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(54) Title: REAL-TIME REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION ASSAY WITH MODIFIED PROBE FOR THE DIAGNOSIS OF RABIES VIRUSES AND OTHER LYSSAVIRUSES

(57) Abstract: A real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay that utilizes multiplex primers and probes with degenerate nucleotides to detect divergent species of lyssavirus is described. The probes used in the RT-PCR assay target a highly conserved region at the 5' end of the lyssavirus genome and are modified with either a minor groove binder (MGB) or locked nucleic acid (LNA) nucleotides to increase their melting temperature. The described assay detects all known lyssavirus species with a sensitivity and specificity superior to traditional hemi-nested PCR and the direct fluorescent antibody (DFA) test.

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REAL-TIME REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION ASSAY WITH MODIFIED PROBE FOR THE DIAGNOSIS OF RABIES VIRUSES AND OTHER LYSSAVIRUSES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 62/339,323, filed May 20, 2016, which is herein incorporated by reference in its entirety.

FIELD

This disclosure concerns a real-time, reverse transcriptase-polymerase chain reaction (RT-PCR) assay capable of detecting divergent lyssavirus species.

BACKGROUND

Rabies is an acute and progressive viral encephalitis characterized by central nervous disorders, ultimately leading to death. This disease is a major public health problem in Asia and Africa with a global annual human mortality of 59,000. Rabies is caused by several different lyssavirus species of the family *Rhabdoviridae*; however, rabies virus (RABV) is responsible for the majority of deaths. The lyssavirus species responsible for causing rabies are grouped into 3 phylogroups. Phylogroup I includes RABV, Aravan virus (ARAV), Khujand virus (KHUV), Bokeloh bat lyssavirus (BBLV), Duvenhage virus (DUVV), European bat lyssavirus-1 (EBLV-1), European bat lyssavirus-2 (EBLV-2), Australian bat lyssavirus (ABLV) and Irkut virus (IRKV); Phylogroup II includes Mokola virus (MOKV), Shimoni bat virus (SHIBV) and Lagos bat virus (LBV); and Phylogroup III includes Ikoma virus (IKOV), West Caucasian bat virus (WCBV) and Lleida bat virus (LLBV) (Marston *et al.*, *Emerg Infect Dis* 18(4):664-667, 2012; Arechiga Ceballos *et al.*, *Emerg Infect Dis* 19(5):793-795, 2013; Voloch *et al.*, *Viruses* 6(11):4465-4478, 2014). The significant diversity of genome sequences among rabies and non-rabies lyssaviruses has previously presented challenges in developing a robust, easy to use, pan-lyssavirus diagnostic assay. Currently, definitive rabies diagnoses in both humans and animals depends on post-mortem laboratory results.

The World Health Organization (WHO) and the World Organization for Animal Health (OIE) have defined the direct fluorescent antibody (DFA) test as the gold standard method for post-mortem detection of rabies. DFA is a rapid and sensitive method for diagnosing rabies, but its accuracy depends on the quality of brain tissue, availability of high-quality anti-rabies diagnostic conjugates, accessibility to a fluorescence microscope, and most importantly, an experienced

diagnostician (Lembo *et al.*, *Emerg Infect Dis* 12(2):310-313, 2006). With the advent of highly sensitive and specific molecular methods, virus-targeted methods have proven useful to detect rabies virus antigen in nuchal skin biopsy, saliva, eyewashes and even in decomposed samples. Polymerase chain reaction (PCR) based assays are particularly useful as sequences of amplified amplicons can assist in phylogenetic analyses and viral typing. However, current rabies reverse transcriptase (RT)-PCR assays have limited sensitivity, and the current hemi-nested RT-PCR assays have frequently been shown to produce non-specific amplification products. Thus, amplicon sequencing is needed to rule out potential false positive results, which is both time-consuming and labor-intensive.

SUMMARY

A need exists for an improved assay for the detection of RABV and non-rabies lyssaviruses. Current assays, including hemi-nested PCR assays and the DFA test, exhibit limited specificity and sensitivity due to the sequence diversity of lyssaviruses, and typically require at least two separate assays to detect a broad range of RABV and lyssaviruses strains. To address this need, disclosed herein is a real-time RT-PCR assay that utilizes multiplex primers and probes with degenerate nucleotides to detect divergent species of lyssavirus. The probes used in the RT-PCR assay target a short, highly conserved region at the 5' end of the lyssavirus genome and are modified to increase their melting temperature. The disclosed assay detects all known lyssavirus species with a sensitivity and specificity superior to traditional hemi-nested PCR and DFA test.

Provided herein is a method for detecting lyssavirus nucleic acid in a sample. The method includes contacting the sample with at least one forward primer and at least one reverse primer to amplify lyssavirus nucleic acid present in the sample, and at least one probe that detects the amplified lyssavirus nucleic acid. In some embodiments, the at least one forward primer and the at least one reverse primer amplify a 5' region of the lyssavirus genome encompassing at least nucleotides 59-75 or 60-76. In some embodiments, the at least one probe is no more than 20 nucleotides and length and comprises the nucleotide sequence of AACACCYCTACAATGGA (SEQ ID NO: 1), AACACTACTACAATGGA (SEQ ID NO: 2) or ACACCYCTACAATGGAT (SEQ ID NO: 3). In some examples, the at least one probe includes modifications that increase the melting temperature of the probe(s).

Also provided is a kit for detecting lyssavirus nucleic acid. The kit includes primers to amplify lyssavirus nucleic acid, and at least one probe that detects the amplified nucleic acid. In some embodiments, the kit includes at least one forward primer and at least one reverse primer capable of amplifying a 5' region of the lyssavirus genome encompassing at least nucleotides 59-75

or 60-76; and at least one probe that is no more than 20 nucleotides and length and comprises the nucleotide sequence of AACACCYCTACAATGGA (SEQ ID NO: 1), AACACTACTACAATGGA (SEQ ID NO: 2) or ACACCYCTACAATGGAT (SEQ ID NO: 3). In some embodiments, the at least one probe includes modifications that increase the melting temperature of the probe(s).

The foregoing and other objects, features, and advantages of the invention will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: Shown is an amplicon sequence alignment of assay LN34 from isolates of 14 species of lyssaviruses (SEQ ID NOs: 11-30). The sequences used in this alignment were selected from over 280 published lyssavirus genome sequences in Genbank. For the LN34 assay, the forward primers target nucleotide position 1 to 25, the reverse primer targets position 140-164, and the probe targets position 59 to 75.

FIG. 2: Shown is a table listing the limit of detection using the droplet digital RT-PCR assay.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file, created on April 19, 2017, 169 KB, which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NO: 1 is the nucleotide sequence of probes LN34 and LN34a.

SEQ ID NO: 2 is the nucleotide sequence of probe LN34lago.

SEQ ID NO: 3 is the nucleotide sequence of probe LN34m.

SEQ ID NO: 4 is the nucleotide sequence of forward primer 1.

SEQ ID NO: 5 is the nucleotide sequence of forward primer 2.

SEQ ID NO: 6 is the nucleotide sequence of reverse primer.

SEQ ID NO: 7 is the nucleotide sequence of a positive control RABV amplicon.

SEQ ID NO: 8 is the nucleotide sequence of a β actin probe.

SEQ ID NO: 9 is the nucleotide sequence of a β actin forward primer.

SEQ ID NO: 10 is the nucleotide sequence of a β actin reverse primer.

SEQ ID NOs: 11-30 are lyssavirus amplicon sequences from assay LN34.

SEQ ID NOs: 31-118 are lyssavirus N gene sequences.

DETAILED DESCRIPTION

I. Abbreviations

ABLV	Australian bat lyssavirus
ARAV	Aravan virus
BBLV	Bokeloh bat lyssavirus
BS	brain stem
ddPCR	droplet digital polymerase chain reaction
DFA	direct fluorescent antibody
DUVV	Duvenhage virus
EBLV-1	European bat lyssavirus-1
EBLV-2	European bat lyssavirus-2
FITC	fluorescein-isothiocyanate
IKOV	Ikoma virus
IRKV	Irkut virus
KHUV	Khujand virus
LBV	Lagos bat virus
LNA	locked nucleic acid
MGB	minor groove binder
MOKV	Mokola virus
N	nucleoprotein
OIE	World Organization for Animal Health
PCR	polymerase chain reaction
RABV	Rabies virus
RT-PCR	reverse transcriptase polymerase chain reaction
SHIBV	Shimoni bat virus
SNP	single nucleotide polymorphism
TCS	tissue culture supernatant
TINA	twisted intercalating nucleic acid
Tm	melting temperature

WCBV	West Caucasian bat virus
WHO	World Health Organization

II. Terms and Methods

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

2,6-Diaminopurine (2-Amino-dA): A base modification that can form three hydrogen bonds when base-paired with dT and can increase the Tm of short oligonucleotides by as much as 1-2°C per insertion.

5-Hydroxybutynl-2'-deoxyuridine: A duplex-stabilizing modified base that increases oligonucleotide Tm. Oligonucleotides containing **5-hydroxybutynl-2'-deoxyuridine** (also known as SUPER T™) can be extended normally by polymerases, including Taq polymerase.

5-Methyl deoxycytidine: A modified base that when substituted for dC increases the Tm of an oligonucleotide by as much as 0.5°C per insertion.

Animal: Living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals. The term "animal" includes both human and veterinary subjects, for example, humans, non-human primates, dogs, cats, horses, raccoons, bats, rats (and other rodents), mice, foxes, squirrels, opossum, coyotes, wolves, lions, shrews and cows.

Contacting: Placement in direct physical association; includes both in solid and liquid form. "Contacting" is often used interchangeably with "exposed." In some cases, "contacting" includes transfecting, such as transfecting a nucleic acid molecule into a cell. In other examples, "contacting" refers to incubating a molecule with a sample, such as a biological sample.

Degenerate variant: A degenerate variant of a probe or primer includes sequences that have altered nucleic acid sequences, but retain their ability to bind to the target sequences (and identify or amplify the target) with sufficient specificity. In some particular examples, no more than about 1, 2, 5, or 10 nucleic acids are changed from the original sequence. In other examples, the probe or primer retains at least 80%, 85%, 90%, 95%, or 98% sequence identity to the original

sequence. Degenerate variants also include probe or primer sequences to which additional sequence has been added, while still retaining the noted specificity of the probe or primer.

Fluorophore: A chemical compound, which when excited by exposure to a particular wavelength of light, emits light (*i.e.*, fluoresces), for example at a different wavelength than that to which it was exposed. Also encompassed by the term "fluorophore" are luminescent molecules, which are chemical compounds which do not require exposure to a particular wavelength of light to fluoresce; luminescent compounds naturally fluoresce. Therefore, the use of luminescent signals eliminates the need for an external source of electromagnetic radiation, such as a laser. An example of a luminescent molecule includes, but is not limited to, aequorin (Tsien, 1998, *Ann. Rev. Biochem.* 67:509).

In some embodiments herein, a probe is labeled with a fluorophore, such as at the 5' end of the probe. Probes used for real-time PCR assays typically include a fluorophore and a quencher. Fluorophores suitable for use with real-time PCR assays, such as TaqMan™ PCR, include, but are not limited to, 6-carboxyfluorescein (FAM), tetrachlorofluorescein (TET), tetramethylrhodamine (TMR), hexachlorofluorescein (HEX), JOE, ROX, CAL Fluor™, Pulsar™, Quasar™, Texas Red™, Cy™3 and Cy™5.

Other examples of fluorophores are provided in U.S. Patent No. 5,866,366. These include: 4-acetamido-4'-isothiocyanatostilbene-2,2'disulfonic acid, acridine and derivatives such as acridine and acridine isothiocyanate, 5-(2'-aminoethyl)amino-naphthalene-1-sulfonic acid (EDANS), 4-amino-N-[3-vinylsulfonyl)phenyl]-naphthalimide-3,5 disulfonate (Lucifer Yellow VS), N-(4-anilino-1-naphthyl)-maleimide, anthranilamide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcouluarin (Coumaran 151); cyanosine; 4',6-diaminidino-2-phenylindole (DAPI); 5', 5"-dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethyl-amino]naphthalene-1-sulfonyl chloride (DNS, dansyl chloride); 4-(4'-dimethyl-aminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin isothiocyanate; ethidium; fluorescein and derivatives such as 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), fluorescein, fluorescein isothiocyanate (FITC), and QFITC (XRITC); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde;

pyrene and derivatives such as pyrene, pyrene butyrate and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron .RTM. Brilliant Red 3B-A); rhodamine and derivatives such as 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride, rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, sulforhodamine B, sulforhodamine 101 and sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid and terbium chelate derivatives.

Other fluorophores include thiol-reactive europium chelates that emit at approximately 617 nm (Heyduk and Heyduk, *Analyt. Biochem.* 248:216-27, 1997; *J. Biol. Chem.* 274:3315-22, 1999).

Other fluorophores include cyanine, merocyanine, stryl, and oxonyl compounds, such as those disclosed in U.S. Patent Nos. 5,627,027; 5,486,616; 5,569,587; and 5,569,766, and in published PCT application no. US98/00475, each of which is incorporated herein by reference. Specific examples of fluorophores disclosed in one or more of these patent documents include Cy3 and Cy5, for instance, and substituted versions of these fluorophores.

Other fluorophores include GFP, Lissamine™, diethylaminocoumarin, fluorescein chlorotriazinyl, naphthofluorescein, 4,7-dichlororhodamine and xanthene (as described in U.S. Patent No. 5,800,996 to Lee *et al.*, herein incorporated by reference) and derivatives thereof. Other fluorophores are known to those skilled in the art, for example those available from Molecular Probes (Eugene, OR).

Hybridization: Oligonucleotides (such as primers and probes) and their analogs hybridize by hydrogen bonding, which includes Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary bases. Generally, nucleic acid consists of nitrogenous bases that are either pyrimidines (cytosine (C), uracil (U), and thymine (T)) or purines (adenine (A) and guanine (G)). These nitrogenous bases form hydrogen bonds between a pyrimidine and a purine, and the bonding of the pyrimidine to the purine is referred to as "base pairing." More specifically, A will hydrogen bond to T or U, and G will bond to C. "Complementary" refers to the base pairing that occurs between two distinct nucleic acid sequences or two distinct regions of the same nucleic acid sequence.

"Specifically hybridizable" and "specifically complementary" are terms that indicate a sufficient degree of complementarity such that stable and specific binding occurs between the oligonucleotide (or its analog) and the DNA or RNA target. The oligonucleotide or oligonucleotide analog need not be 100% complementary to its target sequence to be specifically hybridizable. An

oligonucleotide or analog is specifically hybridizable when there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide or analog to non-target sequences under conditions where specific binding is desired, for example under physiological conditions in the case of *in vivo* assays or systems. Such binding is referred to as specific hybridization.

Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method of choice and the composition and length of the hybridizing nucleic acid sequences. Generally, the temperature of hybridization and the ionic strength (especially the Na^+ and/or Mg^{++} concentration) of the hybridization buffer will determine the stringency of hybridization, though wash times also influence stringency. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed by Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, chapters 9 and 11; and Ausubel *et al. Short Protocols in Molecular Biology*, 4th ed., John Wiley & Sons, Inc., 1999.

For purposes of the present disclosure, "stringent conditions" encompasses conditions under which hybridization will only occur if there is less than 25% mismatch between the hybridization molecule and the target sequence. "Stringent conditions" may be broken down into particular levels of stringency for more precise definition. Thus, as used herein, "moderate stringency" conditions are those under which molecules with more than 25% sequence mismatch will not hybridize; conditions of "medium stringency" are those under which molecules with more than 15% mismatch will not hybridize, and conditions of "high stringency" are those under which sequences with more than 10% mismatch will not hybridize. Conditions of "very high stringency" are those under which sequences with more than 6% mismatch will not hybridize.

Isolated: An "isolated" biological component (such as a nucleic acid, protein or virus) has been substantially separated or purified away from other biological components (such as cell debris, or other proteins or nucleic acids). Biological components that have been "isolated" include those components purified by standard purification methods. The term also embraces recombinant nucleic acids or proteins, as well as chemically synthesized nucleic acids or peptides.

Label: A detectable compound or composition that is conjugated directly or indirectly to another molecule to facilitate detection of that molecule. Specific, non-limiting examples of labels include radioactive isotopes, enzyme substrates, co-factors, ligands, chemiluminescent or fluorescent molecules, haptens and enzymes. Labels may be natural or synthetic, and may also be heterologous in the sense that they do not naturally occur in combination with the molecule to

which it is conjugated. Conjugation can occur, for example, by covalent attachment of the label to the other molecule.

Locked nucleic acid (LNA): A bicyclic nucleic acid where a ribonucleoside is linked between the 2'-oxygen and the 4'-carbon atoms with a methylene unit. This link restricts the flexibility of the ribofuranose ring of the nucleotide, locking the ribose in the 3'-endo conformation. The LNA also induces adjacent bases to adopt a conformation of the more thermodynamically stable form of the A duplex. The locked ribose conformation enhances base stacking and backbone pre-organization, which significantly increases the melting temperature of the nucleic acid. LNA oligonucleotides can be synthesized by standard phosphoramidite chemistry using DNA-synthesizers. In addition, LNA can be mixed with DNA, RNA as well as other nucleic acid analogs.

***Lyssavirus*:** A genus of viruses that is part of the *Rhabdoviridae* family within the order Mononegavirales (viruses with a single-stranded, negative sense genome). Lyssaviruses are the etiological agents of rabies encephalitis in warm-blooded animals and humans. The lyssavirus species responsible for causing rabies are grouped into 3 phylogroups. Phylogroup I includes rabies virus (RABV), Aravan virus (ARAV), Khujand virus (KHUV), Bokeloh bat lyssavirus (BBLV), Duvenhage virus (DUVV), European bat lyssavirus-1 (EBLV-1), European bat lyssavirus-2 (EBLV-2), Australian bat lyssavirus (ABLV) and Irkut virus (IRKV); Phylogroup II includes Mokola virus (MOKV), Shimoni bat virus (SHIBV) and Lagos bat virus (LBV); and Phylogroup III includes Ikoma virus (IKOV), West Caucasian bat virus (WCBV) and Lleida bat virus (LLBV) (Marston *et al.*, *Emerg Infect Dis* 18(4):664-667, 2012; Arechiga Ceballos *et al.*, *Emerg Infect Dis* 19(5):793-795, 2013; Voloch *et al.*, *Viruses* 6(11):4465-4478, 2014).

Melting temperature (Tm): In the context of an oligonucleotide duplex, Tm is the temperature at which half of the oligonucleotide molecules are single-stranded and half are double-stranded. Thus, the melting temperature of an oligonucleotide probe or primer is the temperature at which half of the probe or primer molecules are bound to their target (complementary) oligonucleotides and half are unbound.

Minor groove binder (MGB): A composition or moiety that binds in a non-intercalating manner into the minor groove of double stranded DNA, RNA or hybrids thereof. A variety of minor groove binders are known in the art (see, for example, U.S. Patent Nos. 5,801,155; 6,492,346; 6,084,102; and 6,727,356; and Wemmer and Dervan, *Curr Opin Struct Biol* 7:355-361, 1997). In some embodiments herein, the MGB is a trimer of 1,2-dihydro-(3H)-pyrrolo[3,2-e]indole-7-carboxylate ($CDPI_3$) (Afonina *et al., Nucleic Acids Res* 25(13):2657-2660, 1997) or a pentamer of N-methylpyrrole-4-carbox-2-amide (MPC_5) (U.S. Patent No. 7,943,752).

Probes and primers: A probe comprises an isolated nucleic acid molecule attached to a detectable label or other reporter molecule. Typical labels include radioactive isotopes, enzyme substrates, co-factors, ligands, chemiluminescent or fluorescent agents, haptens, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed, for example, in Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989 and Ausubel *et al. Short Protocols in Molecular Biology*, 4th ed., John Wiley & Sons, Inc., 1999.

Primers are short nucleic acid molecules, for instance DNA oligonucleotides 10 nucleotides or more in length, for example that hybridize to contiguous complementary nucleotides or a sequence to be amplified. Primer oligonucleotides may be about 10, 12, 15, 18, 20, 25, 30, or 50 nucleotides or more in length. Primers can be annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, and then the primer extended along the target DNA strand by a DNA polymerase enzyme. Primer pairs can be used for amplification of a nucleic acid sequence, for example, by the polymerase chain reaction (PCR) or other nucleic-acid amplification methods known in the art. Other examples of amplification include strand displacement amplification, as disclosed in U.S. Patent No. 5,744,311; transcription-free isothermal amplification, as disclosed in U.S. Patent No. 6,033,881; repair chain reaction amplification, as disclosed in WO 90/01069; ligase chain reaction amplification; gap filling ligase chain reaction amplification, as disclosed in 5,427,930; and NASBA™ RNA transcription-free amplification, as disclosed in U.S. Patent No. 6,025,134.

Methods for preparing and using nucleic acid probes and primers are described, for example, in Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989; Ausubel *et al. Short Protocols in Molecular Biology*, 4th ed., John Wiley & Sons, Inc., 1999; and Innis *et al. PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc., San Diego, CA, 1990. Amplification primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

Quencher: A substance that absorbs excitation energy from a fluorophore when in close proximity. Probes used for real-time PCR assays, such as TaqMan™ PCR, typically include a fluorophore and a quencher. Quenchers suitable for use with real-time PCR assays include, but are not limited to, ZEN™, Iowa Black™ FQ, tetramethylrhodamine (TAMRA), black hole quencher (BHQ)1, BHQ2, BHQ3, nonfluorescent quencher (NFQ) and 4-(4'-

dimethylaminophenylazo)benzoic acid (DABCYL). In some cases, a probe contains two quenchers.

Rabies: A viral disease that causes acute encephalitis (inflammation of the brain) in warm-blooded animals. Rabies is zoonotic (transmitted by animals), most commonly by a bite from an infected animal but occasionally by other forms of contact. Rabies is almost frequently fatal if post-exposure prophylaxis is not administered prior to the onset of severe symptoms. Rabies is caused by viruses of the *Lyssavirus* genus.

Reverse-transcription PCR (RT-PCR): A method for detecting, quantifying, or utilizing RNA present in a sample by a procedure wherein the RNA serves as a template for the synthesis of cDNA by a reverse transcriptase followed by PCR to amplify the cDNA. RT-PCR can be used in combination with quantitative real time PCR as a method of measuring the quantity of starting template in the reaction.

Sample: Encompasses a sample obtained from an animal, plant, or the environment, whether unfixed, frozen, or fixed in formalin or paraffin. As used herein, samples include all clinical samples useful for detection of viral infection in subjects, including, but not limited to, cells, tissues, and bodily fluids. In some embodiments, the sample is a biological sample obtained from a human or veterinary subject, such as, for example, a fluid, cell and/or tissue sample. In some examples herein, the biological sample is a fluid sample. Fluid sample include, but are not limited to, serum, blood, plasma, urine, feces, saliva, cerebral spinal fluid (CSF) or other bodily fluid. Biological samples can also refer to cells or tissue samples, such as biopsy samples (for example, skin biopsies), tissue sections (such as brain tissue), corneal tissue samples, or isolated leukocytes. Samples also include samples obtained from inanimate objects or reservoirs within an indoor or outdoor environment, including, but not limited to: soil, water, dust, and air samples.

Sequence identity: The similarity between amino acid or nucleic acid sequences is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are. Homologs or variants of a given gene or protein will possess a relatively high degree of sequence identity when aligned using standard methods.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith and Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman and Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85:2444, 1988; Higgins and Sharp, *Gene* 73:237-244, 1988; Higgins and Sharp, *CABIOS* 5:151-153, 1989; Corpet *et al.*, *Nucleic Acids Research* 16:10881-10890, 1988; and Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85:2444, 1988. Altschul *et al.*, *Nature Genet.* 6:119-129, 1994.

The NCBI Basic Local Alignment Search Tool (BLAST™) (Altschul *et al.*, *J. Mol. Biol.* 215:403-410, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx.

In some embodiments herein, provided are oligonucleotide sequences at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to any one of SEQ ID NOs: 1-6.

Subject: Living multi-cellular vertebrate organisms, a category that includes both human and non-human mammals.

Twisted intercalating nucleic acid (TINA): An intercalator that can stabilize Watson-Crick antiparallel duplex DNA (Schneider *et al.*, *PLoS One* 7(6):e38451, 2012). TINA molecules can be used at the at 5' end of an oligonucleotide to increase Tm.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. "Comprising A or B" means including A, or B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

III. Introduction

Several real-time RT-PCR assays have been previously developed for the detection of RABV or non-rabies lyssaviruses. However, the prior assays exhibit limited specificity and sensitivity due to the sequence diversity of lyssaviruses, and usually require at least two separate assays to detect a broad range of RABV and lyssaviruses strains. SYBR Green based real-time RT-PCR assays are able to detect more RABV and lyssavirus strains than other diagnostic assays, but are relatively prone to generating false positive results due to the non-specific binding of SYBR Green (Dupuis *et al.*, *J Clin Microbiol* 53(9):2983-2989, 2015; Hayman *et al.*, *J Virol Methods*

177(1):87-93, 2011; Coertse *et al.*, *J Clin Microbiol* 48(11):3949-3955, 2010; Vázquez-Morón *et al.*, *J Virol Methods* 135(2):281-287, 2006). To address the need for an improved lyssavirus detection assay, disclosed herein is a new real-time RT-PCR assay that utilizes multiplex primers and probes with degenerate nucleotides, modified to increase their melting temperature (Tm). For example, the modified probes can include, for example, a minor groove binder (MGB) or locked nucleic acid (LNA) nucleotides. The assay disclosed herein detects all known lyssavirus species with a sensitivity and specificity superior to traditional hemi-nested PCR and DFA.

In particular, two assays are described herein, each utilizing a different type of probe. The LN34 assay utilizes nucleic acid probes that are modified by locked nucleic acid (LNA) nucleotides; the LN34m assay uses a probe modified at the 3' end with a minor groove binder (MGB). The probes are capable of hybridizing to divergent lyssavirus species by targeting a very short region of the lyssavirus genome (nucleotides 59-75 or 60-76) that is highly conserved near the ATG translation start site of the nucleoprotein (N) gene (see FIG. 1). Previously described assays have utilized longer probes in order for the probes to have a Tm compatible with RT-PCR. However, the longer the probe, the less likely it is that the probe will hybridize to divergent species of lyssavirus. Therefore, the assays disclosed herein use an alternative approach – modifying the probes to increase their Tm, such as with LNA nucleotides or a MGB, to enable their use in RT-PCR.

To further enable detection of divergent lyssavirus species, the primer and probe sequences were designed with degenerate nucleotides. The sequences of the primers and probes disclosed herein are shown in Table 3.

The LNA probes LN34 and LN34a allowed for the detection of nearly all lyssavirus species tested, except for a subspecies of Lagos virus (LNA probes LN34 and LN34a have the same nucleotide sequence, but differ by the location of the LNA nucleotides). A third LNA probe (LN34lago) was designed to specifically detect Lagos virus. All LNA probes include a 5' fluorescent label (FAM) and a 3' quencher (BHQ1). A single MGB-modified probe (LN34m) was designed for the LN34m assay. In addition to the 3' MGB, this probe includes a 5' fluorescent label (6FAM) and a 3' quencher (NFG). Both RT-PCR assays use a multiplex of two forward primers and a reverse primer that includes several degenerate nucleotides.

The RT-PCR assays disclosed herein were tested using a validation panel of 88 lyssavirus isolates, including 59 variants of RABV from Asia, Africa, Europe, South America and North America; eight non-rabies lyssavirus species from phylogroup I; two non-rabies lyssavirus species from phylogroup II; and one non-rabies lyssavirus species from phylogroup III. It was determined that the LN34 and LN34m assays were both capable of detecting all variants from the validation

panel except for one subspecies of Lagos virus. However, this subspecies was detected using the multiplex LN34 or LN34m assay with the subspecies-specific Lagos virus probe LN34lago. The sensitivity and specificity of the two assays were similar for most of the variants in the panel.

IV. Lyssavirus Detection Assay

Disclosed herein is a real-time RT-PCR assay that utilizes multiplex primers and probes with degenerate nucleotides to detect divergent species of lyssavirus. The probes used in the RT-PCR assay target a highly conserved region at the 5' end of the lyssavirus genome and are modified to increase their melting temperature (Tm). In some examples, the probes are modified with either a MGB or LNA nucleotides to increase their Tm. The disclosed assay detects all known lyssavirus species with a sensitivity and specificity superior to traditional hemi-nested PCR and DFA test. The disclosed assay can be used, for example, to diagnose a rabies virus or non-rabies lyssavirus infection in a subject.

Provided herein is a method for detecting lyssavirus nucleic acid in a sample by contacting the sample with at least one forward primer and at least one reverse primer to amplify lyssavirus nucleic acid present in the sample; and at least one probe that detects the amplified lyssavirus nucleic acid. The at least one forward primer and the at least one reverse primer amplify a 5' region of the lyssavirus genome encompassing at least nucleotides 59-75 or 60-76 (see FIG. 1). In some embodiments, the at least one probe is no more than 20 nucleotides in length, such as no more than 19 nucleotides in length, no more than 18 nucleotides in length, or no more than 17 nucleotides in length, and includes the nucleotide sequence of AACACCYCTACAATGGA (SEQ ID NO: 1), AACACTACTACAATGGA (SEQ ID NO: 2) or ACACCYCTACAATGGAT (SEQ ID NO: 3). In some embodiments, the at least one probe comprises at least one modification to increase its melting temperature (Tm).

In some embodiments, the at least one forward primer is no more than 40 nucleotides in length, such as no more than 35 nucleotides in length, no more than 30 nucleotides in length, or no more than 25 nucleotides in length. In some examples, the nucleotide sequence of the at least one forward primer includes ACGCTTAACAACCAGATCAAAGAA (SEQ ID NO: 4) or ACGCTTAACAACCAGATCAAAGAAG (SEQ ID NO: 5). In specific examples, the at least one forward primer is no more than 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25 or 24 nucleotides in length and/or includes the sequence ACGCTTAACAACCAGATCAAAGAA (SEQ ID NO: 4) or ACGCTTAACAACCAGATCAAAGAAG (SEQ ID NO: 5).

In some embodiments, the at least one reverse primer is no more than 40 nucleotides in length, such as no more than 35 nucleotides in length, no more than 30 nucleotides in length, or no

more than 25 nucleotides in length. In some examples, the nucleotide sequence of the at least one reverse primer includes the sequence CMGGGTAYTTRTAYTCATAYTGRTC (SEQ ID NO: 6). In specific examples, the at least one reverse primer is no more than 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26 or 25 nucleotides in length and/or includes the sequence CMGGGTAYTTRTAYTCATAYTGRTC (SEQ ID NO: 6).

In particular embodiments, the method includes contacting the sample with a first forward primer including the sequence ACGCTTAACAACCAGATCAAAGAA (SEQ ID NO: 4), a second forward primer including the sequence ACGCTTAACAACCAGATCAAAGAAG (SEQ ID NO: 5), and a reverse primer including the sequence CMGGGTAYTTRTAYTCATAYTGRTC (SEQ ID NO: 6).

The at least one probe utilized in the provided assay includes at least one modification to increase melting temperature (Tm). When multiple modifications are present, the modifications can be the same modification or different modifications. Modifications that increase the Tm of an oligonucleotide are well-known in the art, and include but are not limited to, locked nucleic acid (LNA) nucleotides, minor groove binders (MGB), 5-hydroxybutynl-2'-deoxyuridine (SUPER T™), 2,6-diaminopurine (2-Amino-dA), 5-methyl deoxycytidine, and twisted intercalating nucleic acid (TINA).

In some embodiments, the at least one probe is modified to include at least one, at least two, at least three, at least four or at least five LNA nucleotides. In some examples, the at least one probe is modified to include at least 6 or at least 7 LNA nucleotides.

In particular embodiments, the at least one probe includes the nucleotide sequence AACACCYCTACAATGGA (SEQ ID NO: 1), and the probe includes LNA nucleotides at positions 4, 9, 10, 13 and 14. In some examples, the probe further includes LNA nucleotides at positions 3 and 8.

In other particular embodiments, the at least one probe includes the nucleotide sequence AACACTACTACAATGGA (SEQ ID NO: 2), and the probe includes LNA nucleotides at positions 3, 4, 8, 9, 10, 13 and 14.

In some embodiments, the at least one probe is modified to include a MGB.

In particular embodiments, the at least one probe includes the nucleotide sequence ACACCYCTACAATGGAT (SEQ ID NO: 3), and the probe includes a MGB at the 3' terminus.

In some examples of the disclosed method, the method includes contacting the sample with a first forward primer comprising the sequence ACGCTTAACAACCAGATCAAAGAA (SEQ ID NO: 4), a second forward primer comprising the sequence ACGCTTAACAACCAGATCAAAGAA (SEQ ID NO: 5), a reverse primer comprising the

sequence CMGGGTAYTTRTAYTCATAYTGRTC (SEQ ID NO: 6) and a first probe comprising the sequence AACACCYCTACAATGGA (SEQ ID NO: 1), wherein positions 4, 9, 10, 13 and 14 are LNA nucleotides; AACACCYCTACAATGGA (SEQ ID NO: 1), wherein positions 3, 4, 8, 9, 10, 13 and 14 are LNA nucleotides; or ACACCYCTACAATGGAT (SEQ ID NO: 3), wherein the probe comprises a MGB at the 3' terminus. In specific examples, the method further includes contacting the sample with a second probe comprising the sequence AACACTACTACAATGGA (SEQ ID NO: 2), wherein positions 3, 4, 8, 9, 10, 13 and 14 are LNA nucleotides.

In some embodiments, the at least one probe, the first probe, the second probe, or any combination thereof, comprise(s) a 5' fluorescent label, a 3' quencher, or both. In some examples, the fluorescent label is FAM. In some examples, the 3' quencher is BHQ1 or NFQ.

In some embodiments, the sample is a tissue sample, such as brain tissue sample, a skin biopsy or a corneal tissue sample. In other embodiments, the sample is a fluid sample, such as a saliva, blood, serum or cerebral spinal fluid (CSF) sample.

In some embodiments, the sample is a post-mortem sample. In other embodiments, the sample is an ante-mortem sample.

In specific examples, the sample is a post-mortem brain tissue sample.

In other specific examples, the sample is an ante-mortem saliva, serum, blood, CSF, skin biopsy or corneal tissue sample.

In some embodiments, the sample is obtained from a human. In other embodiments, the sample is obtained from a non-human animal. In some examples, the non-human animal is a dog, cat, coyote, skunk, fox, raccoon, mongoose, lion, shrew, rodent or civet.

In some embodiments, the lyssavirus nucleic acid is lyssavirus RNA or cDNA. For detection of lyssavirus RNA, the disclosed detection method further includes contacting the sample with a reverse transcriptase to generate cDNA.

V. Lyssavirus Diagnostic Kits

Further provided herein are kits for detecting lyssavirus nucleic acid, such as lyssavirus nucleic acid in a sample.

In some embodiments, the kit includes at least one forward primer and at least one reverse primer capable of amplifying a 5' region of the lyssavirus genome encompassing at least nucleotides 59-75 or 60-76 (see FIG. 1).

In some embodiments, the kit includes at least one probe that is no more than 20 nucleotides in length, such as no more than 19 nucleotides in length, no more than 18 nucleotides in length, or no more than 17 nucleotides in length, and includes the nucleotide sequence of

AACACCYCTACAATGGA (SEQ ID NO: 1), AACACTACTACAATGGA (SEQ ID NO: 2) or ACACCYCTACAATGGAT (SEQ ID NO: 3). In particular embodiments, the at least one probe comprises at least one modification to increase melting temperature (Tm).

In some embodiments of the kit, the at least one forward primer is no more than 40 nucleotides in length, such as no more than 35 nucleotides in length, no more than 30 nucleotides in length, or no more than 25 nucleotides in length. In some examples, the nucleotide sequence of the at least one forward primer includes ACGCTTAACAACCAGATCAAAGAA (SEQ ID NO: 4) or ACGCTTAACAACCAGATCAAAGAAG (SEQ ID NO: 5). In specific examples, the at least one forward primer is no more than 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25 or 24 nucleotides in length and/or includes the sequence ACGCTTAACAACCAGATCAAAGAA (SEQ ID NO: 4) or ACGCTTAACAACCAGATCAAAGAAG (SEQ ID NO: 5).

In some embodiments of the kit, the at least one reverse primer is no more than 40 nucleotides in length, such as no more than 35 nucleotides in length, no more than 30 nucleotides in length, or no more than 25 nucleotides in length. In some examples, the nucleotide sequence of the at least one reverse primer includes the sequence CMGGGTAYTTRTAYTCATAYTGRTC (SEQ ID NO: 6). In specific examples, the at least one reverse primer is no more than 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26 or 25 nucleotides in length and/or includes the sequence CMGGGTAYTTRTAYTCATAYTGRTC (SEQ ID NO: 6).

In particular embodiments, the kit includes a first forward primer, a second forward primer and a reverse primer, wherein the first forward primer comprises the sequence ACGCTTAACAACCAGATCAAAGAA (SEQ ID NO: 4), the second forward primer comprises the sequence ACGCTTAACAACCAGATCAAAGAA (SEQ ID NO: 5) and the reverse primer comprises the sequence CMGGGTAYTTRTAYTCATAYTGRTC (SEQ ID NO: 6).

The probes of the provided kit include at least one modification to increase their Tm. When multiple modifications are present, the modifications can be the same modification or different modifications. Modifications that increase the Tm of an oligonucleotide are well-known in the art, and include but are not limited to, LNA nucleotides, MGB, 5-hydroxybutynl-2'-deoxyuridine, 2-Amino-dA, 5-methyl deoxycytidine, and TINA.

In some embodiments of the kit, the at least one probe is modified to include at least one, at least two, at least three, at least four or at least five LNA nucleotides. In some examples, the at least one probe is modified to include at least 6 or at least 7 LNA nucleotides.

In particular embodiments, the at least one probe includes the nucleotide sequence AACACCYCTACAATGGA (SEQ ID NO: 1), and the probe includes LNA nucleotides at

positions 4, 9, 10, 13 and 14. In some examples, the probe further includes LNA nucleotides at positions 3 and 8.

In other particular embodiments, the at least one probe includes the nucleotide sequence AACACTACTACAATGGA (SEQ ID NO: 2), and the probe includes LNA nucleotides at positions 3, 4, 8, 9, 10, 13 and 14.

In some embodiments, the at least one probe is modified to include a MGB.

In particular embodiments, the at least one probe includes the nucleotide sequence ACACCYCTACAATGGAT (SEQ ID NO: 3), and the probe includes a MGB at the 3' terminus.

In particular examples, the kit includes a first forward primer comprising the sequence ACGCTTAACAACCAGATCAAAGAA (SEQ ID NO: 4), a second forward primer comprising the sequence ACGCTTAACAACCAGATCAAAGAA (SEQ ID NO: 5), a reverse primer comprising the sequence CMGGGTAYTTRTAYTCATAYTGRTC (SEQ ID NO: 6) and a first probe comprising the sequence AACACCYCTACAATGGA (SEQ ID NO: 1), wherein positions 4, 9, 10, 13 and 14 are LNA nucleotides; AACACCYCTACAATGGA (SEQ ID NO: 1), wherein positions 3, 4, 8, 9, 10, 13 and 14 are LNA nucleotides; ACACCYCTACAATGGAT (SEQ ID NO: 3), wherein the probe comprises a MGB at the 3' terminus. In specific examples, the kit further includes a second probe comprising the sequence AACACTACTACAATGGA (SEQ ID NO: 2), wherein positions 3, 4, 8, 9, 10, 13 and 14 are LNA nucleotides.

In some embodiments, the at least one probe, the first probe, the second probe, or any combination thereof, comprise(s) a 5' fluorescent label, a 3' quencher, or both. In some examples, the fluorescent label is FAM. In some examples, the 3' quencher is BHQ1 or NFQ.

The provided kits enable a user to detect lyssavirus nucleic acid (such as RNA) in a biological sample and/or to diagnose a lyssavirus infection. In some embodiments, the primer(s) and probe(s) are provided in individual containers. In other examples, mixtures of the primers and/or probe(s) are provided in a single container. Kits can optionally include instructions for carrying out the disclosed methods for detection of lyssavirus nucleic acid. In particular examples, the kits further include reagents useful in performing the RT-PCR reactions, such as a DNA polymerase, nucleotides and/or buffers.

The primers and probes provided in disclosed kits may be provided in any form practicable, such as suspended in an aqueous solution or as a freeze-dried or lyophilized powder, for instance. Kits according to this disclosure can also include instructions, usually written instructions, to assist the user in carrying out the detection methods disclosed herein. Such instructions can optionally be provided on a computer readable medium or as a link to an internet page.

The container(s) in which the reagents are supplied can be any conventional container that is capable of holding the supplied form, for instance, microfuge tubes, ampoules, or bottles. In some applications, the primers and probes, DNA polymerase(s), and/or other reagent mixtures useful for performing the RT-PCR detection assay may be provided in pre-measured single use amounts in individual, typically disposable, tubes, microtiter plates, or equivalent containers. The containers may also be compatible with a specific automated liquid handling apparatus.

The amount of a reagent supplied in the kit can be any appropriate amount, depending for instance on the market to which the product is directed. For instance, if the kit is adapted for research or clinical use, the amount of each reagent would likely be an amount sufficient for multiple screening assays. In other examples where the kit is intended for high throughput industrial use, the amounts could be sufficiently increased to accommodate multiple hundreds of assays.

The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the disclosure to the particular features or embodiments described.

EXAMPLES

Example 1: Materials and Methods

This example describes the materials and experimental procedures used for the studies described in Example 2.

Samples

Brain tissue samples from rabid animals were obtained via routine surveillance activity of the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA). Moribund and dead animals, or animals involved in human rabies exposure were collected by the US State Health Departments, US Department of Agriculture and veterinary laboratories during routine surveillance and diagnostic service.

Validation Samples: A total of 88 representative lyssaviruses were selected in the validation panel. The virus isolates and the details about the host species, geographical location and source of extraction are summarized in Table 1. Viruses were amplified in tissue culture only if a limited amount of original material was available. Total RNA was extracted from brain as well as tissue culture supernatant using TRIZOL™ reagent (Invitrogen) according to the manufacturer's instructions.

Table 1. Validation samples – Rabies virus positive samples used to validate the sensitivity of the LN34 Real-Time RT-PCR assay

S. No.	Isolate Origin	Host	Source	Virus Species	LN34m^	LN34^
1	Russia	Dog	TCS	RABV	16.542	ND
2	China	Dog	TCS	RABV	28.989	28.895
3	Argentina	Dog	TCS	RABV	23.143	23.331
4	Texas, USA	Coyote	TCS	RABV	17.206	ND
5	Thailand	Dog	TCS	RABV	17.425	16.504
6	Wisconsin, USA	Skunk	TCS	RABV	19.871	18.374
7	Mexico	Dog	TCS	RABV	25.348	25.341
8	Texas, USA	Coyote	TCS	RABV	19.801	18.572
9	Arizona, USA	Fox	TCS	RABV	19.705	19.063
10	California, USA	Skunk	TCS	RABV	20.904	21.001
11	Texas, USA	Fox	TCS	RABV	16.542	ND
12	Texas, USA	Skunk	TCS	RABV	29.524	14.369
13	Sri Lanka	Dog	TCS	RABV	22.037	22.427
14	Georgia, USA	Raccoon	TCS	RABV	17.206	18.570
15	Puerto Rico, USA	Mongoose	TCS	RABV	20.715	19.749
16	Tunisia	Dog	TCS	RABV	22.318	22.089
17	India	Dog	TCS	RABV	20.767	19.454
18	India	Dog	TCS	RABV	19.801	21.066
19	India	Dog	TCS	RABV	25.703	26.135
20	India	Dog	TCS	RABV	29.210	28.878
21	Arizona, USA	Bat-Lasiurus spp.	BS	RABV	26.892	27.354
22	Brazil	Bat-Desmodus spp.	BS	RABV	25.617	24.447
23	Mexico	Bat-Desmodus spp.	BS	RABV	25.033	23.005
24	Washington, USA	Bat-Lasionycteris spp.	BS	RABV	25.117	25.164
25	Washington, USA	Bat-Myotis spp.	BS	RABV	29.953	28.473
26	Tennessee, USA	Bat-Lasiurus spp.	BS	RABV	23.068	22.452
27	Tennessee, USA	Bat-Lasiurus spp.	BS	RABV	22.096	20.957
28	Tennessee, USA	Bat-Lasiurus spp.	BS	RABV	27.265	26.518
29	Puerto Rico, USA	Mongoose	BS	RABV	31.710	29.956
30	Argentina	Dog	BS	RABV	30.328	30.465
31	Texas, USA	Skunk	BS	RABV	19.979	18.899
32	Alaska, USA	Fox	BS	RABV	30.119	28.494

S. No.	Isolate Origin	Host	Source	Virus Species	LN34m^	LN34^
33	Texas, USA	Fox	BS	RABV	30.963	29.303
34	Thailand	Dog	BS	RABV	26.354	24.969
35	South Dakota, USA	Skunk	BS	RABV	32.672	31.012
36	USA	Laboratory strain	BS	RABV	36.497	36.445
37	Gabon	Dog	BS	RABV	27.304	25.406
38	Texas, USA	Coyote	BS	RABV	27.739	27.046
39	California, USA	Skunk	BS	RABV	25.387	25.036
40	Arizona, USA	Fox	BS	RABV	29.856	28.819
41	Arizona, USA	Bat-Eptesicus spp.	BS	RABV	19.187	18.138
42	Washington, USA	Bat-Eptesicus spp.	TCS	RABV	24.808	25.394
43	Mexico	Dog	TCS	RABV	26.509	24.454
44	Washington, USA	Bat-Eptesicus spp.	BS	RABV	20.956	19.938
45	Alabama, USA	Bat-Tadarida spp.	BS	RABV	33.088	30.792
46	Arizona, USA	Bat-Lasiurus spp.	BS	RABV	20.186	17.338
47	Greece *	Red Fox	BS	RABV	16.859	16.239
48	France *	Red Fox	BS	RABV	15.502	15.363
49	Italy *	Red Fox	BS	RABV	15.963	14.833
50	USA *	Laboratory strain	BS	RABV	16.039	13.123
51	Thailand	Dog	BS	RABV	21.387	21.522
52	Florida, USA	Feline	BS	RABV	20.403	20.296
53	Texas, USA	Dog	BS	RABV	20.205	20.470
54	California, USA	Bat-Antrozous spp.	BS	RABV	21.599	20.024
55	Namibia	Lion	BS	RABV	21.102	21.164
56	Peru	Dog	BS	RABV	18.995	18.427
57	Serbia *	Feline	TCS	RABV	13.443	13.264
58	France *	Red Fox	TCS	RABV	12.039	11.788
59	Europe *	Laboratory strain	TCS	RABV	11.039	11.687
60	Kyrgyzstan	Bat-Myotis spp.	TCS	ARAV	34.127	33.961
61	Tajikistan	Bat-Myotis spp.	TCS	KHUV	36.589	34.605
62	South Africa	Bat-Miniopterus spp.	TCS	DUVV	36.803	ND
63	South Africa	Bat-Nycteris spp.	TCS	DUVV	30.741	31.024
64	South Africa	Human	TCS	DUVV	20.767	19.454
65	South Africa *	Bat-Miniopterus spp.	TCS	DUVV	16.722	15.051
66	Australia	Bat-Pteropus spp.	TCS	ABLV	31.025	28.786
67	Australia	Bat-Pteropus spp.	BS	ABLV	25.515	23.952

S. No.	Isolate Origin	Host	Source	Virus Species	LN34m^	LN34^
68	Europe	Bat-Eptesicus spp.	TCS	EBLV-1	30.484	30.509
69	Denmark	Bat-Eptesicus spp.	TCS	EBLV-1	26.640	26.334
70	France *	Bat-Eptesicus spp.	TCS	EBLV-1	14.772	14.729
71	Europe	Bat-Myotis spp.	TCS	EBLV-2	41.142	38.847
72	United Kingdom *	Bat-Myotis spp.	TCS	EBLV-2	18.826	15.930
73	Neitherlands	Bat-Myotis spp.	TCS	EBLV-2	25.577	24.847
74	United Kingdom *	Bat-Myotis spp.	TCS	EBLV-2	16.263	17.978
75	Russia	Bat-Murina spp.	TCS	IRKV	23.988	13.290
76	Germany *	Bat-Myotis spp.	TCS	BBLV	20.276	19.442
77	Zimbabwe	Feline	TCS	MOKV	35.416	27.557
78	Cameroon	Crocidura	TCS	MOKV	30.726	22.380
79	Central African Republic	Lophuromys	TCS	MOKV	41.764	29.034
80	South Africa	Feline	BS	MOKV	30.130	20.149
81	Nigeria	Crocidura	TCS	MOKV	30.770	28.605
82	South Africa	Bat-Epomophorous	TCS	LBV	37.012	37.912
83	Zimbabwe	Bat-Nycteris spp.	TCS	LBV	26.932	ND
84	Central African Republic	Bat-Nycteris spp.	TCS	LBV	22.985	22.960
85	South Africa	Mongoose	TCS	LBV	23.723	24.441
86	Kenya	Bat-Hipposideros	TCS	SHIBV	18.835	17.042
87	Russia	Bat-Miniopterus spp.	TCS	WCBV	32.087	32.881
88	Tanzania	African civet	TCS	IKOV	43.269	20.916

All 88 samples (except ND=5) in the validation panel were tested using both LN34m and LN34 assays in duplicate. Isolate origin represents the geographical location and/or distribution of the respective sample.

Source of the RNA isolation was either TCS (tissue culture supernatant) or BS (brain stem).

The samples in the validation panel can be divided into 3 phylogroups: Phylogroup I includes RABV, ARAV, KHUV, BBLV, DUVV, EBLV-1, EBLV-2, ABLV and IRKV; Phylogroup II includes MOKV, SHIBV and LBV; and Phylogroup III includes IKOV and WCBV.

* Sample that was part of the ANSES (French Agency for Food, Environmental and Occupational Health & Safety) panel.

^ The mean Ct value of the samples run by both LN34m and LN34 assays.

Clinical Samples: Human ante-mortem as well as post-mortem samples, and those animal samples that had a history of known human exposure received by the Poxvirus and Rabies Branch for testing during November 2014 – November 2015 were defined as clinical samples. A total of 205 clinical samples were included in this study and the details of these samples are included in Table 2.

Table 2: Clinical samples included in the study

Table 2A

	Human		**Animal**		**Total**
	Positive	Negative	Positive	Negative	
Brain	1		5	164	169
Skin Biopsy	2	14			15
Saliva	4	15			21
Cornea	1				
Total	8	29	5	164	205

Table 2B

		Lab ID	**LN34^**	**Beta-actin^**
Human	Skin	A15-2217	37.896	20.149
	Skin	A15-2460	32.207	19.759
	Saliva	A15-2218	32.376	17.307
	Saliva	A15-0728	33.310	19.432
	Saliva	A15-0729	35.347	21.456
	Saliva	A15-2461	32.747	24.4535
	Brain	A15-0731	14.003	18.068
	Cornea	K1	33.622	ND
Animal	Brain	A15-2206	22.998	19.948
	Brain	A15-0755	16.121	13.888
	Brain	A15-1861	17.017	14.048
	Brain	A15-2026	15.098	15.528
	Brain	A15-2190	18.319	16.244

Table 2C

Species		Lab ID	LN34	Beta-actin
Arctic Fox	Brain	A15-2672	26.675	25.673
Bat	Brain	A15-2678	17.787	19.149
Arctic Fox	Brain	A16-0742	20.100	20.617
Arctic Fox	Brain	A16-0743	18.553	17.896
Bat	Brain	A15-0283	14.739	ND
Bat	Brain	A15-0284	34.104	ND
Cow	Brain	A15-0287	15.965	ND
Bat	Brain	A15-0396	16.363	ND
Bat	Brain	A15-0399	19.139	ND
Bat	Brain	A15-0401	17.347	ND
Bat	Brain	A15-0407	15.326	ND
Bat	Brain	A15-0409	19.500	ND
Bat	Brain	A15-1903	14.061	ND
Bat	Brain	A15-1905	18.525	ND
Bat	Brain	A15-1907	24.119	ND
Bat	Brain	A15-1908	30.276	ND
Bat	Brain	A15-1910	24.881	ND
Bat	Brain	A15-1912	28.720	ND

Table 2A: Includes a list of all the clinical samples (both human and animal) tested by the LN34 assay.

Table 2B: Includes Ct values of all the positive samples from both human and animals tested by the LN34 Real-Time RT-PCR assay and β-actin internal control.

Table 2C: Includes Ct values of all the positive animal samples collected from field studies tested by the LN34 Real-Time RT-PCR assay and β-actin internal control.

^ The mean Ct value of the samples run by the LN34 Real-Time RT-PCR assay and β-actin internal control.

ND: Sample was not tested by this assay.

Field Samples: A total of 58 field samples were tested using LN34 as well as β-actin real-time RT-PCR assay. These samples were part of the annual surveillance testing (n=40) and supplemented with samples collected by USDA/APHIS/Wildlife services during passive

surveillance (n=18). All were deemed unfit for testing by DFA test due to limited tissue and preservation conditions.

All clinical samples were tested for the presence of RABV antigens using the DFA test with fluorescein-isothiocyanate (FITC)-conjugated anti-RABV monoclonal antibody (Fujirubio Diagnostics Inc., Malvern, PA, USA) (Dean and Atanasiu, "The fluorescent antibody test," *In*: Meslin FX KM, Koprowski H, editor. Laboratory techniques in rabies. 23. 4th ed. Geneva: World Health Organization monograph series; 1996. p. 88-93). Total RNA was extracted from brain as well as tissue culture supernatant using TRIZOL™ reagent (Invitrogen) according to the manufacturer's instructions.

Reverse transcriptase PCR and sequencing: Reverse transcriptase PCR amplification and sequencing of the N gene were performed as described by Kuzmin *et al.* (*J Clin Microbiol* 46(4):1451-1461, 2008).

Development of Real-Time RT-PCR

Designing Primers and Probes: The probe of the assay was designed by either modified LNA or by a MGB. The differences between those two probes were compared for their sensitivities and specificities in the validation process. All of the probes and primers contain degenerate nucleotides, which is an IUPAC nucleotide ambiguity code. Table 3 contains a list of the primer and probe modifications.

Table 3. Primer and Probe Sequences for the Assay LN34

Name	Length	Sequences[a]	SEQ ID NO:	Position[b]
Probe LN34	17	(FAM) AA+C+ACCY+C+T+ACA+A+TGGA (BHQ1)	1	59 - 75
Probe LN34a	17	(FAM) AAC+ACCYC+T+ACA+A+TGGA (BHQ1)	1	59 - 75
Probe LN34lago	17	(FAM) AA +C +ACTA +C +T +ACA +A +TGGA (BHQ1)	2	59 - 75
Probe LN34m	17	(6FAM)-ACACCYCTACAATGGAT-(MGBNFQ)	3	60 - 76
Primer forward1	24	ACGCTTAACAACCAGATCAAAGAA	4	1 - 24
Primer forward2	25	ACGCTTAACAACAAAATCADAGAAG	5	1 - 25
Primer reverse	25	CMGGGTAYTTRTAYTCATAYTGRTC	6	140 - 164

[a]The probes are labeled by fluorescent FAM at the 5' end, BLACK HOLE QUENCHER™ (BHQ1) at the 3' end, except probe LN34m, which is labeled by MGB and nonfluorescent quencher (NFQ).

LNA nucleotides are indicated by a "+" preceding the base. Degenerate nucleotides are underlined: D = A, G or T; M = A or C; Y = T or C; R = G or A.

[b]The primer and probe positions are given relative to the full lyssavirus genome (FIG. 1).

LN34, LN34a and LN34Lagos: Probes modified by LNA. The name of the probe is followed by the 5' fluorescent label (in parentheses), probe sequence (a plus preceding the location of the LNA base in the sequence (*e.g.* +A, +G, +C, +T)), and 3' quencher (Table 3, in parentheses). LN34Lagos is specific for the Lagos bat virus Nigeria subspecies diagnostics, which is mixed with LN34 in a multiplex format.

LN34m: MGB modified probe with a 5' 6FAM florescent label and a 3' MGB label and NFQ quencher.

The forward primers are a multiplex of two oligos that target the beginning of RABV genome sequences, whereas the reverse primer targets the N gene with 6 degenerate nucleotide modifications.

An artificial positive control for the assay (Hoffmann *et al.*, *J Clin Microbiol* 48(11):3970-8, 2010) was used to minimize potential contamination.

oLPC-rabies3-4: GCA CAG GGT ACT TGT ACT CAT ACT GAT CTG AAT CCA TTG TAG AGG TGT TAG AGC ACG ACA GGT TTC CCG ACT GGA TCT TTC TTT GAT CTG GTT AAG CGT TCG CCC TAT AGT GAG TCG TAT TAC A (SEQ ID NO: 7)

A β-actin real-time PCR assay was used in this study as an internal or negative control (Wakeley *et al.*, *Dev Biol* 126:227-236, 2006).

β_actin probe, (TET)-TCC ACC TTC CAG ATG TGG ATC A-(BHQ1) (SEQ ID NO: 8)

Forward primer: β_act for: CGATGAAGATCAAGATCATTGC (SEQ ID NO: 9)

Reverse primer: β_act rev: AAGCATTTGCGGTGGAC (SEQ ID NO: 10)

Optimization

The reaction conditions were optimized for multiple factors, including annealing temperature, length of reverse transcription and PCR reactions, ratios of primer and probe, and master mix components. The optimized reaction conditions were as follows: Ag-Path ID One-Step RT-PCR Kit (Life Technologies), Assay LN34 primer and probe set, Assay β-actin primer and probe set, artificial positive RNA for assay LN34. One femto gram (about 10,000 copies of the RNA template) was used as positive control. One µl of forward and reverse primer stocks of 10 µM and 1.0 µl of 5 µM probe were used for the 25 µl reaction set up following the directions of the commercial kit.

The cycling conditions were as follows: Reverse transcription at 50°C for 30 minutes, followed by RT inactivation/initial denaturation at 95°C for 10 minutes, and amplification step repeated for 45 cycles at 95°C for 1 second and 56°C for 20 seconds.

Droplet digital PCR (ddPCR) for the limit of detection of LN34: One-step RT-ddPCR advanced kit (Bio-Rad, CA) was used, which provides improved efficiency and specificity for precise RNA target quantification by ddPCR. Details of the reaction set up and cycling conditions are provided in Example 2.

Similarity Matrix

N gene sequence similarity Matrix analysis: Eighty-eight whole N gene nucleotide sequences (SEQ ID NOs: 31-118) included in the validation panel were subjected for multiple sequence alignments using CLUSTALW. All of the sequences were edited to 1350 bp fragments using BioEdit (Hall, *Nucleic Acids Symposium Series* 41:95-98, 1999).

Example 2: Pan-Rabies Real-Time RT-PCR Assay for Detection of Highly Variable Lyssaviruses

Assay LN34 amplifies a 165-nucleotide amplicon including the 58-nucleotide leader sequence, the 12-nucleotide transcription initiation signal, and 95 nucleotides of the nucleoprotein (N) gene (FIG. 1). This leader region and transcription signal sequence are strictly conserved in length among all lyssaviruses (Jackson, *Adv Virus Res* 79:xvii, 2011). The forward primers target the first 25 nucleotides of the leader sequences with a low level of degenerate nucleotides; the reverse primer uses 6 degenerate nucleotides to cover the significant diversity of its targeted sequence. The probe of LN34 targets mostly the transcription initiation signal sequence from position 59 to 75 and utilizes a single degenerate position of pyrimidine (C or T) at position 65. This duplex probe design made the probe sequences match perfectly to all published RABV and other lyssavirus sequences except a few striped skunk RABV variants, MOKV, IKOV, IRKV, and the Nigerian subspecies of LBV. To compensate for the low melting temperature of the 17-nucleotide probe sequences, the probe was modified either by MGB (assay LN34m) or LNA (assay LN34). The melting temperature of the modified probes was calculated using software Primer Express 3.01 for the MGB probe (Life Technology, California) or the Integrated DNA Technologies website (OligoAnalyzer 3.1) for the LNA modified probes. A comparison of LNA modified probes with 5 to 7 LNA nucleotides at different positions of the probe sequences was studied using a number of RABV and other lyssavirus samples. Two formats of the LNA modification, LN34 and LN34a, yielded the overall best results.

The validation panel (n=88) was selected from the CDC RABV repository which includes representative variant samples from around the world (Table 1), including 59 variants of highly divergent RABV isolates from Asia, Africa, Europe, South America and North America. The validation panel also included 13 other lyssavirus species, except LLBV, which is the newest identified lyssavirus species. The assays LN34m and LN34 were able to detect all of the variants from the validation panel except LBV Nigeria subspecies. For the majority of the isolates in the validation panel, assay LN34m and assay LN34 demonstrated similar sensitivity as demonstrated by their similar Ct values (Table 1). The validations of assays LN34m and LN34 were completed separately, which accounts for the Ct value variation for most of the samples in the validation panel. However, for those isolates having sequence variations from the probe sequences, the LN34m and LN34 assays perform considerable differently. The striped Skunk RABV and IRKV have a single nucleotide polymorphism (SNP) at position 69 of the probe sequence (FIG. 1), which reduces the sensitivity of LN34m at least 10 Ct values as compared to that of LN34 for one Texas skunk isolate (Table 1, S. No 12) and one Irkut virus isolate (Table 1 S. No 75). This equates to at least 100-fold differences in the sensitivity. The MGB modified probe is more sensitive to the SNP than the probe modified by LNA. Similarly, MOKV and IKOV have a SNP at position 63 (FIG. 1), which also lead to a Ct value difference of 8-14, also resulting in more than a 100-fold loss in sensitivity for assay LN34m (Table 1, S. No. 75-80 and 88). The Nigeria subspecies of LBV has two SNPs relative to the probe sequences and cannot be detected by either LN34m or LN34. A specific LNA modified probe was synthesized for the Nigerian LBV subspecies and added to the assay LN34; this additional multiplex does not affect the sensitivity and specificity of assay LN34.

The multiplex assay LN34 has overall better sensitivity and specificity. Assay LN34 was able to detect all of the positive samples of the validation panels (n=88) and all of the rabies positive clinical samples confirmed by DFA (n=12), yielding a sensitivity of 100%. Assay LN34 also correctly ruled out about 200 negative clinical samples confirmed by DFA and/or RT-PCR and hemi-nested RT-PCR (n=193), leading to a 100% specificity for this assay. Among those negative samples, many were initially unable to be diagnosed by DFA and were reported as inconclusive due to issues related to the reagent quality. Many of those negative samples produced non-specific amplifications by a conventional RT-PCR assay that was run in parallel with the real-time RT-PCR assays during assay validation. The non-specific amplifications with a similar molecular weight as positive controls from the hemi-nested RT-PCR were ruled out by sequencing the amplicon. These negative samples included skin biopsy (n=14) and saliva (n=15) from patients with neurological symptoms; and DFA-negative or inconclusive animal brain samples (n=164). The positive clinical samples included human ante-mortem samples of skin biopsy (n=2), saliva (n=4), cornea (n=1) and

a brain biopsy (Table 2), as well as the animal brain samples. Comparing to the brain samples, the RABV viral load or RNA levels were much lower for the skin biopsy, saliva, and cornea as indicated by the Ct values. The ante-mortem samples with Lab ID A15-0728, A15-0729, and A15-0731were collected from the same patient and had been stored at room temperature for an extended time due to a transportation delay. Although these samples did not meet the standard for regular DFA test, the results from assay LN34 showed that the brain biopsy had a very high level of viral load. The viral load or viral RNA of all three positive saliva samples of the ante-mortem cases had similar Ct values. The assay LN34 was used to test 58 field samples from US annual surveillance testing and Wildlife services during passive surveillance. Those samples were determined to be unfit to be tested by DFA assay due to limited tissues or unavailable of appropriate conjugates. Eighteen (31.03%) samples were positive (Table 2C).

Two different internal controls were used within the multiplexed assay: (1) detection of a β-actin housekeeping gene assay and (2) a positive control for RNA amplification. The β-actin housekeeping gene assay was adapted from a previous publication with modifications (Wakeley *et al.*, *Dev Biol* 126:227-236, 2006) and used to assess the performance of RNA extraction and has to be positive for the diagnostic results for rabies negative samples (Table 2). The artificial positive controls were also adapted from a previous publication (Hoffmann *et al.*, *J Clin Microbiol* 48(11):3970-8, 2010), with modification to include a short LacZ gene sequence to be used for the identification of the artificial RNA to rule out potential carry-over contamination. The artificially generated RNA positive control can be stored for an extended period by using the stabilization buffer, and can be used as a standard for the comparison of the assay LN34 performance among different laboratories and running conditions. The β-actin assay was labelled by fluorescent HEX and had the same running condition as that of LN34. A diagnostic sample can be run on the sample plate or the same well in a multiplex format. The efficiency of LN34 was estimated to be more than 93% by testing a serial dilution of RNA extracted from RABV strain CVS11. The limit of detection of the LN34 assay was measured by the ddPCR assay using both artificial positive control RNA and a RABV strain ERA. The LN34 assay detected a single copy of RNA for both RNA samples, as detailed below. The results are shown in FIG. 2. The protocol for the ddPCR assay is provided below.

Reaction setup:

1. The reaction mix for the number of reactions needed was prepared according to the table below:

Component	Volume per reaction, µl	final concentration
Supermix	5 µl	1X
Reverse transcriptase	2 µl	20 U/µl
300 mM DTT	1 µl	15 mM
Target Forward primer 34F 10 µM	2 µl	1000 nM
Target Reverse primer 34R 10 µM	2 µl	1000 nM
Target probe 34P 2.5 µM	1 µl	125 nM
RNase-/DNase-free water	2 µl	
	15 µl mix them and then add:	
DNA (different dilution)	5 µl	variable
Total:	20 µl	

2. 20 µl of each reaction mix was mixed thoroughly and loaded into a sample well of a DG8 cartridge for QX100 droplet generator, followed by 70 µl of droplet generator oil for probes into the oil wells. The DG8 cartridge was then put into droplet generator for 2 minutes.

Thermal Cycling Conditions:

3. After droplet generation with the QX100 droplet generator, droplets were transferred into a clean 96-well plate. The plate was heat sealed with foil by the PX1 PCR plate sealer before thermal cycling on an ABI GeneAmp 9700. The ramp rate setting of the thermal cycle was lowered to 3°C/s to allow thorough heating and cooling of the droplets.
4. After PCR, the 96-well plate was placed into a plate holder and loaded into the QX100 droplet digital reader for detection and analysis.

Cycling Conditions:

Cycling step	temperature, ^{0}C	time	number of cycles
Reverse transcription	45^{0}C	60 min	1
Enzyme activation	95^{0}C	10 min	1

Denaturation	95^{0}C	30 sec	40
Annealing/extension	55^{0}C	1 min	40
Enzyme deactivation	98^{0}C	10 min	1
Hold	4^{0}C		

The N gene sequences of all samples used in the validation panel were sequenced and newly generated sequences were deposited in Genbank (set forth herein as SEQ ID NOs: 31-118). The similarity matrix of the N gene among the validation panel samples was calculated using Mega software (5.1). RABV has a similarity range from 82% or higher in which the lowest identities values are found among RABV in southeastern North America, from the vampire bat of Mexico, raccoon and South Central skunk of the U.S., which agrees with previous RABV variant classifications that suggest American indigenous RABV is one of the most divergent strains (Jackson, *Adv Virus Res* 79:xvii, 2011). The non-RABV lyssaviruses have lower similarity values ranging from 68% to 79%. In comparison, the most diverged lyssavirus was IKOV, which also agrees with previous studies (Arechiga Ceballos *et al.*, *Emerg Infect Dis* 19(5):793-795, 2013). The LN34 assay's ability to detect all available lyssaviruses confirms its robustness, and highlights its utility as a diagnostic tool applicable for rabies diagnosis.

Diagnostic Applications

Assay LN34 represents a major improvement over previously published real-time RT-PCR assays for rabies and other lyssavirus diagnostics as it can detect a broad range of phylogenetic diversity with superior sensitivity and specificity in a single step reaction. The assay was validated using a large and highly diverse panel containing major RABV variants and 13 other lyssaviruses. The initial assay development used over 280 published full genome sequences of all lyssavirus species except LLBV, and the RABV sequences covered all major geographic based clades, including American indigenous, India, Asia, Africa 2, Africa 3, Arctic-related and cosmopolitan strains. The forward primer sequences of the assay LN34 only needed degenerate nucleotides near the 3' end as the first 9 nucleotides of the forward primers are identical among all lyssaviruses. The reverse primer also targets a relatively conserved region that has been used for the design of rabies PCR primers (Hayman *et al.*, *J Virol Methods* 177(1):87-93, 2011), but the reverse primer of the assay LN34 is longer compared to previous publications and uses a high level of degenerate nucleotides to balance the universal amplification of lyssaviruses alongside specificity for divergent viruses (Hayman *et al.*, *J Virol Methods* 177(1):87-93, 2011). The probe sequence of the assay LN34 targets one of the most conserved regions among all lyssaviruses (Heaton *et al.*, *J Clin*

Microbiol 35(11):2762-2766, 1997). The LN34 probe has identity with known RABV when a single degenerate site is accounted for (excluding some striped skunk RABV variants). The low level of degeneration of the probe sequences allows the LN34 assay to maintain an optimal degree of sensitivity and specificity for RABV diagnostics. The validation results demonstrated that the Ct values with all RABV variants were highly positive even when all samples used in the validation cohort were diluted more than 10-fold. The non-RABV lyssaviruses are more diverse compared to RABV, but at least 8 lyssavirus species have a perfect match for the LN34 probe sequences (FIG. 1), and can be amplified efficiently as shown in the validation data (Table 1). Since the LBV Nigeria subspecies cannot be detected by the initial design of the probe, a multiplex format with a specific probe sequence for this subspecies of LBV was developed, and additional specific probes can be used for the detection of any newly identified lyssavirus if needed. Both MGB and LNA modified LN34 probes work well for most lyssaviruses except a few virus strains with variations within the probe sequences (FIG. 1). Previous studies showed that LNA and MGB probes have similar sensitivities (Letertre *et al.*, *Mol Cell Probes* 17(5):227-35, 2003). In the validation studies disclosed herein, the MGB probe was very sensitive to a single nucleotide changes in the probe's target sequence, whereas the LNA probe had a higher tolerance to single nucleotide changes.

The validation results showed that the MGB and LNA probes had similar sensitivities for most of the RABV and lyssavirus strains. For those samples with more than 3 Ct value differences, the samples were re-run on the same plate to confirm such differences. Only RABV isolates from Gabon Africa (Table 1 S. No. 37) and a bat associated RABV from the U.S. (Table 1, S. No. 45 and 46) still had more than 2 Ct value differences, which may be due to the primer targeted sequence variations. Only the newest lyssavirus (LLBV) was not available for the validation of LN34 (Arechiga Ceballos *et al.*, *Emerg Infect Dis* 19(5):793-795, 2013). LLBV is grouped with IKOV phylogenetically (Phylogroup 3).

Each year, rabies surveillance in the United States tests over 100,000 suspected rabid animal samples; over 6,000 rabid animals were diagnosed in 2014 (Monroe *et al.*, J *Am Vet Med Assoc* 248(7):777-788, 2016). The inherent high throughput nature of assay LN34 can improve the speed of the testing and reduce the workload for diagnostic laboratories. For those thousands of samples unfit for DFA testing due to limited sample quantities or sample degradation, assay LN34 can be used as the confirmation test of choice, since it requires very little material for the testing and allows passive surveillance using sample stabilization reagents to preserve rabies suspicious samples. By applying the real-time RT-PCR assay LN34, the number of unsuitable samples for testing can be further reduced, which will improve the surveillance and control of rabid animals in the U.S.

The LN34 assay was able to detect a single copy the RNA template of rabies clinical samples. As the majority of RABV and the other lyssaviruses have identical sequences to that of the LN34 probe, the amplification efficiency and the limit of detection for most lyssavirus species should be maintained relatively constant for LN34, as demonstrated during the validation process. The high sensitivity of LN34 is important for the diagnosis of samples that have been stored or transported under non-optimal conditions and cannot to be diagnosed by other technologies.

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only preferred examples of the invention and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

CLAIMS

1. A method for detecting lyssavirus nucleic acid in a sample, comprising contacting the sample with (i) at least one forward primer and at least one reverse primer to amplify lyssavirus nucleic acid present in the sample; and (ii) at least one probe that detects the amplified lyssavirus nucleic acid, wherein:

the at least one forward primer and the at least one reverse primer amplify a 5' region of the lyssavirus genome encompassing at least nucleotides 59-75 or 60-76; and

the at least one probe is no more than 20 nucleotides in length and comprises the nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3, wherein the at least one probe comprises at least one modification to increase melting temperature (Tm).

2. The method of claim 1, wherein the at least one forward primer is no more than 40 nucleotides in length and comprises the nucleotide sequence of SEQ ID NO: 4 or SEQ ID NO: 5.

3. The method of claim 1 or claim 2, wherein the at least one reverse primer is no more than 40 nucleotides in length and comprises the nucleotide sequence of SEQ ID NO: 6.

4. The method of any one of claims 1-3, comprising contacting the sample with a first forward primer comprising SEQ ID NO: 4, a second forward primer comprising SEQ ID NO: 5, and a reverse primer comprising SEQ ID NO: 6.

5. The method of any one of claims 1-4, wherein the at least one probe is modified to include at least one locked nucleic acid (LNA) nucleotide.

6. The method of any one of claims 1-4, wherein the at least one probe is modified to include at least three LNA nucleotides.

7. The method of any one of claims 1-4, wherein the at least one probe is modified to include at least five LNA nucleotides.

8. The method of any one of claims 1-4, wherein the at least one probe is modified to include at least seven LNA nucleotides.

9. The method of any one of claims 1-8 wherein the at least one probe comprises the nucleotide sequence of SEQ ID NO: 1, and wherein the probe comprises LNA nucleotides at positions 4, 9, 10, 13 and 14.

10. The method of claim 9, wherein the probe further comprises LNA nucleotides at positions 3 and 8.

11. The method of any one of claims 1-8, wherein the at least one probe comprises the nucleotide sequence of SEQ ID NO: 2, and wherein the probe comprises LNA nucleotides at positions 3, 4, 8, 9, 10, 13 and 14.

12. The method of any one of claims 1-4, wherein the at least one probe is modified to include a minor groove binder (MGB).

13. The method of any one of claims 1-4 and 12, wherein the at least one probe comprises the nucleotide sequence of SEQ ID NO: 3, and wherein the probe comprises a MGB at the 3' terminus.

14. The method of any one of claims 1-13, comprising contacting the sample with a first forward primer comprising SEQ ID NO: 4, a second forward primer comprising SEQ ID NO: 5, a reverse primer comprising SEQ ID NO: 6, and a first probe comprising:

SEQ ID NO: 1, wherein positions 4, 9, 10, 13 and 14 are LNA nucleotides;

SEQ ID NO: 1, wherein positions 3, 4, 8, 9, 10, 13 and 14 are LNA nucleotides; or

SEQ ID NO: 3, wherein the probe comprises a MGB at the 3' terminus.

15. The method of claim 14, further comprising contacting the sample with a second probe comprising SEQ ID NO: 2, wherein positions 3, 4, 8, 9, 10, 13 and 14 are LNA nucleotides.

16. The method of any one of claims 1-15, wherein the at least one probe, the first probe, the second probe, or any combination thereof, comprise(s) a 5' fluorescent label and a 3' quencher.

17. A kit for detecting lyssavirus nucleic acid, comprising:

at least one forward primer and at least one reverse primer capable of amplifying a 5' region of the lyssavirus genome encompassing at least nucleotides 59-75 or 60-76; and

at least one probe that is no more than 20 nucleotides and length and comprises the nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3, wherein the at least one probe comprises at least one modification to increase melting temperature (Tm).

18. The kit of claim 17, wherein the at least one forward primer is no more than 40 nucleotides in length and comprises the nucleotide sequence of SEQ ID NO: 4 or SEQ ID NO: 5.

19. The kit of claim 17 or claim 18, wherein the at least one reverse primer is no more than 40 nucleotides in length and comprises the nucleotide sequence of SEQ ID NO: 6.

20. The kit of any one of claims 17-19, comprising a first forward primer, a second forward primer and a reverse primer, wherein the first forward primer comprises SEQ ID NO: 4, the second forward primer comprises SEQ ID NO: 5 and the reverse primer comprises SEQ ID NO: 6.

21. The kit of any one of claims 17-20, wherein the at least one probe is modified to include at least one, at least two, at least three, at least four or at least five locked nucleic acid (LNA) nucleotides.

22. The kit of any one of claims 17-20, wherein the at least one probe is modified to include a minor groove binder (MGB).

23. The kit of any one of claims 15-18, wherein the at least one probe is selected from:

SEQ ID NO: 1, wherein positions 4, 9, 10, 13 and 14 are LNA nucleotides;

SEQ ID NO: 1, wherein positions 3, 4, 8, 9, 10, 13 and 14 are LNA nucleotides; and

SEQ ID NO: 3, wherein the probe comprises a MGB at the 3' terminus.

24. The kit of claim 23, further comprising a probe comprising SEQ ID NO: 2, wherein positions 3, 4, 8, 9, 10, 13 and 14 are LNA nucleotides.

25. The kit of claim 23 or claim 24, wherein the at least one probe comprises:

SEQ ID NO: 1, wherein positions 4, 9, 10, 13 and 14 are LNA nucleotides, or SEQ ID NO: 1, wherein positions 3, 4, 8, 9, 10, 13 and 14 are LNA nucleotides; and

SEQ ID NO: 2, wherein positions 3, 4, 8, 9, 10, 13 and 14 are LNA nucleotides.

26. The kit of claim 23 or claim 24, wherein the at least one probe comprises:

SEQ ID NO: 3, wherein the probe comprises a MGB at the 3' terminus; and

SEQ ID NO: 2, wherein positions 3, 4, 8, 9, 10, 13 and 14 are LNA nucleotides.

27. The kit of claim 17, wherein the at least one probe comprises a heterologous or synthetic label.

28. The method of claim 1, wherein the at least one probe comprises a heterologous or synthetic label.

AB608731 Lasiurus-Brazil
JQ685921 Myotis-USA
JN234411 China
JQ944708 Red fox-Russia
JQ685894 Striped skunk-USA
KC196743 Dog Nigeria
KF245925 Bokeloh bat
EU293114 European bat 2
EF614261 Khujand lyssavirus
EF614259 Aravan virus
EU293119 Duvenhage virus
EU293109 European bat 1
EF614260 Irkut virus
EU293108 Lagos bat SEN
EU293110 Lagos bat NGA
GU170201 Shimoni bat virus
EU293117 Mokola virus
EF614258 West Caucasian bat
NC018629 Ikoma lyssavirus

FIG. 1

FIG. 2

RABV strain ERA RNA dilutions	LN34 qPCR CT Value	Estimated RNA copies/µl by ddPCR
1:25	31.15	89.8
1:125	34.3	15.8
1:625	36.48	3.66
1:3125	36.99	1.38
1:15625		0.39
1:78125		0.18
LN34 positive control RNA dilutions	LN34 qPCR CT Value	Estimated RNA copies/µl by ddPCR
1: 390625	28.82	627
1:1953125	31.51	121
1:9765625	33.84	31.2
1:48828125	36.43	3.11
1:244140625	40.59	0.94
1:1220703125		0.28

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/031595

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, EMBASE, FSTA, 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	SUSAN A. NADIN-DAVIS ET AL: "Development of real-time reverse transcriptase polymerase chain reaction methods for human rabies diagnosis", JOURNAL OF MEDICAL VIROLOGY, vol. 81, no. 8, 1 August 2009 (2009-08-01) , pages 1484-1497, XP055381762, US ISSN: 0146-6615, DOI: 10.1002/jmv.21547 abstract; table 1 ----- -/--	1-28

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 June 2017	27/06/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Ripaud, Leslie

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/031595

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/031595

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	Anthony R Fooks ET AL: "Emerging Technologies for the Detection of Rabies Virus: Challenges and Hopes in the 21st Century", PLoS Neglected Tropical Diseases, 29 September 2009 (2009-09-29), XP055382097, San Francisco DOI: 10.1371/journal.pntd.0000530 Retrieved from the Internet: URL:http://journals.plos.org/plosntds/article/file?id=10.1371/journal.pntd.0000530&type=printable [retrieved on 2017-06-16] the whole document	1-28

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/031595

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	B. HOFFMANN ET AL: "Improved Safety for Molecular Diagnosis of Classical Rabies Viruses by Use of a TaqMan Real-Time Reverse Transcription-PCR "Double Check" Strategy", JOURNAL OF CLINICAL MICROBIOLOGY, vol. 48, no. 11, 25 August 2010 (2010-08-25), pages 3970-3978, XP055382098, US ISSN: 0095-1137, DOI: 10.1128/JCM.00612-10 the whole document	1-28
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INTERNATIONAL SEARCH REPORT

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