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(54) Title: ISOTHERMAL AMPLIFICATION ASSAY FOR RAPID AND ACCURATE DETECTION OF HEMORRHAGIC  
FEVER VIRUSES IN CLINICAL SAMPLES

(57) Abstract: The invention relates to a method for diagnosing a Viral Hemorrhagic Fever by isothermal amplification of a target  
sequence of a Hemorrhagic Fever virus nucleic acid located within a gene which codes for a protein of the viral ribonucleocapsid.  
The invention relates also to a kit for performing the method.



**Isothermal amplification assay for rapid and accurate detection  
of Hemorrhagic Fever Viruses in clinical samples**

The invention pertains to the field of Viral Hemorrhagic Fevers (VHFs) diagnosis. The invention relates to a method for detecting a Hemorrhagic  
5 Fever virus (HFV) by isothermal amplification of a target sequence of the HFV nucleic acid. The invention relates also to a kit for performing the method.

Viral Hemorrhagic Fevers (VHFs) are acute infections with high case fatality rates caused by four distinct families of RNA viruses: Arenavirus, Bunyavirus, Filovirus, and Flavivirus (Meltzer *et al.*, Infect. Dis. Clin. N. Am., 2012,  
10 26, 479-496). Infections by Hemorrhagic Fever Viruses (HFVs) are associated with a wide spectrum of clinical manifestations such as diarrhea, myalgia, cough, headache, pneumonia, encephalopathy, and hepatitis. Hemorrhage is the characteristic manifestation, although nonhemorrhagic infections are also common. Although nearly all VHF agents are endemic in tropical or subtropical regions, infections can also  
15 occur outside these regions, for example, in returning travelers.

The Ebola virus disease (EVD) epidemic of 2015 is the largest in history, affecting multiple countries in West Africa and putting neighboring countries at risk (Dixon *et al.*, MMWR Morb. Mortal. Wkly. Rep. 2014, Jun 27; 63(25):548–51). There are five subtypes or species of Ebola virus (or Ebolavirus), a Filovirus. Four  
20 have caused diseases in humans: *Zaire ebolavirus* (Ebola virus (EBOV) or Ebola-Zaire (ZEBOV)), *Tai Forest ebolavirus* (Tai Forest virus (TAFV) or Ebola-Tai Forest (TEBOV), formerly known as Ebola-Ivory Coast), *Sudan ebolavirus* (Sudan virus (SUDV) or Ebola-Sudan (SEBOV)), and *Bundibugyo ebolavirus* (Bundibugyo virus (BDBV) or Ebola-Bundibugyo (BEBOV)). The fifth, *Reston ebolavirus* (Reston virus  
25 (RESTV) or Ebola-Reston (REBOV)), has only caused diseases in nonhuman primates. Marburg hemorrhagic fever is a rare, severe type of HF caused by a Filovirus, which affects both humans and non-humans primates. The structures of filoviruses are very similar. The virus genome is a negative-stranded RNA of 19 kb in length encoding seven viral structural proteins in the order nucleoprotein (NP),  
30 phosphoprotein (VP35), matrix protein (VP40), glycoprotein (GP), replication-transcription protein (VP30), matrix protein (VP24) and RNA-dependent RNA polymerase (L), with an additional soluble glycoprotein produced from an edited GP

mRNA. The viral particle is formed of a Ribonucleocapsid (RNP) comprising the viral RNA genome and the L, NP, VP30 and VP35 proteins, surrounded by a host-membrane derived envelope comprising the glycoprotein and matrix proteins.

Lassa fever virus is caused by an Arenavirus endemic in parts of West Africa. The negative-stranded RNA genome comprises L segment (7.5 kb) and S segment (3.5 kb) encoding four viral structural proteins: nucleoprotein (NP), matrix protein (Z), glycoprotein (GP), and RNA-dependent RNA polymerase (L). The viral particle is formed of a Ribonucleocapsid (RNP) comprising the viral RNA genome and the L and NP proteins, surrounded by an envelope comprising the glycoprotein and matrix protein.

In the absence of bleeding or organ manifestation, a VHF is clinically difficult to diagnose, and the various etiologic agents can hardly be distinguished by clinical tests. Inexpensive, portable, and easy-to-use diagnostic devices are urgently needed to ensure rapid detection of HFVs at the point-of-care. Unfortunately, the availability of laboratory diagnosis in low-resource, high disease-burden areas is currently limited by cost, infrastructure, and personnel constraints (Pai et al., PLoS Med., 2012 Sep 4; 9(9):e1001306). Rapid nucleic acid testing for viral diseases can provide access to much-needed diagnostic methods in low-resource countries, especially for applications requiring fast turnaround times (Niemz et al., Trends Biotechnol., 2011 May; 29(5):240–50).

Common molecular detection methods by end point and real time Polymerase Chain Reaction (PCR) are valuable tools for pathogen detection and are widely used in clinical diagnostics because of high sensitivity and specificity. Various RT-PCR assays for the detection of VHFs are available (Grolla et al., Bull. Société Pathol. Exot., 2005 Sep; 98(3):205–9; Drosten et al., J. Clin. Microbiol., 2002 Jul; 40(7):2323–30; Ogawa *et al.*, J. Virol. Methods, 2011, 171, 310-313). However, they are major problems in implementing these methods for near-patient testing in endemic areas, in particular, the need for trained personal as well as expensive and sophisticated equipment.

In contrast to the polymerase chain reaction (PCR) technology in which the reaction is carried out with a series of alternating temperature steps or cycles, isothermal amplification is carried out at a constant temperature, and does not

require a thermal cycler. Among field-applicable isothermal nucleic acid amplification methods developed for rapid, simple, and cost-effective detection of pathogenic microorganisms in smaller size systems, the RT-LAMP (*reverse-transcription loop-mediated isothermal amplification*) appears to be promising assays, highly suited for on-site detection of EVD (Craw et al., Lab. Chip., 2012 Jun 19; 12(14):2469–86). The RT-LAMP assay uses a DNA polymerization enzyme with high strand-displacement activity and 4 primers, specifically designed to recognize 6 distinct regions on the target gene, to synthesize large amounts of target viral nucleic acids under a constant temperature, usually between 55-65°C (Notomi et al., Nucleic Acids Res., 2000 Jun 15; 28(12):e63). An additional pair of "loop primers" is used to further accelerate the reaction. The LAMP reaction yields high amount of amplification products, which can be detected either visually or by simple detectors. These large amounts of synthesized double strand DNA (dsDNA) yield large amounts of pyrophosphate ion byproducts, which combine with divalent metallic ion (such as  $Mg^{2+}$ ) to form an insoluble salt, resulting in a decrease of  $Mg^{2+}$  ion concentration as the LAMP reaction progresses (Goto et al., BioTechniques, 2009 Mar; 46(3):167–72). In addition, it has been reported that there is a strong correlation between the change in pH and the amplification yield during the LAMP, as hydrogen ions are released during the LAMP procedure. Due to these properties, real-time monitoring of the LAMP reaction can be achieved by: (i) fluorescence, using DNA intercalating dyes, fluorescent molecular beacon probes or a fluorescence metal indicator such as calcein; (ii) colorimetry, using a colored indicator for alkaline metal ions, such as hydroxy naphthol blue (Goto et al., BioTechniques, 2009 Mar; 46(3):167–72) or pH indicators (Tanner et al., BioTechniques, 2015 Feb; 58(2):59–68); (iii) turbidity, as the LAMP reaction produces large amounts of magnesium pyrophosphate (a white precipitate) and dsDNA, which allow visual inspection of results using a turbidimeter (Mori et al., Biochem. Biophys. Res. Commun., 2001 Nov 23; 289(1):150–4); (iv) electrochemically, using a pH meter for direct measurement of released hydrogen ions during the LAMP procedure (Xie et al., Chem. Commun., 2014 Oct 24; 50(100):15932–5), or using integrated electrodes for measuring decreases in current resulting from increasing binding of electrochemically-active DNA-binding redox reporters, such as Methylene Blue, to LAMP reaction products (Xie et al., Biosens.

Bioelectron., 2014 May 15; 55:324–9); (v) enzyme-linked immunosorbent assays (ELISA) or lateral flow immunoassays based on the use of specific probes (Tsai et al., J. Virol. Methods., 2012 Apr; 181(1):117–24; Ravan H and Yazdanparast R., Anal. Biochem., 2013 Aug 15; 439(2):102–8); (vi) bioluminescence, through  
5 bioluminescent output of the coupled conversion of inorganic pyrophosphate produced stoichiometrically during nucleic acid synthesis to ATP by the enzyme ATP sulfurylase (Gandelman et al., PLoS ONE, 2010 Nov 30; 5(11)). In addition to its relative simplicity and low infrastructure costs, the RT-LAMP assay (i) has moderate incubation temperature leading to simplified heating and low power consumption, (ii)  
10 allows direct genetic amplification from target pathogens due to a superior tolerance to well-known PCR inhibitors such as blood (iii) demonstrates high specificity and sensitivity, and (iv) results in rapid detection often within 30 min (Mori Y and Notomi T., J. Infect. Chemother., 2009 Apr 1; 15(2):62–9; Kaneko et al., J. Biochem. Biophys. Methods., 2007 Apr 10; 70(3):499–501). However, RT-LAMP assays designed for  
15 filovirus diagnosis have been shown to be slightly less sensitive compared to equivalent TaqMan RT-PCR assays (Schurtleff *et al.*, frontiers in Microbiology, 2015, 6, article 108).

The inventors have developed an RT-LAMP assay for the species-specific detection of HFVs, in particular major HFV agents including Ebola virus  
20 species, Lassa virus and Marburg virus. The assay which is based on the isothermal amplification of a specific target located within a gene coding for a protein of the viral ribonucleocapsid such as the L or NP gene, demonstrated equivalent analytical sensitivity and at least a 5 times faster time-to-result (15 minutes as compared to 80 minutes) as compared to the currently used reference real-time RT-PCR assay  
25 (RealStar® Ebolavirus RT-PCR Kit 1.0, Altona Diagnostics) as well as other published Ebola virus qRT-PCR assays (Grolla et al., Bull. Société Pathol. Exot., 2005 Sep; 98(3):205–9; Drosten et al., J. Clin. Microbiol., 2002 Jul; 40(7):2323–30) thereby showing great potential for point-of-care applications. Moreover, the RT-LAMP assay demonstrated superior clinical performances as compared to the RT-PCR assay,  
30 probably due to a higher tolerance to inhibitors, making it highly suitable for accurate and reliable diagnostic applications.

Therefore, a first aspect of the present invention relates to a method of detecting a Hemorrhagic Fever Virus (HFV) in a sample, comprising:

- a) subjecting said sample to an isothermal nucleic acid amplification reaction using at least one pair of oligonucleotide primers for amplifying a target sequence of a HFV nucleic acid, wherein the target sequence is located within a gene of the HFV nucleic acid which codes for a protein of the viral ribonucleocapsid, and
- b) detecting the presence of an amplification product for said target sequence.

In step a) of the method of the invention, the oligonucleotide primers which are contacted with the sample suspected of containing HFV are capable of hybridizing with the target sequence present in any HFV target nucleic acid present in said sample. The HFV target nucleic acid present in the sample is used as a template for generating an amplification product (amplicon). In step b) of the method of the invention, the detection of the presence or absence of the amplification product determines the presence or absence of an HFV in the sample.

The method of the invention is used for the detection of any HFV, in particular an RNA virus chosen from a Filovirus, Arenavirus, Bunyavirus, or Flavivirus. Filoviruses, Arenaviruses, and Bunyaviruses have a single-stranded negative sense RNA which is segmented in the case of Arenaviruses. Flaviviruses have a single-stranded positive sense RNA. A Filovirus includes Ebolavirus, Marburgvirus and Cuevavirus. Ebolavirus includes one or more of the different subtypes or species of Ebola virus including Taï Forest virus (TAFV, Ebola-Taï Forest or TEBOV, formerly known as Ivory Coast ebolavirus) of *Taï Forest ebolavirus species*; Reston virus (RESTV, Ebola-Reston or REBOV) of *Reston ebolavirus species*; Sudan virus (SUDV, Ebola-Sudan or SEBOV) of *Sudan ebolavirus species*; Ebola virus (EBOV, Ebola-Zaire or ZEBOV) of *Zaire ebolavirus species* and Bundibugyo virus (BDBV, Ebola-Bundibugyo or BEBOV) of *Bundibugyo ebolavirus species*. Marburgvirus includes Marburg virus (MARV) and Ravn virus (RAVV) of Marburg marburgvirus species. Cuevavirus includes Lloviu virus (LLOV) of Lloviu cuevavirus species. An Arenavirus includes Lassa fever virus (Lassa virus), Lujo virus, Argentina, Bolivian, Brazilian and Venezuelan Hemorrhagic Fever viruses. A Bunyavirus includes Crimean-Congo Hemorrhagic Fever virus (CCHF), Hantavirus,

Garissa virus, Ilesha virus and Rift Valley Fever virus. A Flavivirus includes Dengue virus, Yellow Fever virus, Kyasanur Forest Disease virus and Omsk Hemorrhagic Fever virus. The method of the invention includes the detection of any variant of the above listed viruses.

5 In some preferred embodiments, the HFV is selected from the group consisting of: Ebola virus, Lassa virus and Marburg virus. The Ebola virus is advantageously chosen from: Ebola-Zaire, Ebola-Taï Forest, Ebola-Sudan, Ebola-Bundibugyo and Ebola-Reston, preferably from Ebola-Zaire, Ebola-Taï Forest, Ebola-Sudan and Ebola-Bundibugyo.

10 The method of the invention is performed on any sample suspected of containing an HFV. The sample includes any specimen that may contain an HFV or components thereof such as nucleic acids or fragments of nucleic acids. Samples include biological samples which refer to any material derived from living or dead individual (human or animal) that may contain an HFV or target nucleic acid derived  
15 therefrom, including any tissue or body fluid. Non-limiting examples of body fluids include whole-blood, serum, plasma, urine, cerebral spinal fluid (CSF), and mucosal secretions, such as with no limitations sputa and saliva. Samples include swabs. Samples include also processed samples that have been treated to disrupt tissue or cell structure, thus releasing intracellular components into a solution which may further  
20 contain reagents (buffers, salts, detergents, enzymes and the like) which are used to prepare, using standard methods, a biological sample for analysis. In particular, processed samples include samples that have been treated by standard methods used for the isolation of nucleic acids from biological samples. In some preferred embodiments of the method of the invention, the sample is a clinical sample (*i.e.*, a  
25 sample from living or dead individual (human or animal) suspected of having a VHF). The terms “a”, “an”, and “the” include plural referents, unless the context clearly indicates otherwise. For example “a target” as used herein is understood to represent one or more targets. As such, the term “a” (or “an”), “one or more” or “at least one” can be used interchangeably herein.

30 The target nucleic acid refers to a nucleic acid comprising a target sequence to be amplified. Target nucleic acids may be DNA or RNA and may be

either single-stranded or double-stranded. The target nucleic acid may include other sequences besides the target sequence, which may not be amplified.

In some preferred embodiments of the method of the invention, the target nucleic acid is viral RNA (*i.e.* HFV genomic RNA).

5                   The target sequence refers to the particular nucleotide sequence of the target nucleic acid that is to be amplified and detected. The target sequence includes the sequences to which oligonucleotide primers hybridize during the nucleic acid amplification process. Where the target nucleic acid is originally single-stranded, the term target sequence will also refer to the sequence complementary to the target  
10                   sequence as present in the target nucleic acid. Where the target nucleic acid is originally double-stranded the term target-sequence refers to both the sense (+) and antisense (-) strands.

                  The amplification product includes monomers and concatemers of the target sequence. According to the method of the invention, the target sequence to  
15                   be amplified and detected is located within a HFV gene coding for a protein of the viral ribonucleocapsid (RNP). Filovirus detection comprises the amplification of a target sequence located within the L, NP, VP30 or VP35 gene; Arenavirus detection comprises the amplification of a target sequence located within the L or NP gene.

                  In some preferred embodiments of the method of the invention, the  
20                   target sequence is located within the L or NP genes. Whereas Marburg virus detection comprises the amplification of a target sequence located within the L or NP gene, Ebola virus detection preferably comprises the amplification a target sequence located in the L gene and Lassa virus detection preferably comprises the amplification of a target sequence located in the NP gene.

25                   For Ebola virus detection, the target sequence to be amplified and detected is advantageously located within a region of the L gene of Ebola virus which is selected from the group consisting of:

                  a) a sequence from positions 1671 to 1973 of the coding sequence (CDS) of *Zaire ebolavirus* (ZEBOV or EBOV) L gene of nucleic acid sequence SEQ  
30                   ID NO: 1 (positions 11581 to 18219 of nucleic acid sequence GenBank NC\_002549.1); preferably a sequence selected from positions 1671 to 1876, 1703 to 1973 and 1671 to 1973 of said sequence corresponding to SEQ ID NO: 2, 3, 4,

respectively; more preferably the sequence from positions 1671 to 1876 of said sequence (SEQ ID NO:2);

b) a sequence from positions 3531 to 3745, 4654 to 4940 or 5270 to 5598 of the CDS of *Tai Forest ebolavirus* (TEBOV or TAFV) L gene of nucleic acid sequence SEQ ID NO: 5 (positions 11566 to 18198 of nucleic acid sequence GenBank NC\_014372.1), corresponding to SEQ ID NO: 6, 7 and 8, respectively;

c) a sequence from positions 528-847, 1728-2025 or 2204-2460 of the CDS of *Sudan ebolavirus* (SEBOV or SUDV) L gene of nucleic acid sequence SEQ ID NO: 9 (positions 11535 to 18167 of nucleic acid sequence GenBank NC\_006432.1), corresponding to SEQ ID NO: 10, 11 and 12;

d) a sequence from positions 579-823, 2433 to 2661 or 3570 to 3828 of the CDS of *Bundibugyo ebolavirus* (BEBOV or BDBV) L gene of nucleic acid sequence SEQ ID NO: 13 (positions 11567 to 18199 of nucleic acid sequence GenBank NC\_014373.1), corresponding to SEQ ID NO: 14, 15 and 16; and

e) a sequence from positions 1775 to 2060, 4422 to 4742 or 5922 to 6140 of the CDS of *Reston ebolavirus* (REBOV or RESTV) L gene of nucleic acid sequence SEQ ID NO: 17 (positions 11550 to 18188 of nucleic acid sequence GenBank NC\_004161.1), corresponding to SEQ ID NO: 18, 19, and 20, respectively, and

- a sequence comprising any one of SEQ ID NO: 2-4, 6-8, 10-12, 14-16, 18-20 and up to 20 consecutive nucleotides (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20), preferably up to 15 (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15), up to 10 (2, 3, 4, 5, 6, 7, 8, 9, 10) or up to 5 (2, 3, 4, 5) consecutive nucleotides of 5' and/or 3' flanking sequence from said L gene.

The Ebola virus target sequences listed in a) to e) above are subtype/species specific. Therefore, when the Ebola virus species suspected of being present in the sample is known, the method of the invention comprises the amplification of a target sequence of said Ebola virus species (*i.e.* the target sequence a), b), c), d) or e), respectively for EBOV, TAFV, SUDV, BDBV and RESTV. When the Ebolavirus species suspected of being present in the sample is totally or partially unknown, the method of the invention comprises the amplification of several target sequences specific for several or all different Ebolavirus species as listed above.

According to some more advantageous embodiments, the method of the invention, where being for detecting Ebolavirus, comprises the amplification of two or more of the target sequences a) to e) as listed above, preferably the target sequences a) to d) or a) to e), as listed above.

5 For Marburg virus (MARV) detection, the target sequence to be amplified and detected is advantageously located within a region of the L or NP gene of MARV, which is selected from the group consisting of:

f) a sequence from positions 786 to 1049, 5746 to 5980, 6442 to 6684 of the coding sequence of Marburg virus L gene of nucleic acid sequence SEQ  
10 ID NO: 21 (positions 11481 to 18476 of nucleic acid sequence GenBank NC\_001608.3), corresponding to SEQ ID NO: 22, 23 and 24 respectively;

g) a sequence from positions 862 to 1128 or 1749 to 2038 of the coding sequence of Marburg virus NP gene of nucleic acid sequence SEQ ID NO: 25 (positions 104 to 2191 of nucleic acid sequence GenBank NC\_001608.3),  
15 corresponding to SEQ ID NO: 26 and 27, respectively, and

- a sequence comprising any one of SEQ ID NO 22-24 or 26-27 and up to 20 consecutive nucleotides (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20) , preferably up to 15 (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15), up to 10 (2, 3, 4, 5, 6, 7, 8, 9, 10) or up to 5 (2, 3, 4, 5) consecutive nucleotides of 5' and/or 3' flanking sequence from said L or NP gene.  
20

For Lassa virus (LAV) detection, the target sequence to be amplified and detected is advantageously a sequence located within the NP gene of LAV, which is selected from the group consisting of:

h) a sequence from positions 55 to 293 or 1036 to 1223 of the  
25 coding sequence Lassa virus NP gene of nucleic acid sequence SEQ ID NO: 28 (positions 101 to 1810 of GenBank NC\_004296.1), corresponding to SEQ ID NO: 29 and 30, respectively, and

- a sequence comprising any one of SEQ ID NO 29-30 and up to 20 consecutive nucleotides (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and  
30 20) , preferably up to 15 (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15), up to 10 (2, 3, 4, 5, 6, 7, 8, 9, 10) or up to 5 (2, 3, 4, 5) nucleotides of 5' and/or 3' flanking sequence from said NP gene.

According to the invention, the target region of the target gene consists of any one of the sequences SEQ ID NO: 2-4, 6-8, 10-12, 14-16, 18-20, 22-24, 26-27 and 29-30 and up to 20 consecutive nucleotides of 5' and/or 3' flanking sequence from said target gene. The target sequence of the target gene that is amplified with the oligonucleotide primers consists of or is included, comprised in or contained in the target region. In some preferred embodiment the target sequence consists of or is included in one of the sequences SEQ ID NO: 2-4, 6-8, 10-12, 14-16, 18-20, 22-24, 26-27 and 29-30. The target sequence consists of at least 50 consecutive nucleotides, preferably at least 100 or 150 consecutive nucleotides from the sequence of the target region. Preferably, the target sequence consists of 150 to 350 consecutive nucleotides from the sequence of the target region, more preferably 150 to 250 consecutive nucleotides.

According to another preferred embodiment, the method of the invention comprises assaying different target sequences simultaneously on the same sample, preferably target sequences specific for different VHFs including different types of VHFs and different subtypes or species of the same type of VHF. In some more preferred embodiments, the method of the invention comprises assaying target sequences specific for different types of HFV, for example Ebola, Lassa and Marburg viruses, in order to perform a differential diagnosis of VHFs. In other more preferred embodiments, the method of the invention comprises assaying target sequences specific for different subtypes or species of a type of HFV, for example target sequences specific for different species of Ebola virus as defined above, to perform the HFV species or subtype determination. For detecting the different VHFs, the amplification of the different target sequences are performed in parallel, in separate reaction mixtures or in the same reaction mixture and detected independently.

According to the method of the invention, the target sequence is amplified by isothermal nucleic acid amplification reaction using any of the various natural or engineered enzymes available for this purpose.

These include in particular DNA polymerases having strong strand-displacement activity in isothermal conditions, thereby obviating the need for thermal cycling. Such polymerases are well-known in the art and include DNA polymerase long fragment (LF) of thermophilic bacteria such as *Bacillus stearothermophilus*

(Bst), *Bacillus Smithii* (Bsm), *Geobacillus sp. M* (GspM) and *Thermodesulfatator indicus* (Tin), engineered variants thereof as well as Taq DNA polymerase variants. Non-limiting examples of polymerase which can be used to perform the method of the invention include Bst LF DNA polymerase, GspM LF DNA polymerase, GspSSD LF DNA polymerase, Tin exo-LF DNA polymerase and SD DNA polymerase which are usually used at 50-75°C, more generally at 55 to 65°C. In a preferred embodiment, the isothermal nucleic acid amplification reaction is performed at 65°C or 68°C. In other preferred embodiment, the isothermal nucleic acid amplification reaction is performed using GspSSD LF DNA polymerase, preferably at 65°C or 68°C.

According to another preferred embodiment of the method of the invention, the isothermal amplification is chosen from strand-displacement amplification (SDA), Rolling-circle amplification (RCA), Cross-priming amplification (CPA), nucleic acid sequence-based amplification (NASBA), Recombinase Polymerase Amplification (RPA) and Loop-mediated amplification (LAMP). The principle of LAMP method is disclosed in Notomi *et al.*, Nucleic Acids Res., 2000 Jun 15; 28(12):e63.

When the nucleic acid is RNA, step a) includes a reverse transcription step prior to the isothermal amplification of the target sequence. The reverse transcription reaction is performed using any reverse transcriptase (RT). RT are well-known in the art and include for example Avian Myeloblastosis Virus (AMV) and Moloney Murine Leukemia virus (MMLV) RT. The Reverse transcription and DNA amplification can be performed in the same reaction mixture comprising the DNA polymerase and RT enzymes. In addition, the method of the invention can also use a DNA polymerase having both strong displacement activity and RT activity such as Pyrophage 3173 DNA polymerase.

In some more advantageous embodiments of the method of the invention, the isothermal amplification is LAMP or RT-LAMP.

According to the invention, step a) is performed using at least one pair of oligonucleotide primers for amplifying a target sequence of an HFV nucleic acid, which is located within a gene of the HFV nucleic acid coding for a protein of the viral ribonucleocapsid.

Oligonucleotide refers to a polymer of 5 to 100 nucleotides, preferably from a lower limit of 15 to 20 nucleotides to an upper limit of 45 to 60 nucleotides, wherein the polymer comprise ribonucleotides, deoxyribonucleotides, modified nucleotides or mixtures thereof and may further include modified internucleotide linkages and/or modified 5' and/or 3' termini. Oligonucleotides are usually synthesized using any of a variety of well-known enzymatic or chemical methods. The oligonucleotide primer according to the invention is substantially complementary to the target sequence. Substantially complementary means that the oligonucleotide is at least 80% identical, preferably at least 85%, 90%, 95% and 98% identical to the target sequence. The oligonucleotide may comprise additional sequences (not complementary to the target sequence) at its 5' end. In some embodiments the oligonucleotide comprises a sequence of at least 5, preferably 10 to 15 consecutive nucleotides which is 100% identical to the target sequence. In some more preferred embodiments, the oligonucleotide sequence is 100% identical to the target sequence. Optionally, at least one oligonucleotide of the pair includes a label (detectable moiety).

The oligonucleotides which function as primers are capable of annealing specifically to the target sequence and can be further extended in the presence of a nucleic acid polymerase to specifically amplify the target sequence. At least one oligonucleotide may also function as a probe and further includes a detectable label to detect a target nucleic acid or an amplicon thereof. The detectable label is a moiety that can be detected directly or indirectly by the production of a detectable signal such as colorimetric, fluorescent, chemiluminescent or electrochemoluminescent signal. Directly detectable labels include radioisotopes and fluorophores. Indirectly detectable labels are detected by labeling with additional reagents that enable the detection. Indirectly detectable labels include, for example, chemiluminescent agents, enzymes that produce visible or colored reaction products, and a ligand-detectable ligand binding partner, where a ligand (hapten, antibody, antigen, biotin) may be detected by binding to a labelled ligand-specific binding partner.

The oligonucleotides are designed based on HFV genomic sequences available in sequence databases using general principles for designing

amplification primers. The design of the primers may also include specific principles for designing primers for use in specific isothermal amplification methods. Primer design may be performed using any of the various softwares.

According to another preferred embodiment of the method of the invention, the oligonucleotide primers are selected from the group consisting of: the sequences of 5 to 100 nucleotides, preferably 15 to 60 nucleotides having 80% to 100% identity, preferably at least 85%, 90% 95% or 98% identity with any of SEQ ID NO: 2-4, 6-8, 10-12, 14-16, 18-20, 22-24, 26-27 and 29-30 or with a sequence comprising any of SEQ ID NO: 2-4, 6-8, 10-12, 14-16, 18-20, 22-24, 26-27 and 29-30 and up to 20 consecutive nucleotides of 5' and/or 3' flanking sequence from said L or NP gene as defined above.

In a more preferred embodiment, the primers are selected from the group consisting of the sequences SEQ ID NO: 31 to 162.

The primer pairs are advantageously selected from the group consisting of: SEQ ID NO: 31-32, 33-34, 35-36, 37-38, 39-40, 41-42, 43-44, 45-46, 47-48, 49-50, 51-52, 53-54, 55-56, 57-58, 59-60, 61-62, 63-64, 65-66, 67-68, 69-70, 71-72, 73-74, 75-76, 77-78, 79-80, 81-82, 83-84, 85-86, 87-88, 89-90, 91-92, 93-94, 95-96, 97-98, 99-100, 101-102, 103-104, 105-106, 107-108, 109-110, 111-112, 113-114, 115-116, 117-118, 119-120, 121-122, 123-124, 125-126, 127-128, 129-130, 131-132, 133-134, 135-136, 137-138, 139-140, 141-142, 143-144, 145-146, 147-148, 149-150, 151-152, 153-154, 155-156, 157-158, 159-160, and 161-162 (Table VII).

The primers SEQ ID NO: 31 to 120 are specific for Ebolavirus; SEQ ID NO: 31 to 48 are specific for *Zaire ebolavirus* (Ebola virus or EBOV), SEQ ID NO: 49 to 66 are specific for *Tai Forest ebolavirus* (Tai Forest virus or TAFV); SEQ ID NO: 67 to 84 are specific for *Sudan ebolavirus* (Sudan virus or SUDV); SEQ ID NO: 85 to 102 are specific for *Bundibugyo ebolavirus* (Bundibugyo virus or BDBV); SEQ ID NO: 103 to 120 are specific for *Reston ebolavirus* (Reston virus or RESTV) (Table VII). Preferred primers for *Zaire ebolavirus* are the pairs SEQ ID NO: 43-44, SEQ ID NO: 45-46, and/or SEQ ID NO: 47-48; more preferably the pairs SEQ ID NO: 43-44 and SEQ ID NO: 45-46; even more preferably the pairs SEQ ID NO: 43-44, SEQ ID NO: 45-46 and SEQ ID NO: 47-48.

The primers SEQ ID NO: 121 to 150 are specific for Marburg virus; SEQ ID NO: 121 to 138 are specific for the L gene, while SEQ ID NO: 139 to 150 are specific for the NP gene.

The primers SEQ ID NO: 151 to 162 are specific for Lassa virus.

5 According to another preferred embodiment of the method of the invention, the amplification reaction is a RT-LAMP amplification which is performed with a combination of two or three primer pairs selected from the group consisting of:

- SEQ ID NO: 31-32 and SEQ ID NO: 33-34, and optionally, SEQ ID NO: 35-36, which are specific for *Zaire ebolavirus*;
- 10 - SEQ ID NO: 37-38, and SEQ ID NO: 39-40, and optionally, SEQ ID NO: 41-42, which are specific for *Zaire ebolavirus*;
- SEQ ID NO: 43-44 and SEQ ID NO: 45-46, and optionally, SEQ ID NO: 47-48, which are specific for *Zaire ebolavirus*;
- SEQ ID NO: 49-50 and SEQ ID NO: 51-52, and optionally, SEQ  
15 ID NO: 53-54, which are specific for *Tai Forest ebolavirus*;
- SEQ ID NO: 55-56 and SEQ ID NO: 57-58, and optionally, SEQ ID NO: 59-60, which are specific for *Tai Forest ebolavirus*;
- SEQ ID NO: 61-62 and SEQ ID NO: 63-64, and optionally, SEQ ID NO: 65-66, which are specific for *Tai Forest ebolavirus*;
- 20 - SEQ ID NO: 67-68 and SEQ ID NO: 69-70, and optionally, SEQ ID NO: 71-72, which are specific for *Sudan ebolavirus*;
- SEQ ID NO: 73-74 and SEQ ID NO: 75-76, and optionally, SEQ ID NO: 77-78, which are specific for *Sudan ebolavirus*;
- SEQ ID NO: 79-80 and SEQ ID NO: 81-82, and optionally, SEQ  
25 ID NO: 83-84, which are specific for *Sudan ebolavirus*;
- SEQ ID NO: 85-86 and SEQ ID NO: 87-88, and optionally, SEQ ID NO: 89-90, which are specific for *Bundibugyo ebolavirus*;
- SEQ ID NO: 91-92 and SEQ ID NO: 93-94, and optionally, SEQ ID NO: 95-96, which are specific for *Bundibugyo ebolavirus*;
- 30 - SEQ ID NO: 97-98 and SEQ ID NO: 99-100, and optionally, SEQ ID NO: 101-102, which are specific for *Bundibugyo ebolavirus*;

- SEQ ID NO:103-104 and SEQ ID NO:105-106, and optionally, SEQ ID NO:107-108, which are specific for *Reston ebolavirus* ;
- SEQ ID NO:109-110 and SEQ ID NO:111-112, and optionally, SEQ ID NO:113-114, which are specific for *Reston ebolavirus*;
- 5       - SEQ ID NO:115-116 and SEQ ID NO:117-118, and optionally, SEQ ID NO:119-120, which are specific for *Reston ebolavirus*;
- SEQ ID NO:121-122 and SEQ ID NO:123-124, and optionally, SEQ ID NO:125-126, which are specific for Marburg virus;
- SEQ ID NO:127-128 and SEQ ID NO:129-130, and optionally,
- 10       SEQ ID NO:131-132, which are specific for Marburg virus;
- SEQ ID NO:133-134 and SEQ ID NO:135-136, and optionally, SEQ ID NO:137-138, which are specific for Marburg virus;
- SEQ ID NO:139-140 and SEQ ID NO:141-142, and optionally, SEQ ID NO:143-144, which are specific for Marburg virus; SEQ ID NO:145-146 and
- 15       SEQ ID NO:147-148, and optionally, SEQ ID NO:149-150, which are specific for Marburg virus;
- SEQ ID NO:151-152 and SEQ ID NO:153-154, and optionally, SEQ ID NO:155-156, which are specific for Lassa virus; and
- SEQ ID NO: 157-158 and SEQ ID NO:159-160 and optionally,
- 20       SEQ ID NO:161-162, which are specific for Lassa virus.

In some more preferred embodiment, the RT-LAMP amplification is performed with the three primer pairs as defined above.

In other more preferred embodiment, the RT-LAMP amplification of *Zaire ebolavirus* nucleic acid is performed with the two primer pairs

25       SEQ ID NO: 43-44 and SEQ ID NO: 45-46, preferably with the three primer pairs SEQ ID NO: 43-44, SEQ ID NO: 45-46 and SEQ ID NO: 47-48.

According to another more preferred embodiment, the method of the invention is a method for determining the Ebola virus species in a sample suspected of containing an Ebola virus, wherein the amplification reaction is performed with at

30       least two sets of primers chosen from:

- (i) a first set of primers specific for *Zaire ebolavirus* comprising at least one pair of primers selected from the group consisting of SEQ ID NO: 31-32, 33-

34, 35-36, 37-38, 39-40, 41-42, 43-44, 45-46, 47-48; preferably two or three pairs of primers selected from the group consisting of: SEQ ID NO: 31-32, 33-34, and eventually SEQ ID NO: 35-36; SEQ ID NO: 37-38, 39-40, and eventually SEQ ID NO: 41-42; SEQ ID NO: 43-44, 45-46, and eventually, SEQ ID NO: 47-48; more preferably, SEQ ID NO: 43-44, 45-46, and eventually, SEQ ID NO: 47-48;

(ii) a second set of primers specific for *Tai Forest ebolavirus* comprising at least one pair of primers selected from the group consisting of SEQ ID NO: 49-50, 51-52, 53-54, 55-56, 57-58, 59-60, 61-62, 63-64, 65-66; preferably two or three pairs of primers selected from the group consisting of: SEQ ID NO: 49-50, 51-52, and eventually SEQ ID NO: 53-54; SEQ ID NO: 55-56, 57-58, and eventually SEQ ID NO: 59-60; SEQ ID NO: 61-62, 63-64, and eventually, SEQ ID NO: 65-66;

(iii) a third set of primers specific for *Sudan ebolavirus* comprising at least one pair of primers selected from the group consisting of SEQ ID NO: 67-68, 69-70, 71-72, 73-74, 75-76, 77-78, 79-80, 81-82, 83-84; preferably two or three pairs of primers selected from the group consisting of: SEQ ID NO: 67-68, 69-70, and eventually SEQ ID NO: 71-72; SEQ ID NO: 73-74, 75-76, and eventually SEQ ID NO: 77-78; SEQ ID NO: 79-80, 81-82, and eventually, SEQ ID NO: 83-84;

(iv) a fourth set of primers specific for *Bundibugyo ebolavirus* comprising at least one pair of primers selected from the group consisting of SEQ ID NO: 85-86, 87-88, 89-90, 91-92, 93-94, 95-96, 97-98, 99-100, 101-102 ; preferably two or three pairs of primers selected from the group consisting of: SEQ ID NO: 85-86, 87-88, and eventually SEQ ID NO: 89-90; SEQ ID NO: 91-92, 93-94, and eventually SEQ ID NO: 95-96; SEQ ID NO: 97-98, 99-100, and eventually, SEQ ID NO: 101-102; and

(v) a fifth set of primers specific for *Reston ebolavirus* comprising at least one pair of primers selected from the group consisting of SEQ ID NO: 103-104, 105-106, 107-108, 109-110, 111-112, 113-114, 115-116, 117-118, 119-120; preferably two or three pairs of primers selected from the group consisting of: SEQ ID NO: 103-104, 105-106, and eventually SEQ ID NO: 107-108; SEQ ID NO: 109-110, 111-112, and eventually SEQ ID NO: 113-114; SEQ ID NO: 115-116, 117-118, and eventually, SEQ ID NO: 119-120.

The amplification reaction is advantageously performed with the four set of primers (i) to (iv) or the five set of primers (i) to (v) as defined above.

According to another more preferred embodiment, the method is a method for performing the differential detection of HFVs in a sample, wherein the  
5 amplification reaction is performed with at least two different sets of primers chosen from:

- a first set of primers specific for Ebola virus comprising one or more of the sets (i) to (v) above;
- a second set of primers specific for Margburg virus comprising  
10 at least one pair of primers selected from the group consisting of SEQ ID NO:121-122, 123-124, 125-126, 127-128, 129-130, 131-132, 133-134, 135-136, 137-138, 139-140, 141-142, 143-144, 145-146, 147-148, 149-150; preferably two or three pairs of primers selected from the group consisting of: SEQ ID NO: 121-122, 123-124, and eventually SEQ ID NO: 125-126; SEQ ID NO: 127-128, 129-130, and eventually  
15 SEQ ID NO: 131-132; SEQ ID NO: 133-134, 135-136, and eventually, SEQ ID NO: 137-138; SEQ ID NO: 139-140, 141-142, and eventually SEQ ID NO: 143-144; SEQ ID NO: 145-146, 147-148, and eventually SEQ ID NO: 149-150; and
- a third set of primers specific for Lassa virus comprising at least one pair of primers selected from the group consisting of 151-152, 153-154, 155-  
20 156, 157-158, 159-160, 161-162; preferably two or three pairs of primers selected from the group consisting of: SEQ ID NO: 151-152, 153-154, and eventually SEQ ID NO: 155-156; SEQ ID NO: 157-158, 159-160, and eventually SEQ ID NO: 161-162.

The amplification reaction is advantageously performed with the three sets of primers as defined above to perform the differential detection of Ebola  
25 virus, Lassa virus and Marburg virus.

The method is useful for performing the differential diagnosis of VHF, in particular of Ebola virus Disease, Lassa fever and Marburg Hemorrhagic fever.

According to the invention, the amplification product is detected  
30 using any of the various methods available for this purpose which are well-known in the art. For example, the detection method is fluorescence, bioluminescence, colorimetry, turbidimetry, immunoenzymatic or electrochemical detection. The

detection may be semi-quantitative or quantitative. The detection may also be real-time detection, wherein the signal resulting from the presence of the amplification product is measured during the course of the nucleic acid amplification reaction to monitor the accumulation of specific amplification products. Fluorescence may use  
5 DNA intercalating dyes, fluorescent molecular beacon probes or a fluorescence metal indicator such as calcein. Colorimetry may use a colored indicator for alkaline metal ions, such as hydroxy naphthol blue (Goto et al., *BioTechniques*. 2009 Mar;46(3):167–72) or pH indicators (Tanner et al., *BioTechniques*, 2015 Feb;58(2):59–68). Turbidity is used when the amplification reaction, such as LAMP,  
10 produces large amounts of magnesium pyrophosphate (a white precipitate) and dsDNA, which allow visual inspection of results using a turbidimeter (Mori et al., *Biochem. Biophys. Res. Commun.*, 2001 Nov 23; 289(1):150–4). Electrochemical detection may use a pH meter for direct measurement of released hydrogen ions during the amplification reaction, such as LAMP (Xie S et al., *Chem. Commun.*, 2014  
15 Oct 24; 50(100):15932–5), or integrated electrodes for measuring decreases in current resulting from increasing binding of electrochemically-active DNA-binding redox reporters, such as Methylene Blue, to amplification products (Xie et al., *Biosens. Bioelectron.* 2014 May 15; 55:324–9). Immunoassays include Enzyme-linked immunosorbent assays (ELISA) and lateral flow immunoassays based on the use of  
20 specific probes (Tsai S-M et al., *J. Virol. Methods*. 2012 Apr; 181(1):117–24; Ravan H and Yazdanparast R., *Anal. Biochem.*, 2013 Aug 15; 439(2):102–8). Bioluminescence detection may be through measurement of bioluminescent output of the coupled conversion of inorganic pyrophosphate produced stoichiometrically during nucleic acid synthesis to ATP by the enzyme ATP sulfurylase (Gandelman et  
25 al., *PLoS ONE.*, 2010 Nov 30; 5(11)).

According to another preferred embodiment, the method comprises at least one non-HFV internal control (IC) nucleic acid that is processed as the tested samples and amplified in the same assay reaction mixtures by using amplification oligonucleotide primers specific for a target sequence of the IC nucleic acid sequence.

30 In some embodiments, the IC target sequence is selected from the group consisting of:

- the sequence from positions 26 to 293, 227 to 498, or 387 to 621 of the CDS of human GAPDH gene of nucleic acid sequence SEQ ID NO: 163 (positions 189 to 1196 of GenBank NM\_002046.5.2), corresponding to SEQ ID NO: 164, 165 and 166, respectively;

- 5                   - the sequence from positions 204 to 517, 584 to 869, or 908 to 1148 of the CDS of *Drosophila melanogaster* Sigma virus G gene of nucleic acid sequence SEQ ID NO: 167 (positions 4262 to 5947 of GenBank GQ375258), corresponding to SEQ ID NO: 168, 169 and 170, respectively.

The IC target sequence SEQ ID NO: 165 is preferably amplified  
10 using at least one pair of primers selected from the group consisting of: SEQ ID NO: 171-172, 173-174, 175-176. More preferably, when RT-LAMP is used in the amplification reaction, the IC target sequence is amplified with the pairs SEQ ID NO: 171-172 and 173-174, and optionally SEQ ID NO: 175-176.

The IC target sequence SEQ ID NO: 164 is preferably amplified  
15 using at least one pair of primers selected from the group consisting of: SEQ ID NO: 177-178, 179-180, 181-182. More preferably, when RT-LAMP is used in the amplification reaction, the IC target sequence is amplified with the pairs SEQ ID NO: 177-178 and 179-180, and optionally SEQ ID NO: 181-182.

The IC target sequence SEQ ID NO: 166 is preferably amplified  
20 using at least one pair of primers selected from the group consisting of: SEQ ID NO: 183-184, 185-186, 187-188. More preferably, when RT-LAMP is used in the amplification reaction, the IC target sequence is amplified with the pairs SEQ ID NO: 183-184 and 185-186, and optionally SEQ ID NO: 187-188.

The IC target sequence SEQ ID NO: 169 is preferably amplified  
25 using at least one pair of primers selected from the group consisting of: SEQ ID NO: 189-190, 191-192 and 193-194. More preferably, when RT-LAMP is used in the amplification reaction, the IC target sequence is amplified with the pairs SEQ ID NO: 189-190 and 191-192, and optionally SEQ ID NO: 193-194.

The IC target sequence SEQ ID NO: 170 is preferably amplified  
30 using at least one pair of primers selected from the group consisting of: SEQ ID NO: 195-196, 197-198, 199-200. More preferably, when RT-LAMP is used in the

amplification reaction, the IC target sequence is amplified with the pairs SEQ ID NO: 195-196 and 197-198, and optionally SEQ ID NO: 199-200.

The IC target sequence SEQ ID NO: 168 is preferably amplified using at least one pair of primers selected from the group consisting of: SEQ ID  
5 NO:201-202, 203-204 and 205-206. More preferably, when RT-LAMP is used in the amplification reaction, the IC target sequence is amplified with the pairs SEQ ID NO: 201-202 and 203-204, and optionally SEQ ID NO: 205-206.

According to another preferred embodiment, the method comprises assaying several samples simultaneously. In some advantageous embodiments, the  
10 method comprises assaying multiple samples simultaneously in a high-throughput process.

The method of the invention is useful for the diagnosis of VHFs, in particular the diagnosis of Ebola virus Disease, Lassa fever and Marburg Hemorrhagic fever. The method is also useful for the differential diagnosis of VHFs, in particular  
15 the differential diagnosis of Ebola virus Disease, Lassa fever and Marburg Hemorrhagic fever. The method is also useful for HFV species determination, in particular for determining the Ebola virus species present in a sample suspected of containing an Ebola virus.

Another aspect of the present invention is a kit for the detection of a  
20 HFV, comprising at least one pair of oligonucleotide primers for amplifying a target sequence of a HFV nucleic acid as defined above. At least one oligonucleotide of the pair may comprise a detectable label. The kit optionally comprises reagents for the isothermal amplification of the target sequence and/or the detection of the amplification product. Reagents available for this purpose are well-known in the art  
25 and include the DNA polymerases and RT enzymes described above, buffers for the enzymes, detergents, enhancing agents, nucleic acid binding dyes and probes, preferably labelled probes. In some preferred embodiments of the kit of the invention, the primers, and optional reagents are in lyophilised form to allow ambient storage. The components of the kits are packaged together into any of the various containers  
30 suitable for nucleic acid amplification such as plates, slides, wells, dishes, beads, particles, cups, strands, chips, strips and others. The kit optionally includes instructions for performing at least one specific embodiment of the method of the

invention. In some advantageous embodiments, the kit comprises micro-well plates or microtubes, preferably in a dried format, *i.e.*, wherein the wells of the plates or microtubes comprise a dried composition containing at least the primers, and preferably further comprising all the reagents for the isothermal amplification of the target sequence and/or the detection of the amplification product amplification. In some other advantageous embodiments, the primers and optional reagents are included into any of the devices available for nucleic acid amplification including devices further integrating nucleic acid extraction and/or amplification product detection capacities such as microfluidic devices or other devices.

Another aspect of the invention is a pair of oligonucleotide primers for amplifying a target sequence of an HFV nucleic acid as defined above. The invention encompasses compositions comprising two or more primer pairs, in particular, compositions comprising primer pairs specific for different types of HFV for the differential diagnosis of VHFs and compositions comprising primer pairs specific for different species of a HFV type for the species determination of a VHF, as defined above. The invention encompasses also an oligonucleotide of the pair of oligonucleotides according to the invention.

Another aspect of the invention is the use of an oligonucleotide primer, primer pair or composition as defined above for the *in vitro* detection of HFVs. The oligonucleotides according to the invention are used as primers and /or probes for the detection of HFV target nucleic acid using standard method which are well-known in the art. In particular the primers may be used in any nucleic acid amplification technique including the isothermal amplification methods described above and other amplification methods available for this purpose such as Polymerase Chain Reaction (PCR) and RT-PCR.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques, which are within the skill of the art. Such techniques are explained fully in the literature.

For a better understanding of the invention and to show how the same may be carried into effect, there will now be described by way of example a specific mode contemplated by the Inventors with reference to the accompanying drawings in which:

- **Figure 1:** Evaluation of the analytical sensitivity of the Zaire Ebolavirus RT-LAMP assay. Amplification curves were obtained using 10-fold dilutions of synthetic *Zaire ebolavirus* RNA as template in a real-time *Zaire ebolavirus* qRT-LAMP assay.

5                   - **Figure 2:** Time-to-result obtained by real-time RT-PCR and real-time RT-LAMP for a panel of 87 plasma RNA extracts from patients with confirmed EVD diagnosis.

In the following description numerous specific details are set forth in order to provide a thorough understanding. It will be apparent however, to one skilled  
10 in the art, that the present invention may be practiced without limitation to these specific details. In other instances, well known methods and structures have not been described so as not to unnecessarily obscure the description.

**Example: RT-LAMP assay for rapid and accurate detection of HFVs in clinical samples**

15   **1. Material and methods**

Specific target gene regions were selected by multiple sequence alignments of all genome sequences available in the Genbank database for the 5 known Ebola virus species, namely Ebola virus (*Zaire ebolavirus*); Sudan virus (*Sudan ebolavirus*); Taï Forest virus (*Taï Forest ebolavirus*, formerly *Côte d'Ivoire ebolavirus*); Reston virus (*Reston ebolavirus*) and Bundibugyo virus (*Bundibugyo ebolavirus*). Target gene regions were also selected for two additional viral hemorrhagic fever agents, Marburg virus (*Marburg marburgvirus*) and Lassa virus (*Lassa mammarenavirus*), in order to provide additional diagnostic tools for the differential diagnosis of VHFs. In addition, a target gene region was selected for the  
25 *Drosophila melanogaster sigma virus*, used as internal control for sample inhibition, extraction efficiency and LAMP procedure. Finally, a human housekeeping gene was selected as control for sample integrity. Primers specifically targeting selected gene regions were designed using the LAMP explorer software (PREMIER BIOSOFT). Primer specificity was verified by BLAST analysis. A total of 15 different primer set,  
30 each composed of 6 specific primers, were designed: 3 primer sets targeting Ebola virus; 3 primer sets targeting Sudan virus; 3 primer sets targeting Taï Forest virus; 3

primer sets targeting Reston virus and 3 primer sets targeting Bundibugyo virus (Table VII).

A real-time RT-LAMP protocol was developed for real-time detection of target viral species. Reaction conditions (dNTP concentration, MgSO<sub>4</sub> concentration, Betain concentration, fluorescent dye concentration, enzyme and buffer composition) were compared individually for optimal amplification and reaction time.

A synthetic *Zaire ebolavirus* RNA transcript was prepared, aliquoted and tested in triplicate using various RT-LAMP master mixes to determine conditions for optimal reaction efficiency:

10                   - Master mix 1: 1.4 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 0.8 M, 40 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB primers, EvaGreen® dye 0.5X, 8 units of Bst 2.0 DNA Polymerase, 5 units of AMV Reverse Transcriptase.

15                   - Master mix 2: 1.4 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 0.8 M, 40 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB primers, EvaGreen® dye 0.5X, 8 units of Bst 2.0 DNA Polymerase, 5 units of WarmStart® RTx Reverse Transcriptase.

20                   - Master mix 3: 1.4 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 0.8 M, 40 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB primers, EvaGreen® dye 0.5X, 8 units of Bst 2.0 WarmStart™ DNA Polymerase, 5 units of AMV Reverse Transcriptase.

25                   - Master mix 4 : 1.4 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 0.8 M, 40 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB primers, EvaGreen® dye 0.5X, 8 units of Bst 2.0 WarmStart™ DNA Polymerase, 5 units of WarmStart® RTx Reverse Transcriptase.

30                   - Master mix 5 : 1.4 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 0.8 M, 40 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB

primers, EvaGreen® dye 0.5X, 8 units of GspSSD LF DNA polymerase, 5 units of AMV Reverse Transcriptase.

- Master mix 6: 1.4 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 0.8 M, 40  
5 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB primers, EvaGreen® dye 0.5X, 8 units of GspSSD LF DNA polymerase, 5 units of WarmStart® RTx Reverse Transcriptase.

- Master mix 7: 1X Isothermal Mastermix ISO001 (OPTIGENE), 40 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB  
10 primers, EvaGreen® dye 0.5X, 5 units of AMV reverse transcriptase.

- Master mix 8: 1X Isothermal Mastermix ISO001 (OPTIGENE), 40 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB primers, EvaGreen® dye 0.5X, 5 units of WarmStart® RTx Reverse Transcriptase.

- Master mix 9: 1.4 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM  
15 KCl, 8 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 0.8 M, 40 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB primers, EvaGreen® dye 0.5X, 8 units of GspSSD LF DNA Polymerase, 2.5 units of AMV Reverse Transcriptase.

- Master mix 10: 1.4 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM  
20 KCl, 8 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 0.8 M, 40 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB primers, EvaGreen® dye 0.5X, 8 units of GspSSD LF DNA Polymerase, 1.25 units of AMV Reverse Transcriptase.

- Master mix 11: 1.4 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM  
25 KCl, 8 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 0.8 M, 40 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB primers, SYTO® 1.5 µM, 8 units of GspSSD LF DNA Polymerase, 1.25 units of AMV Reverse Transcriptase.

- Master mix 12: 1.4 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM  
30 KCl, 8 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 0.8 M, 40 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB

primers, SYTO® 9 dye 10  $\mu$ M, 8 units of GspSSD LF DNA Polymerase, 1.25 units of AMV Reverse Transcriptase.

- Master mix 13: 1.4 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 0.8 M, 40  
5 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB primers, EvaGreen® dye 0.5X, 8 units of GspSSD LF DNA Polymerase, 5 units of AMV Reverse Transcriptase.

- Master mix 14: 1.4 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 12 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 0.8 M, 40  
10 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB primers, EvaGreen® dye 0.5X, 8 units of GspSSD LF DNA Polymerase, 5 units of AMV Reverse Transcriptase.

- Master mix 15: 1.4 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 0.6 M, 40  
15 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB primers, EvaGreen® dye 0.5X, 8 units of GspSSD LF DNA Polymerase, 5 units of AMV Reverse Transcriptase.

- Master mix 16: 1.4 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 1.0 M, 40  
20 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB primers, EvaGreen® dye 0.5X, 8 units of GspSSD LF DNA Polymerase, 5 units of AMV Reverse Transcriptase.

- Master mix 17: 1.2 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 0.8 M, 40  
25 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB primers, EvaGreen® dye 0.5X, 8 units of GspSSD LF DNA Polymerase, 5 units of AMV Reverse Transcriptase.

- Master mix 18: 1.6 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton™ X-100, Betaine 0.8 M, 40  
30 pmol of FIP and BIP primers, 5 pmol of F3 and B3 primers, 20 pmol of LF and LB primers, EvaGreen® dye 0.5X, 8 units of GspSSD LF DNA Polymerase, 5 units of AMV Reverse Transcriptase.

All RT-LAMP reactions were carried out in a 25- $\mu$ L reaction volume containing 20  $\mu$ L reaction Mastermix and 5  $\mu$ L of RNA extract. Amplification was performed at a constant temperature of 65°C for 30 minutes. The resulting amplification efficiency for the different RT-LAMP reaction mastermixes were compared using the purified synthetic EBOV RNA.

Following the selection of the most efficient mastermix composition, reaction temperatures (63°C, 65°C and 68°C) were compared individually for optimal amplification and reaction time using the synthetic EBOV RNA transcript tested in triplicate.

Finally, the designed primers sets targeting Zaire Ebola virus were compared individually for optimal amplification and reaction time using a patient plasma RNA extract tested in triplicate.

All reactions were conducted in a LightCycler® 480 (Roche) or a LightCycler® Nano (Roche) or a Genie® III (OptiGene) instrument.

Serial dilutions (10-fold) of *Zaire ebolavirus* synthetic RNA transcripts and genomic RNA from *Zaire ebolavirus* strain H.sapiens-tc/GIN/2014/Gueckedou-C05 were prepared and used to determine the analytical sensitivity of the *Zaire ebolavirus* RT-LAMP assay, as compared to real-time RT-PCR (RealStar® Ebolavirus RT-PCR Kit 1.0 (Altona Diagnostics)).

The analytical specificity of the *Zaire ebolavirus* RT-LAMP assay was verified using a panel of genomic RNA and DNA extracts from closely-related viruses and from pathogens causing symptoms similar to those of EVD.

A monocentric retrospective clinical evaluation of the *Zaire ebolavirus* RT-LAMP assay was conducted within the laboratory facilities of the Macenta Ebola Treatment Center in Guinea. As there is no universally accepted reference test for EVD diagnosis, clinically suspected EVD patients were confirmed based on the results of the reference molecular diagnostic test, the RealStar® Ebolavirus RT-PCR Kit 1.0 (Altona Diagnostics), authorized by the U.S. Food and Drug Administration under an Emergency Use Authorization. Archived EDTA plasma RNA extracts or oral swab RNA extracts, positive for EBOV genome detection by the RealStar Ebolavirus RT-PCR, were used for performance evaluation of the optimized *Zaire ebolavirus* RT-LAMP assay. Negative controls included RNA extracts from

febrile patients negative for EBOV genome detection but diagnosed with one of the conditions that can present symptoms similar to EBOV infection. All EBOV-positive and -negative oral swab extracts were obtained from decedents with suspected EVD. The assay was performed blindly on the selected sample extract panel. Obtained  
 5 results were collected at the end of each testing session using a standard report datasheet, and combined into a final database. Results obtained for the RT-LAMP assay were compared in a 2x2 table with results from the reference Altona RT-PCR assay, in order to estimate indices of sensitivity, specificity, and corresponding 97.5% confidence intervals (97.5% CI). Confidence intervals were calculated using binomial  
 10 exact methods. Statistical analysis was carried out using STATA/SE version 12.0 (StataCorp, College Station, USA).

## 2. Results

The *L* gene, encoding the RNA-dependent RNA polymerase, was selected as target gene for the detection of the 5 known Ebola virus species and  
 15 for the detection of Marburg virus. In addition, genes encoding the nucleoprotein were selected as target for the detection of Marburg virus and Lassa virus. For the detection of *Drosophila melanogaster sigma virus*, used as internal control, the G glycoprotein gene was selected as target gene. In addition, LAMP primers targeting the human *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) housekeeping gene were  
 20 designed as control for sample integrity.

RT-LAMP reaction conditions were optimized using different master mix compositions and different temperatures for the RT-LAMP reaction (Table I).

**Table I: Comparison of RT-LAMP assay efficiency (time-to-result) for different mastermix compositions**

| Master Mix N° | Time to result (min) |
|---------------|----------------------|
| 1             | 17.24 ± 0.78         |
| 2             | 16.26 ± 2.02         |
| 3             | 27.94 ± 14.56        |
| 4             | 16.47 ± 1.32         |
| 5             | 12.42 ± 0.55         |
| 6             | 14.27 ± 8.36         |
| 7             | <b>10.55 ± 0.44</b>  |
| 8             | 11.44 ± 1.83         |
| 9             | 11.94 ± 2.74         |
| 10            | 14.09 ± 2.00         |

|    |              |
|----|--------------|
| 11 | 11.60 ± 1.38 |
| 12 | 12.19 ± 0.43 |
| 13 | 13.19 ± 1.01 |
| 14 | 16.74 ± 1.74 |
| 15 | 11.70 ± 0.37 |
| 16 | 12.53 ± 0.49 |
| 17 | 14.26 ± 0.23 |
| 18 | 15.51 ± 2.90 |

Among the tested RT-LAMP mastermixes, mastermixes n° 7 and n°11 showed optimal amplification of target RNA as compared to other mastermix compositions. Consequently, mastermix n° 7 was used for all subsequent RT-LAMP reactions.

**Table II: Comparison of RT-LAMP assay efficiency (time-to-result) for different reaction temperatures**

| Reaction temperature (°C) | Time to result (min) |
|---------------------------|----------------------|
| 63                        | 12.05 ± 0.62         |
| 65                        | 12.03 ± 2.19         |
| 68                        | 10.11 ± 0.09         |

A reaction temperature of 68°C was identified as optimal (Table II); however, as little variation of efficiency was observed as compared to amplification at 65°C, a reaction temperature of 65°C was kept for all subsequent RT-LAMP assays.

**Table III: Comparison of RT-LAMP assay efficiency (time-to-result) for different *Ebola Zaire virus* primer sets**

| Primer Set N°        | Time to result (min) |
|----------------------|----------------------|
| 1 (SEQ ID NO: 31-36) | 9.36 ± 0.23          |
| 2 (SEQ ID NO: 37-42) | 8.18 ± 2.02          |
| 3 (SEQ ID NO: 43-48) | 6.36 ± 0.00          |

In addition, primer set n° 3 was identified as best performing for *Zaire ebolavirus* detection (Table III), and was therefore used for the analytical and clinical validation of the *Zaire ebolavirus* RT-LAMP assay.

Serial dilutions of *Zaire ebolavirus* synthetic and genomic RNA were used to determine the analytical sensitivity of the *Zaire ebolavirus* RT-LAMP assay. Amplification curves obtained by RT-LAMP for 10-fold serial dilutions of *Zaire ebolavirus* synthetic RNA are shown in **Figure 1**. The *Zaire ebolavirus* RT-

LAMP assay demonstrated a limit of detection of 5 genome copies per reaction, with a time-to-result of less than 15 minutes.

The analytical specificity of the *Zaire ebolavirus* RT-LAMP assay was assessed using a panel of 27 genomic RNA and DNA extracts from closely-  
5 related viruses and from pathogens causing symptoms similar to those of EVD (Table IV).

**Table IV: Viral and bacterial strains analyzed by the *Zaire ebolavirus* RT-LAMP assay**

| Agent    | Species                                           | Strain                     |
|----------|---------------------------------------------------|----------------------------|
|          | <i>Adenovirus 2</i>                               | Unknown                    |
|          | <i>Crimean-Congo Hemorrhagic Fever Virus</i>      | Afg09-2990 / BNI           |
|          | <i>Crimean-Congo Hemorrhagic Fever Virus</i>      | Kosovo Hoti / BNI          |
|          | <i>Chikungunya virus</i>                          | A26 Strain                 |
|          | <i>Chikungunya virus</i>                          | Chik/06/21 La Réunion 2006 |
|          | <i>Cytomegalovirus</i>                            | Unknown                    |
|          | <i>Dengue virus 1</i>                             | VR344 (Thai 1958 strain)   |
|          | <i>Dengue virus 2</i>                             | VR345 (TH-36 strain)       |
|          | <i>Dengue virus 3</i>                             | VR216 (H87 strain)         |
|          | <i>Dengue virus 4</i>                             | VR217 (H241 strain)        |
|          | <i>Influenza A virus H3N2</i>                     | Flu/A/Panama/2007/1999     |
|          | <i>Lassa virus</i>                                | Lib 1580/121 / BNI         |
|          | <i>Lassa virus</i>                                | Josiah /BNI                |
|          | <i>Marburg Virus</i>                              | Musoke /BNI                |
|          | <i>Measles Virus</i>                              | Schwartz                   |
|          | <i>Rift Valley Fever Virus</i>                    | Strain ZH548               |
|          | <i>Yellow fever virus 17D</i>                     | RKI #142/94/1              |
|          | <i>Yellow fever virus Asibi</i>                   | AY640589.1 /RKI            |
|          | <i>Varicella-zoster virus</i>                     | Unknown                    |
|          | <i>West Nile virus</i>                            | WN-143                     |
|          | <i>West Nile virus</i>                            | WN-56-2 Camargue 2002      |
|          | <i>Zika virus</i>                                 | MR766                      |
|          | <i>Zika virus</i>                                 | 7630 Côte d'Ivoire 1980    |
| Bacteria | <i>Bacillus subtilis</i>                          | 52.65                      |
|          | <i>Listeria monocytogenes</i>                     | CIP82.110T                 |
|          | <i>Leptospira interrogans icterohaemorrhagiae</i> | CIP6.1204                  |
|          | <i>Neisseria meningitidis</i>                     | CIP73.10T                  |

None of the tested viral and bacterial RNA and DNA extracts were  
5 detected by the *Zaire Ebolavirus* RT-LAMP assay, confirming the specificity of the  
assay.

The RT-LAMP assay was evaluated using a panel of RNA extracts  
from 174 patient plasma samples including 87 samples from acute and convalescent  
phase patients with confirmed EVD diagnosis and 87 samples from febrile patients  
10 who presented with symptoms compatible for EVD. Patients with symptoms  
compatible to EVD included 12 (13.7%) patients diagnosed with typhoid fever and 11  
(12.6%) patients diagnosed with malaria.

All 87 EBOV-positive RNA extracts were found positive by RT-LAMP. A comparison of the time-to-results observed between the reference RT-PCR and the RT-LAMP assay is shown for each sample from patients with confirmed EVD diagnosis in **Figure 2**.

5 The time-to-result ranged from 4.66 to 11.09 minutes for the real-time RT-LAMP assay and from 56.40 to 102.60 minutes for the reference real-time RT-PCR assay. Among the 87 extracts from febrile patients negative for EBOV genome detection by the reference real-time RT-PCR, 3 were found positive by the RT-LAMP assay. Presence of *Zaire ebolavirus* genome was confirmed by testing the  
10 extracts again in duplicate using two different *Zaire ebolavirus* RT-LAMP primer sets. These samples were therefore classified as true positives for *Zaire ebolavirus* genome detection (false-negative for RT-PCR). When compared to the results of the reference *Zaire ebolavirus* genome detection method (real-time RT-PCR), the RT-LAMP assay demonstrated a clinical sensitivity of 100.0% (one-sided 97.5% CI =  
15 96.0 to 100.0 %) and a clinical specificity of 100.0 % (one-sided 97.5% CI = 95.7 to 100.0 %) on plasma RNA extracts (Table V).

**Table V: Overall clinical performances of the *Zaire ebolavirus* RT-LAMP assay on patient plasma RNA extracts**

| Gold standard              | Parameter              | New test          |
|----------------------------|------------------------|-------------------|
|                            |                        | RT-LAMP           |
| Reference test<br>(RT-PCR) | No. of samples         | 174               |
|                            | True positive          | 90*               |
|                            | False negative         | 0                 |
|                            | True negative          | 84                |
|                            | False positive         | 0                 |
|                            | <b>Sensitivity</b>     | <b>100.0%</b>     |
|                            | Sensitivity (97.5% CI) | (96.0 to 100.0 %) |
|                            | <b>Specificity</b>     | <b>100.0%</b>     |
|                            | Specificity (97.5% CI) | (95.7 to 100.0 %) |
|                            |                        |                   |

20 \* Three samples with false negative results for the reference RT-PCR were classified as true positives after retesting

§ one-sided 97.5 % CI

In addition, a panel of 50 oral swab RNA extracts from decedents, 27 of which were positive by the reference real-time RT-PCR, were tested by RT-LAMP. All 27 EBOV-positive RNA extracts were found positive by RT-LAMP. The  
25 time-to-result ranged from 6.26 to 13.45 minutes for the real-time RT-LAMP assay and from 60.80 to 93.80 minutes for the reference real-time RT-PCR assay. Among the 23 swab extracts negative for EBOV genome detection by the reference real-time RT-PCR, 2 were found positive by the RT-LAMP assay. Presence of *Zaire ebolavirus*

genome was confirmed by testing the extracts again in duplicate using two different *Zaire ebolavirus* RT-LAMP primer sets. These samples were therefore also classified as true positives for *Zaire ebolavirus* genome detection. When compared to the results of the reference *Zaire ebolavirus* genome detection method (real-time RT-PCR), the RT-LAMP assay demonstrated a clinical sensitivity of 100.0% (one-sided 97.5% CI = (88.1 to 100.0 %) and a clinical specificity of 100.0 % (one-sided 97.5% CI = 83.9% to 100.0 %) on oral swab RNA extracts (Table VI).

**Table VI: Overall clinical performances of the *Zaire ebolavirus* RT-LAMP assay on RNA extracts from oral swabs collected from decedents**

| Gold standard              | Parameter              | New test<br>RT-LAMP |
|----------------------------|------------------------|---------------------|
| Reference test<br>(RT-PCR) | No. of samples         | 50                  |
|                            | True positive          | 29*                 |
|                            | False negative         | 0                   |
|                            | True negative          | 21                  |
|                            | False positive         | 0                   |
|                            | <b>Sensitivity</b>     | <b>100.0%</b>       |
|                            | Sensitivity (97.5% CI) | (88.1 to 100.0 %)§  |
|                            | <b>Specificity</b>     | <b>100.0%</b>       |
|                            | Specificity (97.5% CI) | (83.9 to 100.0 %)§  |

\* Two samples with false negative results for the reference RT-PCR were classified as true positives after retesting  
§ one-sided 97.5 % CI

### 3. Conclusions

In conclusion, the developed RT-LAMP method demonstrated equivalent analytical sensitivity and at least a 5 times faster time-to-result (15 minutes as compared to 80 minutes) as compared to the currently used reference real-time RT-PCR assay as well as other published Ebola virus qRT-PCR assays (16,17), thereby showing great potential for point-of-care applications. Moreover, the RT-LAMP assay demonstrated superior clinical performances as compared to the RT-PCR assay, probably due to a higher tolerance to inhibitors, making it highly suitable for accurate and reliable diagnostic applications.

The developed system, a highly accurate, fast, and easy-to-use molecular diagnostic test will be applicable not only in all well-equipped hospitals and field laboratories but also directly at the point-of-care in decentralized healthcare facilities in high-burden areas. Put together, this easily interpretable, rapid turn-around field tool will provide critical information to impact clinical management including triage, referral, treatment decisions, and decision to discharge. In addition to medical, national defense, and homeland security applications, the proposed technology is applicable in pharmaceutical, industrial, veterinary and environmental testing.

Table VII: RT-LAMP assays primer list (SEQ ID NO: 31 to 162)

| Target species<br>(Accession Nr)                | Target<br>gene | Target<br>Region | Oligo Name         | Sequence 5' to 3'                            | SEQ<br>ID NO: | Primer position      |
|-------------------------------------------------|----------------|------------------|--------------------|----------------------------------------------|---------------|----------------------|
| <i>Zaire ebolavirus</i><br><br>(NC_002549)      | <i>L</i>       | SEQ ID NO: 3     | EBOV-ZAIRE-1-F3    | ATCCGACTCGCAATGTC                            | 31            | 1703-1720            |
|                                                 |                |                  | EBOV-ZAIRE-1-B3    | ACACCATAGCAACGGTTG                           | 32            | 1973-1956            |
|                                                 |                |                  | EBOV-ZAIRE-1-FIP   | TTGCTCAGCTCCGTAACCTACC-AACACTTTGTGAAGCTCTGT  | 33            | 1803-1782, 1722-1741 |
|                                                 |                |                  | EBOV-ZAIRE-1-BIP   | TCAAGCATCATGGCACCACA-AATCAGTTACAAAGCTACTCCC  | 34            | 1821-1840, 1897-1876 |
|                                                 |                |                  | EBOV-ZAIRE-1-LoopF | GCTTTAGCAAGACCATCAGC                         | 35            | 1763-1744            |
|                                                 |                |                  | EBOV-ZAIRE-1-LoopB | TGGTGAACATGCCACAGTTA                         | 36            | 1854-1873            |
|                                                 | <i>L</i>       | SEQ ID NO: 4     | EBOV-ZAIRE-2-F3    | GAATGTAGGTAGAACCTTCGG                        | 37            | 1671-1691            |
|                                                 |                |                  | EBOV-ZAIRE-2-B3    | ACACCATAGCAACGGTTG                           | 38            | 1973-1956            |
|                                                 |                |                  | EBOV-ZAIRE-2-FIP   | TTGCTCAGCTCCGTAACCTACC-TTGTGAAGCTCTGTAGCTG   | 39            | 1803-1782, 1728-1747 |
|                                                 |                |                  | EBOV-ZAIRE-2-BIP   | TCAAGCATCATGGCACCACA-AATCAGTTACAAAGCTACTCCC  | 40            | 1821-1840, 1897-1876 |
|                                                 |                |                  | EBOV-ZAIRE-2-LoopF | GGAATGCTTTAGCAAGACCAT                        | 41            | 1769-1748            |
|                                                 |                |                  | EBOV-ZAIRE-2-LoopB | TGGTGAACATGCCACAGTTA                         | 42            | 1854-1873            |
| <i>Tai Forest ebolavirus</i><br><br>(NC_014372) | <i>L</i>       | SEQ ID NO: 2     | EBOV-ZAIRE-3-F3    | GAATGTAGGTAGAACCTTCGG                        | 43            | 1671-1691            |
|                                                 |                |                  | EBOV-ZAIRE-3-B3    | CTCTAACTGTGGCATGTTCA                         | 44            | 1876-1857            |
|                                                 |                |                  | EBOV-ZAIRE-3-FIP   | TGCTTTAGCAAGACCATCAGCT-CTTATCCGACTCGCAATGT   | 45            | 1764-1743, 1700-1718 |
|                                                 |                |                  | EBOV-ZAIRE-3-BIP   | GGTAGTTACGGAACGTGAGCAA-CCAAATCATCATCTTGTGTGG | 46            | 1782-1803, 1856-1836 |
|                                                 |                |                  | EBOV-ZAIRE-3-LoopF | ACAGAGCTTCACAAAGTTTG                         | 47            | 1741-1720            |
|                                                 |                |                  | EBOV-ZAIRE-3-LoopB | ATTGCATCAAGCATCATGCG                         | 48            | 1815-1834            |
|                                                 | <i>L</i>       | SEQ ID NO: 8     | EBOV-TAI-1-F3      | TCTGCGACAGAAATTGACTG                         | 49            | 5270-5289            |
|                                                 |                |                  | EBOV-TAI-1-B3      | TGCTCAATGCTGTGTTCT                           | 50            | 5598-5580            |
|                                                 |                |                  | EBOV-TAI-1-FIP     | CTATCCCGTGAAGCGGCAATA-GACAACAGAGATTGTAAGGCT  | 51            | 5428-5407, 5349-5369 |
|                                                 |                |                  | EBOV-TAI-1-BIP     | GCTGGATGAAGTCTTTGGGAA-GTAATGCACCCGAACCTT     | 52            | 5448-5469, 5527-5510 |
|                                                 |                |                  | EBOV-TAI-1-LoopF   | TGTGGTGTCTGGTATTGCC                          | 53            | 5385-5403            |
|                                                 |                |                  | EBOV-TAI-1-LoopB   | TCTGCTATAACACTTGCAGAA                        | 54            | 5485-5505            |
| <i>Zaire ebolavirus</i><br><br>(NC_002549)      | <i>L</i>       | SEQ ID NO: 6     | EBOV-TAI-2-F3      | GCCTGATCAATCACGACTT                          | 55            | 3531-3549            |
|                                                 |                |                  | EBOV-TAI-2-B3      | TTAAGTGGTCTCTATGAAGG                         | 56            | 3745-3725            |
|                                                 |                |                  | EBOV-TAI-2-FIP     | GGCTGGTGGCCCAATCTTA-GGACAAATTGGAGATGGCAT     | 57            | 3618-3600, 3554-3572 |
|                                                 |                |                  | EBOV-TAI-2-BIP     | AAATGCCCTTCAGCAGCCTTA-GCTCCACCTTGAGTAACC     | 58            | 3628-3648, 3701-3684 |
|                                                 |                |                  | EBOV-TAI-2-LoopF   | TCCTCTGTTGAGATCCGATA                         | 59            | 3599-3579            |

|                                                  |                  |                                            |    |                      |
|--------------------------------------------------|------------------|--------------------------------------------|----|----------------------|
| <i>L</i>                                         | EBOV-TAI-2-LoopB | CGTGAAGCAATTGAGTTGACAT                     | 60 | 3649-3670            |
|                                                  | EBOV-TAI-3-F3    | AGAACTGCCATTACTGTCTTC                      | 61 | 4654-4674            |
|                                                  | EBOV-TAI-3-B3    | GATCGACTTCTCAGTATGC                        | 62 | 4940-4921            |
|                                                  | EBOV-TAI-3-FIP   | AACAATGCGATGACGTGGTGTA-TTCCACTGTGGGTCACT   | 63 | 4799-4778, 4718-4735 |
|                                                  | EBOV-TAI-3-BIP   | CAACATGAGTCTCCACGATC-AATGCTCTCCACTCGACTGT  | 64 | 4801-4822, 4862-4844 |
|                                                  | EBOV-TAI-3-LoopF | ACTGTTGATCGGACTAGGACT                      | 65 | 4773-4753            |
|                                                  | EBOV-TAI-3-LoopB | GTTTGTGCTGCAGAAGCC                         | 66 | 4825-4842            |
| <i>Sudan<br/>ebolavirus<br/>(NC_006432)</i>      | EBOV-SUD-1-F3    | CCTCTGCGAAGCAATTACTT                       | 67 | 1728-1746            |
|                                                  | EBOV-SUD-1-B3    | CATGTAACATTGCGGAATTAGG                     | 68 | 2025-2004            |
|                                                  | EBOV-SUD-1-FIP   | TCATCATTGTATGTGTCAGG-CAAGCAATATGATGGTTGTAC | 69 | 1853-1832, 1772-1793 |
|                                                  | EBOV-SUD-1-BIP   | GCCACAGTTCGTGGAAGTAGTT-TGAAGGGAGCTGTGAATTC | 70 | 1867-1888, 1948-1930 |
|                                                  | EBOV-SUD-1-LoopF | ATGCTTGGTGAAGGAGGC                         | 71 | 1831-1814            |
|                                                  | EBOV-SUD-1-LoopB | AATACAATCTGGCCTTCAGGT                      | 72 | 1907-1927            |
|                                                  | EBOV-SUD-2-F3    | AATTGCGATCAGCATCAT                         | 73 | 2204-2222            |
| <i>L</i>                                         | EBOV-SUD-2-B3    | TGCTGTCTTGAGTGTAG                          | 74 | 2460-2441            |
|                                                  | EBOV-SUD-2-FIP   | GCCAAAGCTAGCAGCCACT-CTAGAATCTAGTCCGAATGAGC | 75 | 2333-2316, 2263-2284 |
|                                                  | EBOV-SUD-2-BIP   | GTCAACAGTGCCTGTGGAT-GATAAAGCCTGAGTACGAA    | 76 | 2338-2357, 2400-2380 |
|                                                  | EBOV-SUD-2-LoopF | GCTGCATTGCTCTTCTGCG                        | 77 | 2312-2295            |
|                                                  | EBOV-SUD-2-LoopB | ATTCCTCAAGCCTGATGAGAC                      | 78 | 2358-2378            |
|                                                  | EBOV-SUD-3-F3    | AACGAATGAGGTGTTGACA                        | 79 | 528-547              |
|                                                  | EBOV-SUD-3-B3    | AGTCTCTCGTTATACAGA                         | 80 | 847-828              |
| <i>L</i>                                         | EBOV-SUD-3-FIP   | TGTGTCTCTTGAATAGCCTCC-AACACAGCTAATGTTCCACA | 81 | 681-660, 604-623     |
|                                                  | EBOV-SUD-3-BIP   | GTCTCTACAGCCGAGGTCTTA-GGCTAACTOAGCAATCAGAA | 82 | 694-715, 774-755     |
|                                                  | EBOV-SUD-3-LoopF | TTAGGTTGGTACCAGTCAGTTG                     | 83 | 650-629              |
|                                                  | EBOV-SUD-3-LoopB | AAGGATCTTGTCACAAGTCGTT                     | 84 | 724-745              |
|                                                  | EBOV-BUN-1-F3    | ATTGCCACAATCACTCAAGA                       | 85 | 2433-2452            |
|                                                  | EBOV-BUN-1-B3    | TTGACCTAGATCGGTTCTCTT                      | 86 | 2661-2642            |
|                                                  | EBOV-BUN-1-FIP   | TGCGTTCTCTATACTAGCCAGA-CTGCTACCAGGATTGCTC  | 87 | 2529-2508, 2453-2470 |
| <i>Bundibugyo<br/>ebolavirus<br/>(NC_014373)</i> | EBOV-BUN-1-BIP   | TATACCTTCCCGGTGGTT-CAAGGTGGTGTATTGGAG      | 88 | 2561-2580, 2632-2614 |
|                                                  | EBOV-BUN-1-LoopF | CCTGAAGGTCAATCAAGATTGC                     | 89 | 2503-2482            |
|                                                  | EBOV-BUN-1-LoopB | GCGCATTCATATACATTCTTC                      | 90 | 2581-2601            |
|                                                  | EBOV-BUN-2-F3    | TGTACCTTACATCGGGTCTC                       | 91 | 3570-3569            |
|                                                  | EBOV-BUN-2-B3    | GCTGTATTGGTCAATTATACCGA                    | 92 | 3828-3807            |
|                                                  | EBOV-BUN-2-FIP   | GCTTACGTAAGCGCAGCAGA-GAACACAGGACAAGATTGGG  | 93 | 3656-3637, 3590-3609 |
|                                                  | EBOV-BUN-2-BIP   | CCAAGGTGGTCCCAATAGTGAT-AAGGATCTCCTGCACACT  | 94 | 3690-3711, 3768-3751 |
|                                                  | EBOV-BUN-2-LoopF | TTAGGCTTGATTGCTGGCT                        | 95 | 3629-3611            |

|                                         |               |                  |                                                |     |                      |
|-----------------------------------------|---------------|------------------|------------------------------------------------|-----|----------------------|
| <i>Reston ebolavirus</i><br>(NC_004161) | <i>L</i>      | EBOV-BUN-2-LoopB | TTTAGAGGACGAGTAAACC                            | 96  | 3728-3748            |
|                                         |               | EBOV-BUN-3-F3    | ACCGTTGTCATTGTTGCA                             | 97  | 579-597              |
|                                         |               | EBOV-BUN-3-B3    | TAGAGATTGTTTACGGTTGTGG                         | 98  | 823-802              |
|                                         |               | EBOV-BUN-3-FIP   | GTGTACCTTGAACCGCTTCT-GATTCTCATGAGCTAAGG        | 99  | 677-657, 612-631     |
|                                         | SEQ ID NO: 14 | EBOV-BUN-3-BIP   | TGTACGTTTCCACTGCAGATGT-GCTGCAATGAGTGTGGTAT     | 100 | 686-707, 764-746     |
|                                         |               | EBOV-BUN-3-LoopF | TGAAGATTGATCGGTGATACCA                         | 101 | 655-634              |
|                                         |               | EBOV-BUN-3-LoopB | AAGGACATCATAACTGTGTT                           | 102 | 721-742              |
|                                         | <i>L</i>      | EBOV-RES-1-F3    | TTACTTGGCTCCATTATTGGG                          | 103 | 5922-5942            |
|                                         |               | EBOV-RES-1-B3    | GTAGCATACTGTGCGATGT                            | 104 | 6140-6122            |
|                                         | SEQ ID NO: 20 | EBOV-RES-1-FIP   | ATGGCGGATCTCTGTTAGTA-ACCGATTACATCAAGTGCC       | 105 | 6042-6021, 5961-5979 |
| <i>Marburg virus</i><br>(NC_001608)     |               | EBOV-RES-1-BIP   | TCACAAGGCATGCTCTGGTGT-GACTCAACCGACGACGC        | 106 | 6048-6069, 6121-6104 |
|                                         |               | EBOV-RES-1-LoopF | AGGTACCATTCACTTGACCG                           | 107 | 5999-5980            |
|                                         |               | EBOV-RES-1-LoopB | AAGCAAGTCCAGCGAG                               | 108 | 6086-6103            |
|                                         | <i>L</i>      | EBOV-RES-2-F3    | AGGCAATACCTATCTATGGACT                         | 109 | 4422-4443            |
|                                         |               | EBOV-RES-2-B3    | CTTCAAGCGGATATACTATCC                          | 110 | 4742-4721            |
|                                         | SEQ ID NO: 19 | EBOV-RES-2-FIP   | TTAATCGGGACATCACACTGGC-ACCAGCTGCACAACTTAC      | 111 | 4564-4543, 4484-4501 |
|                                         |               | EBOV-RES-2-BIP   | TGGCGGACATCTGGAGA-CGATTGCTGTCCGAAGAA           | 112 | 4596-4613, 4660-4643 |
|                                         |               | EBOV-RES-2-LoopF | CACGGAGTGTCTCGATGAG                            | 113 | 4519-4502            |
|                                         |               | EBOV-RES-2-LoopB | CTGATGCTGCTCGACTGT                             | 114 | 4625-4642            |
|                                         | <i>L</i>      | EBOV-RES-3-F3    | ACATGATGGTAGTAAGTGAAG                          | 115 | 1775-1796            |
| <i>Marburg virus</i><br>(NC_001608)     |               | EBOV-RES-3-B3    | TTAACAATTGTAGGGCGATT                           | 116 | 2060-2041            |
|                                         | SEQ ID NO: 18 | EBOV-RES-3-FIP   | ACTCCTCGAAGCGTAGCA-CTTCTTGTATGAGGCATCAT        | 117 | 1881-1863, 1812-1831 |
|                                         |               | EBOV-RES-3-BIP   | CGCTATGAGTTCACTGCACCAT-AAGACATTACGCACACCAT     | 118 | 1921-1942, 1985-1967 |
|                                         |               | EBOV-RES-3-LoopF | TCATCACTGGTGTGTGTC                             | 119 | 1850-1833            |
|                                         |               | EBOV-RES-3-LoopB | TGAGTACTGCAACCATTTGCT                          | 120 | 1947-1966            |
|                                         | <i>L</i>      | MARV-L-1-F3      | CGAAGACAGTGTGGAGC                              | 121 | 786-803              |
|                                         |               | MARV-L-1-B3      | CACCTACGCTTATTGTCTGAA                          | 122 | 1049-1029            |
|                                         | SEQ ID NO: 22 | MARV-L-1-FIP     | CGTCTCCAGGTACATGGC-CTCCTCTTGTAGTGTAGGTG        | 123 | 880-862, 812-832     |
|                                         |               | MARV-L-1-BIP     | TTAGAACCCTTGTGTGTCAGCT-ATCATCTGTGATTTGGAACCAAG | 124 | 901-922, 980-960     |
|                                         |               | MARV-L-1-LoopF   | AATTGGTCTCCCTGGTTGTAT                          | 125 | 858-837              |
| <i>Marburg virus</i><br>(NC_001608)     |               | MARV-L-1-LoopB   | CACATGGCATCTTTACACCAAG                         | 126 | 932-953              |
|                                         | <i>L</i>      | MARV-L-2-F3      | AGCAATCAATCTGTGCGTT                            | 127 | 5746-5765            |
|                                         |               | MARV-L-2-B3      | ACAACATTCGGTATTACCC                            | 128 | 5980-5960            |
|                                         | SEQ ID NO: 23 | MARV-L-2-FIP     | CCCTTCGGCAAAACAAATTCG-GTCTCATCATGATGATTAAGC    | 129 | 5853-5833, 5776-5797 |
|                                         |               | MARV-L-2-BIP     | AAGGAAGTGTGCTCGGTTAC-GTTGTGAATCCGTAGCCAA       | 130 | 5855-5875, 5935-5917 |

|                                     |                 |                                                |     |                      |
|-------------------------------------|-----------------|------------------------------------------------|-----|----------------------|
| <i>L</i>                            | MARV-L-2-LoopF  | TTTACCTGGAGGCAAAAGATCA                         | 131 | 5823-5802            |
|                                     | MARV-L-2-LoopB  | TTGAAGTGGAAAGGAAACGGAT                         | 132 | 5878-5898            |
|                                     | MARV-L-3-F3     | AGACAAGCAATTCCCTACTGG                          | 133 | 6442-6462            |
|                                     | MARV-L-3-B3     | TCTCAAGTCTAGTAGAGTGAA                          | 134 | 6684-6663            |
|                                     | MARV-L-3-FIP    | ACGATGGCAGAAAGATGACTCT-ATCCTGCTTTCATTACACGAG   | 135 | 6555-6534, 6485-6504 |
|                                     | MARV-L-3-BIP    | TCCATTGAGTGAAGGAAAGGCT-AAATGTTGTTTGCCTTGACG    | 136 | 6564-6585, 6632-6613 |
|                                     | SEQ ID NO: 24   | AGGAAACCCCAAGTTAGGAAA                          | 137 | 6528-6507            |
|                                     | MARV-L-3-LoopF  | TTCAAGGTCAGTCTTATGT                            | 138 | 6590-6611            |
|                                     | MARV-L-3-LoopB  | AACAACCTCGAACATGGAC                            | 139 | 862-880              |
|                                     | MARV-NP-1-F3    | GACGGCTAGTGTCTGACT                             | 140 | 1128-1111            |
| <i>NP</i>                           | MARV-NP-1-B3    | GTTCCGCAACATTTGACACCAG-ATCCTCAGCTTTTCAGCAATT   | 141 | 961-941, 884-903     |
|                                     | SEQ ID NO: 26   | CTACGAGAGGGCGCACATG-CCTCGGCAATAGCTTGAAT        | 142 | 973-991, 1051-1033   |
|                                     | MARV-NP-1-BIP   | AATGTACTGCCGTGTGCT                             | 143 | 938-921              |
|                                     | MARV-NP-1-LoopF | GGCGACATGAACATCAGGA                            | 144 | 1013-1031            |
|                                     | MARV-NP-1-LoopB | CGGCAGTATTGGTATGTAA                            | 145 | 1749-1768            |
|                                     | MARV-NP-2-F3    | AATCTGCTTGAAGCTCCTTAG                          | 146 | 2038-2018            |
|                                     | MARV-NP-2-B3    | AGGCGACAATTCATAGGCTTCC-CCTTCTTCCACCATCTGCTC    | 147 | 1860-1839, 1798-1816 |
|                                     | SEQ ID NO: 27   | AGCAGAATTGGCCACAAAGAGT-GAGGATTTGTTTGCAGAAAGATC | 148 | 1886-1907, 1966-1845 |
|                                     | MARV-NP-2-BIP   | CTCATCCTTGTGTCTTCTCTGAG                        | 149 | 1838-1817            |
|                                     | MARV-NP-2-LoopF | GGTGACAAAGAGGGTAGAACT                          | 150 | 1908-1929            |
| <i>Lassa virus</i><br>(NC_004296)   | MARV-NP-2-LoopB | TCATCTGAGGTTAGAGTCCC                           | 151 | 293-274              |
|                                     | LASV-NP-1-F3    | GAATTATCTGGTTACTGTCTCCA                        | 152 | 55-76                |
|                                     | LASV-NP-1-B3    | CGCAAGGAGAGAAGGGATGAC-ACAAGATTGTTGACCGCTT      | 153 | 163-183, 233-215     |
|                                     | LASV-NP-1-FIP   | GACTTCGGAGAAGTCAAGTCCA-ACATCAAACTACAGGTGGTG    | 154 | 141-120, 77-96       |
|                                     | SEQ ID NO: 29   | AACGGTTGAGGGACCTAAATC                          | 155 | 194-214              |
|                                     | LASV-NP-1-BIP   | AAAGAGCTGGGCATCTTT                             | 156 | 115-97               |
|                                     | LASV-NP-1-LoopF | CCTGAACCTGGTTGATAGAGG                          | 157 | 1223-1203            |
|                                     | LASV-NP-1-LoopB | AAAGCTGACAGCAACAATTC                           | 158 | 1036-1055            |
|                                     | LASV-NP-2-F3    | GGATGCAATGCTGCAACTTGA-TTCAGGTCTTCTTCAATGTC     | 159 | 1122-1142, 1185-1165 |
|                                     | SEQ ID NO: 30   | TTGAGGGTTCATCAGCTGAGAAT-GTAAATCCCTGCAGTCGG     | 160 | 1100-1121, 1058-1075 |
| <i>Homo sapiens</i><br><i>GAPDH</i> | LASV-NP-2-BIP   | CCCAATGCTAAGACCTGGA                            | 161 | 1143-1162            |
|                                     | LASV-NP-2-LoopF | TAGCCCCAGCGGTAAACC                             | 162 | 1096-1079            |
|                                     | LASV-NP-2-LoopB | TCTTCCAGGACGAGATC                              | 171 | 227-244              |
|                                     | HUM-GAPDH-1-F3  | GTCATGGATGACCTTGGC                             | 172 | 488-481              |
|                                     | HUM-GAPDH-1-B3  | GCAAATGAGCCCCAGCCTTGATGACGTGCTGGAGTC           | 173 | 337-320, 276-293     |
|                                     | SEQ ID NO: 31   |                                                |     |                      |
|                                     | HUM-GAPDH-1-FIP |                                                |     |                      |
|                                     | SEQ ID NO: 32   |                                                |     |                      |
|                                     | HUM-GAPDH-1-BIP |                                                |     |                      |
|                                     | SEQ ID NO: 33   |                                                |     |                      |

|                                                |                |                    |                                              |     |                      |
|------------------------------------------------|----------------|--------------------|----------------------------------------------|-----|----------------------|
| (NM_002046.5)                                  | SEQ ID NO: 165 | HUM-GAPDH-1-BIP    | TGTTTCGTGCGGTGTGAACCGAGGCATTGCTGATGATCT      | 174 | 389-409, 452-434     |
|                                                |                | HUM-GAPDH-1-LoopF  | CTCCATGGTGGTGAAGACG                          | 175 | 318-300              |
|                                                |                | HUM-GAPDH-1- LoopB | TGAGAAATATGACAAACAGCCTC                      | 176 | 411-432              |
|                                                |                | HUM-GAPDH-2-F3     | ACGGATTGGTGGTATTGG                           | 177 | 26-44                |
|                                                |                | HUM-GAPDH-2-B3     | GACTCCACGACGTACTCA                           | 178 | 293-276              |
|                                                |                | HUM-GAPDH-2-FIP    | TGAAATTTGCCATGGGTGGAATGTTGCCATCAATGACCC      | 179 | 170-150, 90-107      |
|                                                |                | HUM-GAPDH-2-BIP    | GCACCGTCAAGGCTGAGAAGATCTCGCTCCTCGAAGA        | 180 | 173-191, 244-227     |
|                                                |                | HUM-GAPDH-2-LoopF  | ACCATGTAGTTGAGGTCAATGA                       | 181 | 131-110              |
|                                                |                | HUM-GAPDH-2- LoopB | ATCAATGGAAATCCCATCACCA                       | 182 | 205-226              |
|                                                |                | HUM-GAPDH-3-F3     | CATGTTGTCATGGGTGT                            | 183 | 387-404              |
| Drosophila melanogaster sigma virus (GQ375258) | SEQ ID NO: 166 | HUM-GAPDH-3-B3     | GATGATGTTCTGGAGAGCC                          | 184 | 621-603              |
|                                                |                | HUM-GAPDH-3-FIP    | GGTGCTAAGCAGTTGGTGATGAGAAAGTATGACAAACAGCC    | 185 | 476-457, 410-430     |
|                                                |                | HUM-GAPDH-3-BIP    | GCCAAAGGTCATCCATGACAACTCAGTGATGGCATGGACTG    | 186 | 481-502, 547-530     |
|                                                |                | HUM-GAPDH-3-LoopF  | AGGCATTGCTGATGATCTTGA                        | 187 | 451-431              |
|                                                |                | HUM-GAPDH-3- LoopB | GGTATCGTGAAGGACTCATG                         | 188 | 505-525              |
|                                                |                | SIGV-G-1-F3        | GGACCATTTCATGACGATATGAT                      | 189 | 584-605              |
|                                                |                | SIGV-G-1-B3        | ACTTGGAAATGTCACAGAGTTAG                      | 190 | 869-848              |
|                                                |                | SIGV-G-1-FIP       | GACTTCCAGCATCGGCTCC-CTGGGTGGCAACAAAGTAT      | 191 | 717-699, 644-662     |
|                                                |                | SIGV-G-1-BIP       | GGCTCCATCCACATCGAGG-CCTATCTCACCACAGAAAGGA    | 192 | 718-736, 794-775     |
|                                                |                | SIGV-G-1-LoopF     | CTCTCCATACAGCCGAGATG                         | 193 | 696-677              |
| G                                              | SEQ ID NO: 169 | SIGV-G-1- LoopB    | CAGGACAAAGAACTCCTCACITTA                     | 194 | 741-762              |
|                                                |                | SIGV-G-2-F3        | AAACTGCCGTTGTGGAAA                           | 195 | 908-925              |
|                                                |                | SIGV-G-2-B3        | GTGTTCACTGTACTGTGTCTA                        | 196 | 1148-1127            |
|                                                |                | SIGV-G-2-FIP       | AGAGTCCGAAAGCCAGAT-AGCTCACTGTCAACATGATG      | 197 | 1023-1005, 947-966   |
|                                                |                | SIGV-G-2-BIP       | TCAAGTGCAGGAGGGACCT-GTATTGGTACATTCCGACACT    | 198 | 1047-1065, 1125-1105 |
|                                                |                | SIGV-G-2-LoopF     | GAGATGGTCTCTTGACACTTCA                       | 199 | 995-974              |
|                                                |                | SIGV-G-2- LoopB    | GAGAGAGGGTGTCTTGTTACC                        | 200 | 1081-1102            |
|                                                |                | SIGV-G-3-F3        | CAAGAAGCACGATCCTG                            | 201 | 204-222              |
|                                                |                | SIGV-G-3-B3        | CCAACTTACAGGATGGTCTT                         | 202 | 517-497              |
|                                                |                | SIGV-G-3-FIP       | GCGGAGAGATGAGTGTGAAAT-ATGCCATAAGGTGAAGTATGAG | 203 | 361-340, 282-303     |
| G                                              | SEQ ID NO: 168 | SIGV-G-3-BIP       | AGAGCACCAATTGGGCACC-GAGTTCACAAAGACAGTCCTC    | 204 | 399-417, 461-442     |
|                                                |                | SIGV-G-3-LoopF     | TACCATGGCATATCACAGATGG                       | 205 | 326-305              |
|                                                |                | SIGV-G-3- LoopB    | ATGTCTCGCTGAGCTTTCC                          | 206 | 419-437              |

## CLAIMS

1. A method of detecting a Hemorrhagic Fever Virus (HFV) in a sample, comprising:
  - a) subjecting said sample to an isothermal nucleic acid amplification reaction using at least one pair of oligonucleotide primers for amplifying a target sequence of a HFV nucleic acid, wherein the target sequence is located within the L gene of the HFV nucleic acid, and
  - b) detecting the presence of an amplification product for said target sequence.
2. The method according to claim 1, wherein the HFV is selected from the group consisting of: Ebola virus, Lassa virus and Marburg virus.
3. The method according to claim 2, wherein the Ebola virus is chosen from Ebola-Zaire, Ebola-Taï Forest, Ebola-Sudan, Ebola-Bundibugyo and Ebola-Reston.
4. The method according to any one of claims 1 to 3, wherein the target sequence is located within a region of the L gene selected from the group consisting of: a sequence of any of SEQ ID NO: 2-4, 6-8, 10-12, 14-16, 18-20 and 22-24 and a sequence comprising any of SEQ ID NO: 2-4, 6-8, 10-12, 14-16, 18-20 and 22-24 and up to 20 consecutive nucleotides of 5' and/or 3' flanking sequence from said L gene.
5. The method according to any one of claims 1 to 4, wherein the oligonucleotide primers are selected from the group consisting of: the sequences of 5 to 100 nucleotides, preferably 15 to 60 nucleotides having 80% to 100% identity, preferably at least 85%, 90%, 95% or 98% identity with any of SEQ ID NO: 2-4, 6-8, 10-12, 14-16, 18-20 and 22-24.
6. The method according to claim 5, wherein the primers are selected from the group consisting of the sequences SEQ ID NO: 31 to 138.
7. The method according to any one of claims 1 to 6, wherein the HFV is Ebola-Zaire and the isothermal nucleic acid amplification reaction is

performed with at least one pair of primers of SEQ ID NO: 43-44, SEQ ID NO: 45-46, and/or SEQ ID NO: 47-48.

8. The method according to any one of claims 1 to 7, wherein the isothermal amplification is Loop-mediated Amplification (LAMP) or Reverse-transcription-Loop-mediated Amplification (RT-LAMP).

9. The method according to claim 8, which is performed with a combination of two or three primer pairs selected from the group consisting of: SEQ ID NO: 31-32 and SEQ ID NO: 33-34, and optionally, SEQ ID NO: 35-36; SEQ ID NO: 37-38, and SEQ ID NO: 39-40, and optionally, SEQ ID NO: 41-42; SEQ ID NO: 43-44 and SEQ ID NO: 45-46, and optionally, SEQ ID NO: 47-48; SEQ ID NO: 49-50 and SEQ ID NO: 51-52, and optionally, SEQ ID NO: 53-54; SEQ ID NO: 55-56 and SEQ ID NO: 57-58, and optionally, SEQ ID NO: 59-60; SEQ ID NO: 61-62 and SEQ ID NO: 63-64, and optionally, SEQ ID NO: 65-66; SEQ ID NO: 67-68 and SEQ ID NO: 69-70, and optionally, SEQ ID NO: 71-72; SEQ ID NO: 73-74 and SEQ ID NO: 75-76, and optionally, SEQ ID NO: 77-78; SEQ ID NO: 79-80 and SEQ ID NO: 81-82, and optionally, SEQ ID NO: 83-84; SEQ ID NO: 85-86 and SEQ ID NO: 87-88, and optionally, SEQ ID NO: 89-90; SEQ ID NO: 91-92 and SEQ ID NO: 93-94, and optionally, SEQ ID NO: 95-96; SEQ ID NO: 97-98 and SEQ ID NO: 99-100, and optionally, SEQ ID NO: 101-102; SEQ ID NO: 103-104 and SEQ ID NO: 105-106, and optionally, SEQ ID NO: 107-108; SEQ ID NO: 109-110 and SEQ ID NO: 111-112, and optionally, SEQ ID NO: 113-114; SEQ ID NO: 115-116 and SEQ ID NO: 117-118, and optionally, SEQ ID NO: 119-120; SEQ ID NO: 121-122 and SEQ ID NO: 123-124, and optionally, SEQ ID NO: 125-126; SEQ ID NO: 127-128 and SEQ ID NO: 129-130, and optionally, SEQ ID NO: 131-132; SEQ ID NO: 133-134 and SEQ ID NO: 135-136, and optionally, SEQ ID NO: 137-138.

10. The method according to any one of claims 1 to 9, wherein the detection in step b) is fluorescence, bioluminescence, colorimetry, turbidimetry, immunoenzymatic or electrochemical detection.

11. The method according to any one of claims 1 to 10, which comprises assaying multiple samples simultaneously or multiple target sequences simultaneously on the same sample.

12. The method according to claim 11, which is for the differential  
5 diagnosis of Viral Hemorrhagic fevers or HFV subtype or species determination.

13. The method according to any one of claims 1 to 12, wherein the isothermal nucleic acid amplification reaction is performed at 65°C or 68°C.

14. The method according to any one of claims 1 to 13, wherein the isothermal nucleic acid amplification reaction is performed using GspSSD LF DNA  
10 polymerase.

15. A kit for the detection of an HFV, comprising at least one pair of oligonucleotide primers for amplifying a target sequence of an HFV nucleic acid as defined in any one of claims 6, 7 and 9, and optionally reagents for the isothermal amplification of the target sequence and/or the detection of the amplification product.

16. The kit according to claim 15, wherein the primers and optional  
15 reagents are in lyophilised form.

17. A pair of oligonucleotide primers as defined in claim 7 or 9.

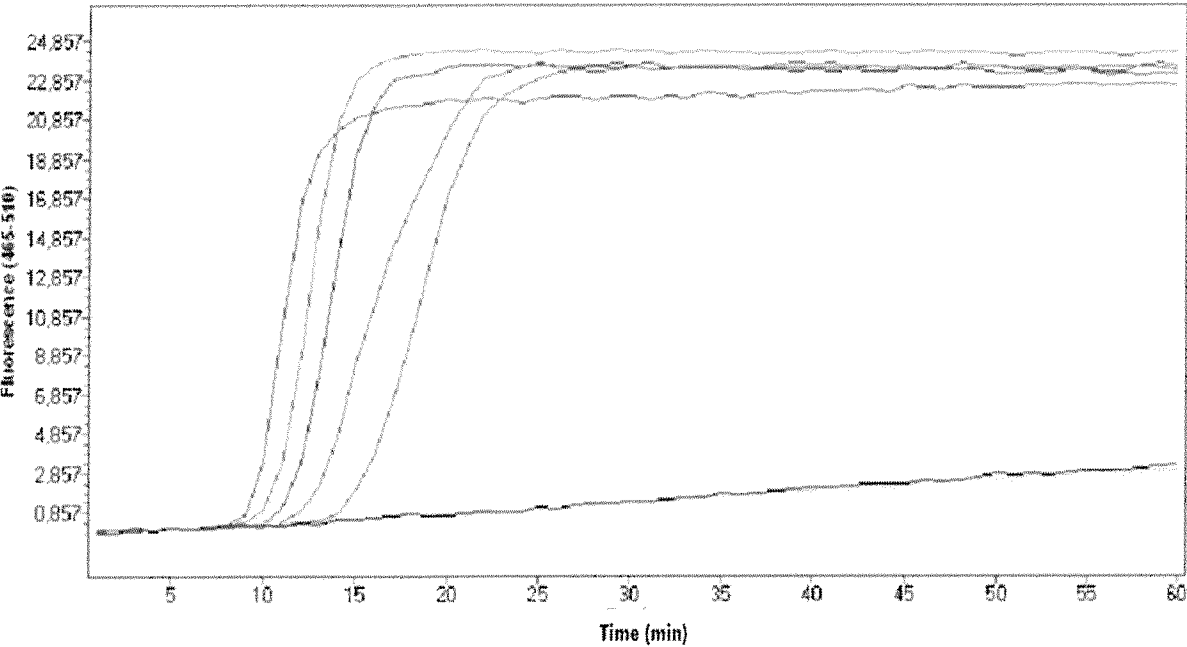


FIGURE 1

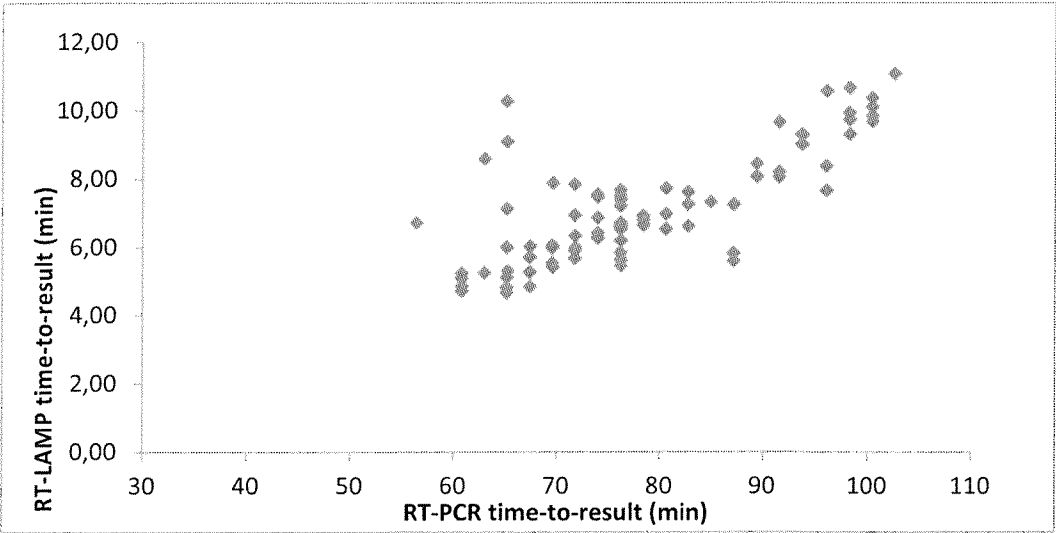


FIGURE 2

# INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2016/053089

## A. CLASSIFICATION OF SUBJECT MATTER

INV. C12Q1/70

ADD.

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

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q

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

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

EPO-Internal, BIOSIS, CHEM ABS Data, Sequence Search, EMBASE, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                | Relevant to claim No. |
|-----------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Y         | <p>AIKO FUKUMA ET AL: "Rapid detection of Lassa virus by reverse transcription-loop-mediated isothermal amplification", MICROBIOLOGY AND IMMUNOLOGY, vol. 55, no. 1, 22 December 2010 (2010-12-22), pages 44-50, XP055202919, ISSN: 0385-5600, DOI: 10.1111/j.1348-0421.2010.00286.x page 46, table 1; page 47, figure 1; abstract; page 49, Discussion</p> <p>-----<br/>-/--</p> | 1-17                  |



Further documents are listed in the continuation of Box C.



See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

16 September 2016

Date of mailing of the international search report

05/10/2016

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# INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2016/053089

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                                                       |                       |
|------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                    | Relevant to claim No. |
| Y                                                    | Y. KUROSAKI ET AL: "Development and Evaluation of a Simple Assay for Marburg Virus Detection Using a Reverse Transcription-Loop-Mediated Isothermal Amplification Method", JOURNAL OF CLINICAL MICROBIOLOGY, vol. 48, no. 7, 1 July 2010 (2010-07-01), pages 2330-2336, XP055202923, ISSN: 0095-1137, DOI: 10.1128/JCM.01224-09 page 2331, "Materials and methods"; figure 1, table 1 | 1                     |
| Y                                                    | -----<br>CN 104 313 179 A (ZHEJIANG CT DISEASE CONTROL & PREVENTION)<br>28 January 2015 (2015-01-28)<br>claims                                                                                                                                                                                                                                                                        | 1                     |
| Y                                                    | -----<br>CECILIA ANDERSSON ET AL: "rolling circle amplification detection of Crimean Congo hemorrhagic fever virus (CCHFV) complementary and viral RNA", VIROLOGY, ELSEVIER, AMSTERDAM, NL, vol. 426, no. 2, 26 January 2012 (2012-01-26), pages 87-92, XP028403698, ISSN: 0042-6822, DOI: 10.1016/J.VIROL.2012.01.032 [retrieved on 2012-01-31] abstract                             | 1                     |
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