAACATTACCAAgT*gAAgTAAATCTCTgCAATgTTgACATATTCAATCCCAAATATgATTgTAAAATTA**gTTTgCAgTACACACgTCAAAgAAACTCA**gTTATCACATCTCTAggAgCCAT*TgTgTCATgCTATggCAAAAC*

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Table 10: Rhino Virus

Oligo Name	Sequence (5' to 3')
Forward Primer RV-forward	AgSCTgCgTggCKgCC
Reverse Primer RV-reverse	ACACCCAAAgTAgTYggT
RV-linker-1	AgCCTgCgTggCTgCCTgCACACCCTATgggTgTgAAgCCAAACAATggACAAggTgTgAAgAgCCCCgTgTgCTCgCTTTgAgACggCAATCTCTCggCCgATAgCCgCC
RV-linker-2	ACACCCAAAgTAgTTggTCCCATCCCgCAATTgCTCATTACgACTAgATACACATTgggTTACgTgCTCTAgCTgCAgggTTAAggCggCTATCggCCgAgAgATTgCCgT
Virus-Specific RV-WT-TW probe	TCCTCCggCCCCTgAATgYggCTAAYC
IC-Specific RV-IC-TM probe	ACggCAATCTCTCggCCgATAgCCgCC
Internal Control	*AgCCTgCgTggCTgCC*TgCACACCCTATgggTgTgAAgCCAAACAATggACAAggTgTgAAgAgCCCCgTgTgCTCgCTTTgAg**ACggCAATCTCTCggCCgATAgCCgCC**TTAACCCTgCAgCTAgAgCACgTAACCCAATgTgTATCTAgTCgTAATgAgCAATTgCgggATggg*ACCAACTACTTTgggTgT*

Table 11: Adenovirus

Oligo Name	Sequence (5' to 3')
Forward Primer Ad19548F	CAggACgCCTCggRgTAYCTSAg
Reverse Primer Ad19652R	ggAgCCACVgTgggRTT
Ad-linker-1	CAggACgCCTCggAgTACCTgAgCCgATgTgTCCgCCgTggTCCCCTggACCgAgACgTACTT
Ad-linker-2	ggAgCCACCgTggggTTTCTAAACTTgTTATTCAggCTgAAgTACgTCTCggTCCAggggACCACgg
Virus-Specific Adeno-WT-TM probe	CCgggTCTggTgCAgTTTgCCCgC
IC-Specific Adeno-IC-TM probe	gATgTgTCCgCCgTggTCCCCTgg
Internal Control	*CAggACgCCTCggAgTACCTgAg*CC**gATgTgTCCgCCgTggTCCCCTgg**ACCgAgACgTACTTgTCggACTTATTgTTCAAATCT*TTggggTgCCACCgAgg*

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Table 12: Parainfluenza Virus 1

Oligo Name	Sequence (5' to 3')
Forward Primer Para-1-F	ATCTCATTATTACCYggACCAAgTCTACT
Reverse Primer Para-1-R	CATCCTTgAgTgATTAAgTTTgATgAATA
Para-linker-1	ATCTCATTATTACCTggACCAAgTCTACTTTCTggATCCACCACAATTTCCTCgAAgAACTATCAggACATATTCATATTACgCTTgT
Para-linker-2	CATCCTTgAgTgATTAAgTTTgATgAATACgCATATATTgACAAgCgTAATATgAATATgTCCTgATAgTTCTTCgAg
Virus-Specific Para-1-WT-TM probe	AggATgTgTTAgAYTACCTTCATTATCAATTggTgA
IC-Specific Para-1-IC-TM probe	CTCgAAgAACTATCAggACATATTCATATTACgCTTgT
Internal Control	*ATCTCATTATTACCTggACCAAgTCTACT*TTCTggATCCACCACAATTTC**CTCgAAgAACTATCAggACATATTCATATTACgCTTgT**CAATATATgCg*TATTCATCAAACTTAATCACTCAAggATg*

Table 13: Enterovirus

Oligo Name	Sequence (5' to 3')
Forward Primer ID#1	CCCTGAATGCGGCTAAT
Reverse Primer ID#2	ATT GTC ACC ATA AGC AGC C
Virus-Specific EV-specific probe	GCG GAA CCG ACT ACT TTG GGT
IC-specific probe	CTT GAG ACG TGC GTG GTA ACC
Internal Control	CCC TGA ATG CGG CTA ATC CTA ACC ACG GAA CAG GCG GTC GCG AAC CAG TGA CTG GCT TGT CGT AAC GCG CAA GTC TGT GCT TGA GAC GTG CGT GGT AAC CGT CCG TGT TTC CTG TTA TTT TTA TCA TGG CTG CTT ATG GTG ACA AT

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Table 14. PCR sensitivity for armoured AV DNA and IC DNA control phages

DNA type and no. of Copies/200µl of throat swab	No. of DNA copies in the PCR mixture (1/10)	Proportion positive
AV DNA		
2500	250	12/12 (100%)
1250	125	12/12 (100%)
620	62	12/12 (100%)
310	31	10/12 (83,3%)
160	16	10/12 (83,3%)
80	8	6/12 (50%)
40	4	1/12 (8,3%)
0	0	0/12 (0%)
IC DNA		
2500	250	12/12 (100%)
1250	125	12/12 (100%)
620	62	11/12 (91,7%)
310	31	10/12 (83,3%)
160	16	5/12 (50%)
80	8	3/12 (25%)
40	4	2/13 (15,4%)
0	0	0/11 (0%)

Table 15. Linearity of AV Taqman PCR

No. of AV DNA Copies/200µl of sputum[a]	No. of AV DNA copies in the PCR mixture (1/10)[b]	Proportion positive[c]
1,25 E9	1,25 E8	8/8
1,25 E8	1,25 E7	8/8
1,25 E7	1,25 E6	8/8
1,25 E6	1,25 E5	8/8
1,25 E5	1,25 E4	8/8
1,25 E4	1250	8/8
1250	125	8/8
125	12,5	5/8

[a] In each sample 10.000 copies of IC DNA per 200 µl of sputum.

[b] In each PCR mixture 1000 copies of IC DNA

[c] Linearity standard curve: exclusive 12,5 copies input slope= –3,35 and R^2=0,998; inclusive 12,5 copies input slope= -3,38 and R^2=0,990

Table 16. Sensitivity and specificity of AV PCR for prototype strains of AVs and controls

Virus	Source	Result
Species A (type 12, 18, 31)	Cell culture	Positive
Species B (type 3, 7, 11, 14, 16, 21, 34, 35, 50)	Cell culture	Positive
Species C (type 1, 2, 5, 6)	Cell culture	Positive
Species D (type 8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51)	Cell culture	Positive
Species E (type 4)	Cell culture	Positive
Species F (type 40, 41)	Cell culture	Positive
Other virus	Enterovirus	Negative
Negative control	Calf-thymus DNA	Negative
HPC	Plasmid DNA	Positive
LPC	Plasmid DNA	Positive
IC	Plasmid DNA	Positive

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Table 17: Comparison of results of Adeno PCR, virus culture, and ELISA with different clinical specimens from patients with clinically suspected Adeno infections

				ELISA +		
		culture +	*culture -*	*culture -*	*culture +*	*culture -*
	number	*PCR +*	*PCR +*	*PCR +*	*PCR -*	*PCR -*
sample						
throat wash	33	14	4	-	0	15
sputum	6	2	1	-	0	3
nasopharynx wash	18	4	2	-	1	11
nasopharynx secretion.	3	1	1	-	0	1
BAL	3	2	0	-	0	1
faeces	90	36	4	10	0	36
CSF	1	0	0	-	0	1
throat swab	2	1	0	-	0	1
number (%)	152	60 (39,5)	12 (7,8)	10 (6,5)	1 (0,7)	69 (45,4)

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Table.18.: Adenovirus serotypes 1 to 51 isolated by MagNApure

'old' non-degenerated primerpair			
serotype	Ct-WT	Ct-IC	subgroup
1	22.37	undet.	C
2	21.37	undet.	C
3	37.83	35.12	B
4	20.89	undet.	E
5	19.90	undet.	C
6	21.72	undet.	C
7	27.46	39.83	B
8	22.17	undet.	D
9	20.38	undet.	D
10	19.64	undet.	D
11	38.05	35.05	B
12	28.60	35.83	A
13	18.57	undet.	D
14	undet.	33.29	B
15	20.81	undet.	D
16	22.83	undet.	B
17	20.28	undet.	D
18	undet.	34.53	A
19	18.92	undet.	D

(degenerated) primerpair of the invention			
serotype	Ct-WT	Ct-IC	subgroup
1	20.09	38.02	C
2	21.53	undet.	C
3	29.54	35.73	B
4	17.35	38.33	E
5	19.13	37.79	C
6	20.50	37.20	C
7	28.05	35.36	B
8	22.22	44.24	D
9	20.46	undet.	D
10	19.40	42.70	D
11	29.12	36.52	B
12	25.47	36.55	A
13	18.02	44.96	D
14	37.37	34.04	B
15	19.38	undet.	D
16	21.52	undet.	B
17	19.94	undet.	D
18	27.71	35.55	A
19	18.33	41.55	D

20	21.45	44.64	
21	27.97	39.54	B
22	19.36	undet.	D
23	18.43	undet.	D
24	18.51	undet.	D
25	18.55	undet.	
26	19.37	undet.	D
27	19.19	undet.	D
28	18.01	undet.	
29	19.11	undet.	
30	14.47	undet.	D
31	33.72	33.67	A
32	20.79	undet.	D
33	20.58	undet.	D
34	undet.	33.19	B
35	undet.	32.36	B
36	21.36	undet.	D
37	14.80	undet.	D
38	19.96	undet.	D
39	18.31	undet.	D
40	23.76	39.88	F
41	34.59	32.99	F
42	20.26	undet.	D
43	16.47	undet.	D
44	18.69	undet.	D

20	20.69	41.22	
21	23.28	40.09	B
22	19.49	undet.	D
23	17.75	undet.	D
24	17.95	undet.	D
25	18.05	undet.	
26	18.80	undet.	D
27	18.63	42.99	D
28	17.83	undet.	
29	18.00	undet.	
30	11.65	undet.	D
31	19.97	undet.	A
32	19.62	undet.	D
33	18.70	42.43	D
34	28.83	34.05	B
35	30.75	34.20	B
36	20.58	39.14	D
37	13.32	undet.	D
38	20.12	undet.	D
39	18.13	undet.	D
40	15.88	undet.	F
41	17.24	43.88	F
42	19.85 '	undet.	D
43	16.80	undet.	D
44	17.48	44.86	D

45	19.05	41.79	D
46	19.05	undet.	D
47	21.23	undet.	D
48	20.15	undet.	D
49	18.45	undet.	D
50*	26.58	42.97	B
51	19.77	undet.	D
*annealing at 60°C			

45	17.87	undet.	D
46	16.77	undet.	D
47	20.20	undet.	D
48	19.28	undet.	D
49	16.43	undet.	D
50	22.57	42.02	B
51	18.61	undet.	D

Table 19
AMC primer and probe sequences

Primer	Sequence	Position
AMC Entero 1	5'–CCC.TgA.Atg.Cgg.CTA.AT- 3'	(452 – 468)#
AMC Entero 2	5'-ATT.gTC.ACC.ATA.AgC.AgC.C- 3'	(579 – 597)#
AMC wildtype probe	5'-gCg.gAA.CCg.ACT.ACT.TTg.ggT- 3'	(531 – 551)#
AMC IC probe	5'-CTT.gAg.ACg.TgC.gTg.gTA.ACC- 3'	(531 – 551)#

= numbering according to Toyoda et al. 1984.

Table 20

Sample	Sampling Date	Material	Test Result
20471597	01-04-2004	CEREBRO SPINAL FLUID	Pos
20471918	21-04-2004	CEREBRO SPINAL FLUID	Pos
20472186	10-05-2004	CEREBRO SPINAL FLUID	Pos
20472242	12-05-2004	CEREBRO SPINAL FLUID	Pos
20472716	07-06-2004	CEREBRO SPINAL FLUID	Pos
20472951	20-06-2004	CEREBRO SPINAL FLUID	Pos
20473149	24-06-2004	CEREBRO SPINAL FLUID	Pos
20473147	28-06-2004	CEREBRO SPINAL FLUID	Pos
20473626	28-07-2004	CEREBRO SPINAL FLUID	Pos
20473678	02-08-2004	CEREBRO SPINAL FLUID	Pos
20473732	03-08-2004	CEREBRO SPINAL FLUID	Pos
20473838	09-08-2004	CEREBRO SPINAL FLUID	Pos
20473909	13-08-2004	CEREBRO SPINAL FLUID	Pos
20473903	13-08-2004	CEREBRO SPINAL FLUID	Pos
20474038	23-08-2004	CEREBRO SPINAL FLUID	Pos
20474219	28-08-2004	CEREBRO SPINAL FLUID	Pos
20474298	03-09-2004	CEREBRO SPINAL FLUID	Pos
20474455	09-09-2004	CEREBRO SPINAL FLUID	Pos
20475062	27-09-2004	CEREBRO SPINAL FLUID	Pos
20474736	29-09-2004	CEREBRO SPINAL FLUID	Pos
20474964	12-10-2004	CEREBRO SPINAL FLUID	Pos
20475007	12-10-2004	CEREBRO SPINAL FLUID	Pos
20475125	18-10-2004	CEREBRO SPINAL FLUID	Pos
20475155	20-10-2004	CEREBRO SPINAL FLUID	Pos
20475249	26-10-2004	CEREBRO SPINAL FLUID	Pos
20475515	09-11-2004	CEREBRO SPINAL FLUID	Pos
20475515	09-11-2004	CEREBRO SPINAL FLUID	Pos
20475690	18-11-2004	CEREBRO SPINAL FLUID	Pos
20570105	01-01-2005	CEREBRO SPINAL FLUID	Pos
20570671	02-02-2005	CEREBRO SPINAL FLUID	Pos
20571027	21-02-2005	CEREBRO SPINAL FLUID	Pos
20571314	05-03-2005	CEREBRO SPINAL FLUID	Pos
20471618	03-04-2004	CEREBRO SPINAL FLUID	Neg
20471664	05-04-2004	CEREBRO SPINAL FLUID	Neg
20450754	05-04-2004	CEREBRO SPINAL FLUID	Neg
20471690	06-04-2004	CEREBRO SPINAL FLUID	Neg
20471732	06-04-2004	CEREBRO SPINAL FLUID	Neg
20471692	07-04-2004	CEREBRO SPINAL FLUID	Neg
20471770	08-04-2004	CEREBRO SPINAL FLUID	Neg
20471720	08-04-2004	CEREBRO SPINAL FLUID	Neg
20471734	09-04-2004	CEREBRO SPINAL FLUID	Neg
20471762	14-04-2004	CEREBRO SPINAL FLUID	Neg
20471766	14-04-2004	CEREBRO SPINAL FLUID	Neg
20471768	14-04-2004	CEREBRO SPINAL FLUID	Neg
20471840	16-04-2004	CEREBRO SPINAL FLUID	Neg
20471838	18-04-2004	CEREBRO SPINAL FLUID	Neg
20471841	18-04-2004	CEREBRO SPINAL FLUID	Neg

20471839	19-04-2004	CEREBRO SPINAL FLUID	Neg
20471876	20-04-2004	CEREBRO SPINAL FLUID	Neg
20471943	21-04-2004	CEREBRO SPINAL FLUID	Neg
20471891	21-04-2004	CEREBRO SPINAL FLUID	Neg
20471919	21-04-2004	CEREBRO SPINAL FLUID	Neg
20472090	29-04-2004	CEREBRO SPINAL FLUID	Neg
20472077	03-05-2004	CEREBRO SPINAL FLUID	Neg
20472115	04-05-2004	CEREBRO SPINAL FLUID	Neg
20450984	04-05-2004	CEREBRO SPINAL FLUID	Neg
20472199	05-05-2004	CEREBRO SPINAL FLUID	Neg
20472114	05-05-2004	CEREBRO SPINAL FLUID	Neg
20472203	06-05-2004	CEREBRO SPINAL FLUID	Neg
20472184	08-05-2004	CEREBRO SPINAL FLUID	Neg
20472330	13-05-2004	CEREBRO SPINAL FLUID	Neg
20472323	14-05-2004	CEREBRO SPINAL FLUID	Neg
20472322	14-05-2004	CEREBRO SPINAL FLUID	Neg
20472377	17-05-2004	CEREBRO SPINAL FLUID	Neg
20472354	17-05-2004	CEREBRO SPINAL FLUID	Neg
20472344	18-05-2004	CEREBRO SPINAL FLUID	Neg
20472485	21-05-2004	CEREBRO SPINAL FLUID	Neg
20472431	23-05-2004	CEREBRO SPINAL FLUID	Neg
20472508	27-05-2004	CEREBRO SPINAL FLUID	Neg
20472534	28-05-2004	CEREBRO SPINAL FLUID	Neg
20472539	28-05-2004	CEREBRO SPINAL FLUID	Neg
20472537	30-05-2004	CEREBRO SPINAL FLUID	Neg
20472533	30-05-2004	CEREBRO SPINAL FLUID	Neg
20472581	30-05-2004	CEREBRO SPINAL FLUID	Neg
20472540	31-05-2004	CEREBRO SPINAL FLUID	Neg
20472675	31-05-2004	CEREBRO SPINAL FLUID	Neg
20472676	31-05-2004	CEREBRO SPINAL FLUID	Neg
20472674	02-06-2004	CEREBRO SPINAL FLUID	Neg
20472715	05-06-2004	CEREBRO SPINAL FLUID	Neg
20472670	06-06-2004	CEREBRO SPINAL FLUID	Neg
20472673	07-06-2004	CEREBRO SPINAL FLUID	Neg
20472705	08-06-2004	CEREBRO SPINAL FLUID	Neg
20472755	10-06-2004	CEREBRO SPINAL FLUID	Neg
20472809	11-06-2004	CEREBRO SPINAL FLUID	Neg
20472811	12-06-2004	CEREBRO SPINAL FLUID	Neg
20472808	14-06-2004	CEREBRO SPINAL FLUID	Neg
20472856	15-06-2004	CEREBRO SPINAL FLUID	Neg
20472857	15-06-2004	CEREBRO SPINAL FLUID	Neg
20472858	15-06-2004	CEREBRO SPINAL FLUID	Neg
20472963	16-06-2004	CEREBRO SPINAL FLUID	Neg
20472859	16-06-2004	CEREBRO SPINAL FLUID	Neg
20472899	17-06-2004	CEREBRO SPINAL FLUID	Neg
20472964	18-06-2004	CEREBRO SPINAL FLUID	Neg
20472960	21-06-2004	CEREBRO SPINAL FLUID	Neg
20473030	22-06-2004	CEREBRO SPINAL FLUID	Neg
20472998	22-06-2004	CEREBRO SPINAL FLUID	Neg
20472999	23-06-2004	CEREBRO SPINAL FLUID	Neg
20473093	23-06-2004	CEREBRO SPINAL FLUID	Neg

20472978	23-06-2004	CEREBRO SPINAL FLUID	Neg
20472997	23-06-2004	CEREBRO SPINAL FLUID	Neg
20473095	23-06-2004	CEREBRO SPINAL FLUID	Neg
20473089	27-06-2004	CEREBRO SPINAL FLUID	Neg
20473175	28-06-2004	CEREBRO SPINAL FLUID	Neg
20473091	28-06-2004	CEREBRO SPINAL FLUID	Neg
20473644	29-06-2004	CEREBRO SPINAL FLUID	Neg
20473133	29-06-2004	CEREBRO SPINAL FLUID	Neg
20473128	30-06-2004	CEREBRO SPINAL FLUID	Neg
20473168	01-07-2004	CEREBRO SPINAL FLUID	Neg
20473169	01-07-2004	CEREBRO SPINAL FLUID	Neg
20473318	03-07-2004	CEREBRO SPINAL FLUID	Neg
20473301	04-07-2004	CEREBRO SPINAL FLUID	Neg
20473230	04-07-2004	CEREBRO SPINAL FLUID	Neg
20473363	09-07-2004	CEREBRO SPINAL FLUID	Neg
20473371	09-07-2004	CEREBRO SPINAL FLUID	Neg
20473359	09-07-2004	CEREBRO SPINAL FLUID	Neg
20473365	11-07-2004	CEREBRO SPINAL FLUID	Neg
20473357	11-07-2004	CEREBRO SPINAL FLUID	Neg
20473366	12-07-2004	CEREBRO SPINAL FLUID	Neg
20473400	13-07-2004	CEREBRO SPINAL FLUID	Neg
20473470	16-07-2004	CEREBRO SPINAL FLUID	Neg
20473482	17-07-2004	CEREBRO SPINAL FLUID	Neg
20473471	18-07-2004	CEREBRO SPINAL FLUID	Neg
20473485	19-07-2004	CEREBRO SPINAL FLUID	Neg
20473523	20-07-2004	CEREBRO SPINAL FLUID	Neg
20473589	21-07-2004	CEREBRO SPINAL FLUID	Neg
20473520	21-07-2004	CEREBRO SPINAL FLUID	Neg
20473548	22-07-2004	CEREBRO SPINAL FLUID	Neg
20473594	23-07-2004	CEREBRO SPINAL FLUID	Neg
20473549	23-07-2004	CEREBRO SPINAL FLUID	Neg
20473599	26-07-2004	CEREBRO SPINAL FLUID	Neg
20473590	26-07-2004	CEREBRO SPINAL FLUID	Neg
20473628	26-07-2004	CEREBRO SPINAL FLUID	Neg
20473598	27-07-2004	CEREBRO SPINAL FLUID	Neg
20473631	28-07-2004	CEREBRO SPINAL FLUID	Neg
20473627	28-07-2004	CEREBRO SPINAL FLUID	Neg
20473686	31-07-2004	CEREBRO SPINAL FLUID	Neg
20473722	31-07-2004	CEREBRO SPINAL FLUID	Neg
20473685	02-08-2004	CEREBRO SPINAL FLUID	Neg
20473681	02-08-2004	CEREBRO SPINAL FLUID	Neg
20473731	03-08-2004	CEREBRO SPINAL FLUID	Neg
20473733	03-08-2004	CEREBRO SPINAL FLUID	Neg
20473723	03-08-2004	CEREBRO SPINAL FLUID	Neg
20473735	04-08-2004	CEREBRO SPINAL FLUID	Neg
20473797	06-08-2004	CEREBRO SPINAL FLUID	Neg
20473837	07-08-2004	CEREBRO SPINAL FLUID	Neg
20473799	08-08-2004	CEREBRO SPINAL FLUID	Neg
20473803	09-08-2004	CEREBRO SPINAL FLUID	Neg
20473802	09-08-2004	CEREBRO SPINAL FLUID	Neg
20473839	11-08-2004	CEREBRO SPINAL FLUID	Neg

20473948	17-08-2004	CEREBRO SPINAL FLUID	Neg
20473940	18-08-2004	CEREBRO SPINAL FLUID	Neg
20473941	18-08-2004	CEREBRO SPINAL FLUID	Neg
20474048	19-08-2004	CEREBRO SPINAL FLUID	Neg
20474049	21-08-2004	CEREBRO SPINAL FLUID	Neg
20474041	23-08-2004	CEREBRO SPINAL FLUID	Neg
20474094	24-08-2004	CEREBRO SPINAL FLUID	Neg
20474089	24-08-2004	CEREBRO SPINAL FLUID	Neg
20474081	25-08-2004	CEREBRO SPINAL FLUID	Neg
20474093	25-08-2004	CEREBRO SPINAL FLUID	Neg
20474218	29-08-2004	CEREBRO SPINAL FLUID	Neg
20474174	30-08-2004	CEREBRO SPINAL FLUID	Neg
20474213	31-08-2004	CEREBRO SPINAL FLUID	Neg
20474212	31-08-2004	CEREBRO SPINAL FLUID	Neg
20474605	01-09-2004	CEREBRO SPINAL FLUID	Neg
20474211	01-09-2004	CEREBRO SPINAL FLUID	Neg
20474580	02-09-2004	CEREBRO SPINAL FLUID	Neg
20474300	03-09-2004	CEREBRO SPINAL FLUID	Neg
20474302	03-09-2004	CEREBRO SPINAL FLUID	Neg
20474304	04-09-2004	CEREBRO SPINAL FLUID	Neg
20474607	05-09-2004	CEREBRO SPINAL FLUID	Neg
20474339	06-09-2004	CEREBRO SPINAL FLUID	Neg
20474335	07-09-2004	CEREBRO SPINAL FLUID	Neg
20474337	07-09-2004	CEREBRO SPINAL FLUID	Neg
20474333	08-09-2004	CEREBRO SPINAL FLUID	Neg
20474358	10-09-2004	CEREBRO SPINAL FLUID	Neg
20474449	10-09-2004	CEREBRO SPINAL FLUID	Neg
20474405	11-09-2004	CEREBRO SPINAL FLUID	Neg
20474404	12-09-2004	CEREBRO SPINAL FLUID	Neg
20474410	12-09-2004	CEREBRO SPINAL FLUID	Neg
20474448	14-09-2004	CEREBRO SPINAL FLUID	Neg
20474437	15-09-2004	CEREBRO SPINAL FLUID	Neg
20474436	15-09-2004	CEREBRO SPINAL FLUID	Neg
20474479	16-09-2004	CEREBRO SPINAL FLUID	Neg
20474531	16-09-2004	CEREBRO SPINAL FLUID	Neg
20474476	16-09-2004	CEREBRO SPINAL FLUID	Neg
20474524	20-09-2004	CEREBRO SPINAL FLUID	Neg
20474579	21-09-2004	CEREBRO SPINAL FLUID	Neg
20474577	21-09-2004	CEREBRO SPINAL FLUID	Neg
20474578	21-09-2004	CEREBRO SPINAL FLUID	Neg
20474608	22-09-2004	CEREBRO SPINAL FLUID	Neg
20474614	23-09-2004	CEREBRO SPINAL FLUID	Neg
20474660	23-09-2004	CEREBRO SPINAL FLUID	Neg
20474606	23-09-2004	CEREBRO SPINAL FLUID	Neg
20474609	23-09-2004	CEREBRO SPINAL FLUID	Neg
20474656	24-09-2004	CEREBRO SPINAL FLUID	Neg
20474649	24-09-2004	CEREBRO SPINAL FLUID	Neg
20474648	25-09-2004	CEREBRO SPINAL FLUID	Neg
20474740	01-10-2004	CEREBRO SPINAL FLUID	Neg
20474778	03-10-2004	CEREBRO SPINAL FLUID	Neg
20474783	04-10-2004	CEREBRO SPINAL FLUID	Neg

20474822	06-10-2004	CEREBRO SPINAL FLUID	Neg
20474906	10-10-2004	CEREBRO SPINAL FLUID	Neg
20474963	11-10-2004	CEREBRO SPINAL FLUID	Neg
20474912	11-10-2004	CEREBRO SPINAL FLUID	Neg
20474966	13-10-2004	CEREBRO SPINAL FLUID	Neg
20475002	14-10-2004	CEREBRO SPINAL FLUID	Neg
20475190	23-10-2004	CEREBRO SPINAL FLUID	Neg
20475193	25-10-2004	CEREBRO SPINAL FLUID	Neg
20475248	26-10-2004	CEREBRO SPINAL FLUID	Neg
20475292	27-10-2004	CEREBRO SPINAL FLUID	Neg
20475334	29-10-2004	CEREBRO SPINAL FLUID	Neg
20475333	01-11-2004	CEREBRO SPINAL FLUID	Neg
20475418	03-11-2004	CEREBRO SPINAL FLUID	Neg
20475465	03-11-2004	CEREBRO SPINAL FLUID	Neg
20475509	08-11-2004	CEREBRO SPINAL FLUID	Neg
20475539	11-11-2004	CEREBRO SPINAL FLUID	Neg
20452386	11-11-2004	CEREBRO SPINAL FLUID	Neg
20475592	11-11-2004	CEREBRO SPINAL FLUID	Neg
20475591	12-11-2004	CEREBRO SPINAL FLUID	Neg
20475588	12-11-2004	CEREBRO SPINAL FLUID	Neg
20475586	14-11-2004	CEREBRO SPINAL FLUID	Neg
20475577	15-11-2004	CEREBRO SPINAL FLUID	Neg
20475627	16-11-2004	CEREBRO SPINAL FLUID	Neg
20475693	18-11-2004	CEREBRO SPINAL FLUID	Neg
20475740	19-11-2004	CEREBRO SPINAL FLUID	Neg
20475686	19-11-2004	CEREBRO SPINAL FLUID	Neg
20475685	20-11-2004	CEREBRO SPINAL FLUID	Neg
20475721	23-11-2004	CEREBRO SPINAL FLUID	Neg
20475736	24-11-2004	CEREBRO SPINAL FLUID	Neg
20475795	26-11-2004	CEREBRO SPINAL FLUID	Neg
20475796	26-11-2004	CEREBRO SPINAL FLUID	Neg
20475801	29-11-2004	CEREBRO SPINAL FLUID	Neg
20475840	30-11-2004	CEREBRO SPINAL FLUID	Neg
20475865	02-12-2004	CEREBRO SPINAL FLUID	Neg
20475911	03-12-2004	CEREBRO SPINAL FLUID	Neg
20475917	03-12-2004	CEREBRO SPINAL FLUID	Neg
20475914	03-12-2004	CEREBRO SPINAL FLUID	Neg
20476054	03-12-2004	CEREBRO SPINAL FLUID	Neg
20475905	04-12-2004	CEREBRO SPINAL FLUID	Neg
20475910	06-12-2004	CEREBRO SPINAL FLUID	Neg
20476053	10-12-2004	CEREBRO SPINAL FLUID	Neg
20476103	13-12-2004	CEREBRO SPINAL FLUID	Neg
20476122	16-12-2004	CEREBRO SPINAL FLUID	Neg
20476127	16-12-2004	CEREBRO SPINAL FLUID	Neg
20476166	17-12-2004	CEREBRO SPINAL FLUID	Neg
20476293	22-12-2004	CEREBRO SPINAL FLUID	Neg
20476286	25-12-2004	CEREBRO SPINAL FLUID	Neg
20476285	27-12-2004	CEREBRO SPINAL FLUID	Neg
20476312	28-12-2004	CEREBRO SPINAL FLUID	Neg
20570031	31-12-2004	CEREBRO SPINAL FLUID	Neg
20570029	01-01-2005	CEREBRO SPINAL FLUID	Neg

20570027	01-01-2005	CEREBRO SPINAL FLUID	Neg
20570106	05-01-2005	CEREBRO SPINAL FLUID	Neg
20570257	05-01-2005	CEREBRO SPINAL FLUID	Neg
20570255	06-01-2005	CEREBRO SPINAL FLUID	Neg
20570128	07-01-2005	CEREBRO SPINAL FLUID	Neg
20570258	07-01-2005	CEREBRO SPINAL FLUID	Neg
20570168	08-01-2005	CEREBRO SPINAL FLUID	Neg
20570256	10-01-2005	CEREBRO SPINAL FLUID	Neg
20570167	10-01-2005	CEREBRO SPINAL FLUID	Neg
20570346	12-01-2005	CEREBRO SPINAL FLUID	Neg
20570350	14-01-2005	CEREBRO SPINAL FLUID	Neg
20570343	15-01-2005	CEREBRO SPINAL FLUID	Neg
20570440	18-01-2005	CEREBRO SPINAL FLUID	Neg
20570403	18-01-2005	CEREBRO SPINAL FLUID	Neg
20570480	19-01-2005	CEREBRO SPINAL FLUID	Neg
20570483	20-01-2005	CEREBRO SPINAL FLUID	Neg
20570481	20-01-2005	CEREBRO SPINAL FLUID	Neg
20570473	21-01-2005	CEREBRO SPINAL FLUID	Neg
20570631	26-01-2005	CEREBRO SPINAL FLUID	Neg
20570576	26-01-2005	CEREBRO SPINAL FLUID	Neg
20570632	28-01-2005	CEREBRO SPINAL FLUID	Neg
20570629	28-01-2005	CEREBRO SPINAL FLUID	Neg
20570622	29-01-2005	CEREBRO SPINAL FLUID	Neg
20570729	31-01-2005	CEREBRO SPINAL FLUID	Neg
20570730	01-02-2005	CEREBRO SPINAL FLUID	Neg
20570670	01-02-2005	CEREBRO SPINAL FLUID	Neg
20570726	03-02-2005	CEREBRO SPINAL FLUID	Neg
20570821	03-02-2005	CEREBRO SPINAL FLUID	Neg
20570820	07-02-2005	CEREBRO SPINAL FLUID	Neg
20570847	08-02-2005	CEREBRO SPINAL FLUID	Neg
20570806	08-02-2005	CEREBRO SPINAL FLUID	Neg
20570850	10-02-2005	CEREBRO SPINAL FLUID	Neg
20570883	11-02-2005	CEREBRO SPINAL FLUID	Neg
20570882	11-02-2005	CEREBRO SPINAL FLUID	Neg
20570889	14-02-2005	CEREBRO SPINAL FLUID	Neg
20570944	14-02-2005	CEREBRO SPINAL FLUID	Neg
20570881	14-02-2005	CEREBRO SPINAL FLUID	Neg
20570976	17-02-2005	CEREBRO SPINAL FLUID	Neg
20570975	17-02-2005	CEREBRO SPINAL FLUID	Neg
20571028	18-02-2005	CEREBRO SPINAL FLUID	Neg
20571083	19-02-2005	CEREBRO SPINAL FLUID	Neg
20571174	23-02-2005	CEREBRO SPINAL FLUID	Neg
20571072	23-02-2005	CEREBRO SPINAL FLUID	Neg
20571077	23-02-2005	CEREBRO SPINAL FLUID	Neg
20571118	24-02-2005	CEREBRO SPINAL FLUID	Neg
20571166	25-02-2005	CEREBRO SPINAL FLUID	Neg
20571232	27-02-2005	CEREBRO SPINAL FLUID	Neg
20571226	01-03-2005	CEREBRO SPINAL FLUID	Neg
20571222	01-03-2005	CEREBRO SPINAL FLUID	Neg
20571259	03-03-2005	CEREBRO SPINAL FLUID	Neg
20571315	04-03-2005	CEREBRO SPINAL FLUID	Neg

20571361	04-03-2005	CEREBRO SPINAL FLUID	Neg
20571313	04-03-2005	CEREBRO SPINAL FLUID	Neg
20571312	07-03-2005	CEREBRO SPINAL FLUID	Neg
20571362	08-03-2005	CEREBRO SPINAL FLUID	Neg
20571384	09-03-2005	CEREBRO SPINAL FLUID	Neg
20571439	10-03-2005	CEREBRO SPINAL FLUID	Neg
20571450	12-03-2005	CEREBRO SPINAL FLUID	Neg
20571511	14-03-2005	CEREBRO SPINAL FLUID	Neg
20571541	16-03-2005	CEREBRO SPINAL FLUID	Neg
20571510	16-03-2005	CEREBRO SPINAL FLUID	Neg
20571595	17-03-2005	CEREBRO SPINAL FLUID	Neg
20571590	18-03-2005	CEREBRO SPINAL FLUID	Neg
20571586	18-03-2005	CEREBRO SPINAL FLUID	Neg
20571591	20-03-2005	CEREBRO SPINAL FLUID	Neg
20571629	20-03-2005	CEREBRO SPINAL FLUID	Neg
20571654	22-03-2005	CEREBRO SPINAL FLUID	Neg
20571656	23-03-2005	CEREBRO SPINAL FLUID	Neg
20571658	24-03-2005	CEREBRO SPINAL FLUID	Neg

Claims

1. A method for reverse transcribing an RNA target molecule comprising incubating said RNA target molecule with a reverse transcriptase, preferably a moloney Murine Leukemia Virus reverse transcriptase or SuperScript II or SuperScript III or a functional part, derivative and/or analogue thereof in a solution comprising between 25 and 75 mM of a suitable salt, between 0.05 % and 0.2 % of a non-ionic detergent, between 60 and 240 µM per nucleotide or equivalent thereof and a suitable buffer that buffers said solution at a pH between about 8 or 10, said method further comprising incubating said solution at a temperature of between 35 and 50 ^{0}C.

2. A method according to claim 1, wherein the solution is buffered at a pH between about 8 and 9.

3. A method according to claim 1 or claim 2, wherein the solution is buffered at a pH between about 8.1 and 8.5.

4. A method according to claim 3, wherein the solution is buffered at a pH of about 8.3.

5. A method according to any one of claims 1-4, wherein said solution further comprises random hexamer primers.

6. A method according to any one of claims 1-5, wherein said RNA target molecule is derived from a faecal sample.

7. A method according to claim 6, wherein said RNA target molecule comprises an enterovirus and/or an influenza virus.

8. A method according to any one of claims 1-7, wherein said solution further comprises an internal control RNA molecule.

9. A method according to claim 8, wherein said internal control RNA molecule comprises a sequence 5'-CCC TGA ATG CGG CTA ATC CTA ACC ACG GAA CAG GCG GTC GCG AAC CAG TGA CTG GCT TGT CGT AAC GCG CAA GTC TGT GCT TGA GAC GTG CGT GGT AAC CGT CCG TGT TTC CTG TTA TTT TTA TCA TGG CTG CTT ATG GTG ACA AT-3'.

10. A method for testing a sample for the presence therein of a nucleic acid derived from a virus, comprising providing a nucleic acid amplification solution with said sample and with a first and a second primer, said first primer comprising a nucleotide sequence of a forward primer depicted in any one of tables 6-13, or a functional equivalent thereof, and said second primer comprising a nucleotide sequence of the reverse primer depicted in the same table as said forward primer, or a functional equivalent thereof, and incubating said amplification solution to allow for amplification of said nucleic acid when present.

11. A method according to claim 10, wherein said virus comprises an enterovirus, adenovirus or influenza virus.

12. A method according to claim 10 or 11, wherein said first primer comprises a nucleotide sequence ID#1: 5'-CCC TGA ATG CGG CTA AT-3'; or a functional equivalent thereof and said second primer comprises a nucleotide sequence ID#2: 5'-ATT GTC ACC ATA AGC AGC C-3' or a functional equivalent thereof.

13. A method according to claim 10 or 11, wherein said first primer comprises the sequence 5'-CAGGACGCCTCGGRGTAYCTSAG-3' or a functional equivalent thereof and said second primer comprises the sequence 5'-GGAGCCACVGTGGGRTT-3' or a functional equivalent thereof.

14. A method according to claim 10 or 11, wherein said first primer comprises the sequence 5'-GACAAGACCAATCCTGTCACYTCTG-3' or a functional equivalent thereof and said second primer comprises the sequence 5'-AAGCGTCTACGCTGCAGTCC-3' or a functional equivalent thereof.

15. A method according to any one of claims 10-12 or 14, wherein said sample comprises product of a method for reverse transcribing an RNA target molecule according to any one of claims 1-9.

16. A method according to any one of claims 10-15, further comprising analysing the presence or absence of said nucleic acid by means of a probe.

17. A method according to claim 16, wherein said probe comprises a nucleotide sequence of the virus-specific probe depicted in the same table as

said forward primer and/or reverse primer, or a functional equivalent of said virus-specific probe.

18. A method according to claim 16 or 17, wherein the presence of nucleic acid derived from an enterovirus is analysed with a probe comprising a sequence ID#3: EV-specific probe; 5'-GCG GAA CCG ACT ACT TTG GGT-3'or a functional equivalent thereof.

19. A method according to claim 16 or 17, wherein the presence of nucleic acid derived from an adenovirus is analysed with a probe comprising a sequence 5'-CCGGGTCTGGTGCAGTTTGCCCGC-3'or a functional equivalent thereof.

20. A method according to claim 16 or 17, wherein the presence of nucleic acid derived from an influenza A virus is analysed with a probe comprising a sequence 5'-TTCACGCTCACCGTGCCCAGTGAGC-3'or a functional equivalent thereof.

21. A method according to any one of claims 10-20, wherein said nucleic acid amplification solution further comprises an internal control nucleic acid molecule.

22. A method according to claim 21, wherein said internal control nucleic acid molecule comprises a nucleotide sequence of the internal control depicted in the same table as said forward primer and/or reverse primer, or a functional equivalent of said internal control.

23. A method according to claim 21 or 22, wherein said internal control comprises the sequence 5'-CCC TGA ATG CGG CTA ATC CTA ACC ACG GAA CAG GCG GTC GCG AAC CAG TGA CTG GCT TGT CGT AAC GCG CAA GTC TGT GCT TGA GAC GTG CGT GGT AAC CGT CCG TGT TTC CTG TTA TTT TTA TCA TGG CTG CTT ATG GTG ACA AT-3'.

24. A method according to any one of claims 21-23, further comprising analysing the presence or absence of said internal control nucleic acid by means of a probe comprising a nucleotide sequence of the IC-specific probe depicted in the same table as said internal control, or a functional equivalent of said IC-specific probe.

25. A method according to claim 24, wherein said IC-specific probe comprises the sequence 5'-CTT GAG ACG TGC GTG GTA ACC-3' , 5'-GATGTGTCCGCCGTGGTCCCCTGG-3', or 5'-TTCACTGGGCCCGACTCGCACTGAC-3', or a functional equivalent thereof.

26. A method according to any one of claims 10-25, wherein a product of said amplification is measured in real-time.

27. A method according to any one of claims 10-26, wherein a sample is tested for the presence of a first virus as depicted in any one of Tables 6-13 and for the presence of a second virus as depicted in any one of Tables 6-13, using at least two primers depicted in the same Tables as said first and said second virus.

28. An aqueous solution for reverse transcribing an RNA target molecule comprising between 25 and 75 mM of a suitable salt, between 0.05 % and 0.2 % of a non-ionic detergent and a suitable buffer that buffers said solution at a pH between about 8 or 10.

29. An aqueous solution according to claim 28, further comprising between 60 and 240 µM of at least one nucleotide or equivalents thereof and a reverse transcriptase, preferably a Moloney Murine Leukemia Virus reverse transcriptase or SuperScript II or SuperScript III or a functional part, derivative and/or analogue thereof.

30. A concentrate of an aqueous solution according to claim 28 or 29.

31. An isolated or recombinant oligonucleotide comprising a sequence as depicted in any one of tables 6-13.

32. An oligonucleotide according to claim 31 comprising a sequence 5'-CCC TGA ATG CGG CTA AT-3'; or 5'-ATT GTC ACC ATA AGC AGC C-3' ; or 5'-GCG GAA CCG ACT ACT TTG GGT-3'; or 5'-CTT GAG ACG TGC GTG GTA ACC-3' or 5'-CCC TGA ATG CGG CTA ATC CTA ACC ACG GAA CAG GCG GTC GCG AAC CAG TGA CTG GCT TGT CGT AAC GCG CAA GTC TGT GCT TGA GAC GTG CGT GGT AAC CGT CCG TGT TTC CTG TTA TTT TTA TCA TGG CTG CTT ATG GTG ACA AT-3.

33. A recombinant virus particle comprising a sequence as depicted in any one of Tables 6-13 or a functional equivalent thereof, or the complement thereof.

34. A recombinant virus particle according to claim 33, comprising the sequence 5'-CCC TGA ATG CGG CTA ATC CTA ACC ACG GAA CAG GCG GTC GCG AAC CAG TGA CTG GCT TGT CGT AAC GCG CAA GTC TGT GCT TGA GAC GTG CGT GGT AAC CGT CCG TGT TTC CTG TTA TTT TTA TCA TGG CTG CTT ATG GTG ACA AT-3' and/or ID#4 5'-CTT GAG ACG TGC GTG GTA ACC-3' or the complement thereof.

35. A kit for detecting viral nucleic acid comprising at least one and preferably at least two, more preferably at least three oligonucleotides comprising a sequence as depicted in any one of tables 6-13, or a functional equivalent of said sequence.

36. A kit according to claim 35 for detecting enteroviral RNA comprising at least one and preferably at least two, more preferably at least three oligonucleotides according to claim 32.

37. A kit according to claim 36, comprising an internal control nucleic acid, comprising a sequence according to claim 32, or a functional equivalent thereof.

38. A kit according to any one of claims 35-37, comprising an armoured internal control RNA.

39. A kit according to claim 35 for detecting adenoviral DNA comprising at least one and preferably at least two, more preferably at least three oligonucleotides comprising a sequence as depicted in table 11, or a functional equivalent thereof.

40. A kit according to claim 35 for detecting influenza A RNA comprising at least one and preferably at least two, more preferably at least three oligonucleotides comprising a sequence as depicted in table 6, or a functional equivalent thereof.

41. A kit according to claim 39 or 40, further comprising an internal control nucleic acid, comprising a sequence as depicted in table 11 or table 6, or a functional equivalent thereof.

42. A kit according to any one of claims 35-41, further comprising an aqueous solution according to claim 28 or claim 29 and/or a concentrate according to claim 30.

43. A kit according to any one of claims 35-42, further comprising SuperScript II or SuperScript III.

44. A primer pair comprising an oligonucleotide comprising a sequence of a forward primer as depicted in any one of Tables 6-13, or a functional equivalent thereof, and an oligonucleotide comprising a sequence of the reverse primer depicted in the same table as said forward primer, or a functional equivalent thereof.