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(54) Title: AMPLIFYING AND DETECTING COCONUT CADANG-CADANG VIROID RNA

(57) Abstract: Provided herein are methods and compositions for amplifying CCCVd nucleic acids.

Amplifying and Detecting Coconut Cadang-Cadang viroid RNA

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application and 62/258,197 filed November 20, 2015, which is hereby incorporated by reference in its entirety.

BACKGROUND

Viroids are small, single-stranded RNA molecules, that typically range from 246 to 375 nucleotides in lengrt. Viroids do not have protein coats but contain genes for autonomous replication. They have the abilitiy to cause serious disease among plants, but they are more commonly found in a latent stage. Cadang-cadang is a disease caused by Coconut cadang-cadang viroid (CCCVd). Every year more than one million coconut palms are killed by CCCVd and over 30 million coconut palms have been killed since Cadang-cadang has been discovered. Due to the complex secondary structure of the CCCVd genome, detection of CCCVd using nucleic acid amplification assays has proven challenging. Thus, there is a need for improved methods of screening CCCVd.

SUMMARY

Provided herein are compositions and methods for nucleic acid based diagnostic assays for the detection of CCCVd nucleic acids. For example, disclosed herein are compositions (*e.g.*, primers, probes, kits, reaction solutions), and methods for improved CCCVd nucleic acid extraction and handling, hybridization, reverse transcription, amplification (*e.g.*, using symmetric PCR, asymmetric PCR, LATE-PCR, or other amplification methods), and/or detection (*e.g.*, homogenous detection).

In some aspects, provided herein are methods of amplifying and/or detecting CCCVd nucleic acids (*e.g.,* CCCVd RNA). In some embodiments, the amplification methods disclosed herein comprise forming a reaction mixture. In some embodiments, the reaction mixture comprises a CCCVd nucleic acid target molecule with a CCCVd nucleic acid target sequence. In some embodiments, amplification methods include a pre-incubation step (*e.g.*, forming a reaction mixture and incubating the mixture at one or more temperatures for one or more periods of time) at a point when the reaction mixture does not comprise a nucleic acid polymerase during the pre-incubation step. In some embodiments, during the pre-incubation step, the reaction mixture is incubated (*e.g.*, for at least 5 seconds, 10 seconds, 15 seconds, 20 seconds, 25 seconds or 30 seconds) at a temperature between 50 °C and the melting temperature of the one or more primers (*e.g.,* a primer in Table 1) hybridized to

target sequence (*e.g.*, at a temperature of between 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C or 85 °C, and the melting temperature of the one or more primers hybridized to target sequence). In some embodiments, during the pre-incubation step the reaction mixture is incubated (*e.g.*, for at least 5 seconds, 10 seconds, 15 seconds, 20 seconds, 25 seconds or 30 seconds) at two or more temperatures between 50 °C and the melting temperature of the one or more primers hybridized to target sequence (*e.g.*, at two or more temperatures of between 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C or 85 °C, and the melting temperature of the one or more primers hybridized to target sequence). In some embodiments, the pre-incubation step is performed under conditions such that the one or more primers that hybridize to the CCCvd nucleic acid target sequence in the absence of a nucleic acid polymerase. In some embodiments, the pre-incubation step is followed by the lowering of the temperature of the reaction mixture (*e.g.,* to a temperature of no more than 30 °C, 25 °C or 20 °C). In some embodiments, the methods further comprise adding one or more nucleic acid polymerases (*e.g.*, a reverse transcriptase, a DNA polymerase and/or a RNA polymerase) to the reaction mixture following the lowering of the temperature. In some embodiments, the reaction mixture is then incubated under conditions such that the one or more nucleic acid primers is extended by the one or more nucleic acid polymerases (*e.g.*, a reverse transcriptase, an RNA polymerase and/or a DNA polymerase) to create an amplification product comprising the CCCVd nucleic acid target sequence or a complement thereof.

In some aspects, provided herein are methods for amplifying CCCVd nucleic acids using an extensible nucleic acid primer (*e.g.*, a primer comprising a random sequence of nucleotides or a sequence specific primer) and a non-extensible oligonucleotides (*e.g.,* a non-extensible oligonucleotide in Table 3) that hybridize to regions of a CCCVd nucleic acid target sequence and in which the non-extensible oligonucleotide hybridized to the CCCVd nucleic acidtarget sequence has a predicted melting temperature that is higher (*e.g.*, at least 5 °C higher, at least 10 °C higher, at least 15 °C higher) than the predicted melting temperature of the extensible nucleic acid primer hybridized to the CCCVd nucleic acid target sequence. In some embodiments, the method includes the step of forming a reaction mixture comprising the non-extensible oligonucleotide, the extensible nucleic acid primer (*e.g.,* a primer in Table 1) and a CCCVd nucleic acid molecule comprising the CCCVd nucleic acid target sequence. In some embodiments, the non-extensible oligonucleotide has a predicted melting temperature of at least 85 °C, at least 90 °C, or at least 95 °C. In some embodiments, the non-extensible oligonucleotide comprises one or more chemical modifications (*e.g.*, a 2'-

O-methyl nucleoside). In some embodiments, the reaction mixture is incubated at one or more temperatures (*e.g.*, at a temperature of at least 65 °C, 70 °C, 75 °C, 80 °C or 85 °C) for a period of time (*e.g.*, at least about 1, 2, 3, 4, 5, 10, 15, 20, 25 or 30 minutes) sufficient to hybridize the non-extensible oligonucleotide and the extensible nucleic acid primer to the CCCVd nucleic acid target sequence. In some embodiments, the method further comprises adding one or more nucleic acid polymerases (*e.g.*, a reverse transcriptase, an RNA polymerase and/or a DNA polymerase) to the reaction mixture. In some embodiments, reaction mixture is incubated under conditions such that the extensible nucleic acid primer is extended by the nucleic acid polymerase to create an amplification product comprising the CCCVd target nucleic acid sequence or a complement thereof. Exemplary primers and probes for use with the methods disclosed herein can be found in Table 1.

Table 1: Exemplary CCCVd-Specific Primer and Probe Sequences

SEQ ID NO:	Sequence (5′ to 3′)
1	TTCTGCAGTAGTTTTTGGGGTGCCCTGTAGAT
2	TTGGCCTCTCCTGCAGTAGTTTTTGGGGTGCCCTGTAGAT
3	TTGGGAGACTACCCGGTGGATACA
4	TTCGCACGATCGACCCAGGTACGCT
5	TCTTTGTAGCCTCTCTGGGGAAATCTACAG
6	TTGGTAGTACGAAGAA
7	ATGTAAGAGCCGCGTGAGAT
8	AAGCCTCTCCTGCAGTAGTTTTTGTT

In certain aspects, the methods provided herein relate to amplifying CCCVd nucleic acids using primers having high melting temperatures when hybridized to a CCCVd nucleic acid target. In certain embodiments, the method comprises forming a reaction mixture comprising a CCCVd nucleic acid target molecule comprising a CCCVd nucleic acid target sequence, one or more primers that hybridize to the CCCVd nucleic acid target sequence and that comprise at least one primer that has a predicted melting temperature of at least 85 °C, and one or more nucleic acid polymerases (*e.g.*, a reverse transcriptase, an RNA polymerase and/or a DNA polymerase). In some embodiments, the reaction mixture is incubated under conditions such that the one or more nucleic acid primers are extended by the nucleic acid polymerase to create an amplification product comprising the CCCVd nucleic acid target sequence or a complement thereof.

In some aspects, provided herein are methods of amplifying CCCVd RNA that comprise the step of lysing cells (*e.g.*, plant cells, animal cells, fungus cells, bacterial cells or parasite cells) in a solution comprising a chaotrope, a reducing agent, a detergent, a chelator and a buffer and application of mechanical disruption to form a nucleic acid solution comprising a CCCVd nucleic acid target molecule. In some embodiments, the nucleic acid solution is diluted without performing a nucleic acid purification step to form a reaction mixture comprising the CCCVd nucleic acid target molecule, one or more primers that hybridize to the CCCVd nucleic acid target molecule and one or more nucleic acid polymerases (*e.g.*, a reverse transcriptase, an RNA polymerase and/or a DNA polymerase). In some embodiments, the nucleic acid solution comprising the CCCVd nucleic acid target molecule is diluted by an amount sufficient to reduce the concentration of the chaotrope, the reducing agent, the detergent, the chelator and the buffer to a level whereby the nucleic acid polymerase has at least 20% of the activity it has in a reaction mixture that does not comprise the chaotrope, the reducing agent, the detergent, the chelator and the buffer. In some embodiments, the nucleic acid solution is diluted by at least 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 40-fold, 45-fold or 50-fold. In some embodiments, the dilution is accomplished in one step. In some embodiments, the dilution is accomplished in two or more steps. In some embodiments, the first dilution step is carried out in a buffer that contains at least one DNA oligonucleotide primer and wherein said first-dilution step is followed by heating to at least 85 °C followed by gradual cooling, prior to a second dilution step. In some embodiments, the method comprises incubating the reaction mixture under conditions such that the one or more nucleic acid primers are extended by the nucleic acid polymerase to create an amplification product comprising the CCCVd nucleic acid target sequence or a complement thereof.

In certain embodiments of the methods provided herein, a CCCVd nucleic acid target molecule is isolated from a sample and prepared in a solution comprising a chaotrope, a reducing agent, a detergent, a chelator and a buffer. In some embodiments, the reducing agent is 2 mercaptoethanol, tris(2-carboxyethyl)phosphine, dithiothreitol, dimethylsulfoxide, or any combination thereof. In some embodiments, the chaotrope is guanidine thiocyanate, guanidine isocyanate, guanidine hydrochloride, or any combination thereof. In some embodiments, the detergent is sodium dodecyl sulfate, lithium dodecyl sulfate, sodium taurodeoxycholate, sodium taurocholate, sodium glycocholate, sodium deoxycholate, sodium cholate, sodium alkylbenzene sulfonate, N-lauroyl sarcosine, or any combination thereof. In

some embodiments, the chelator is ethylene glycol tetraacetic acid, hydroxyethylethylenediaminetriacetic acid, diethylene triamine pentaacetic acid, N,N-bis(carboxymethyl)glycine, ethylenediaminetetraacetic, citrate anhydrous, sodium citrate, calcium citrate, ammonium citrate, ammonium bicitrate, citric acid, diammonium citrate, ferric ammonium citrate, lithium citrate, or any combination thereof. In some embodiments, the buffer is tris(hydroxymethyl)aminomethane, citrate, 2-(N-morpholino)ethanesulfonic acid, N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 1,3-bis(tris(hydroxymethyl)methyl amino)propane, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 3-(N-morpholino) propanesulfonic acid, bicarbonate, phosphate, or any combination thereof.

In some embodiments, the methods provided herein comprise the addition of at least one mispriming prevention reagent to a reaction mixture.

In some embodiments, the at least one mispriming prevention reagents comprises a mispriming prevention reagent that comprises a nucleic acid molecule comprising, in 5′ to 3′ order: (i) a first condition-dependent stem region comprising a 5′ terminal covalently linked moiety and a first stem nucleic acid sequence, wherein the first stem nucleic acid sequence is at least 6 nucleotides in length and wherein the 5′ terminal covalently linked moiety comprises a cyclic or polycyclic planar moiety that does not have a bulky portion; (ii) a condition-dependent loop region comprising a loop nucleic acid sequence of at least 3 nucleotides in length; and (iii) a second condition-dependent stem region comprising a second stem nucleic acid sequence and a 3′ terminal covalently linked moiety, wherein the second stem nucleic acid sequence is at least 6 nucleotides in length and is complementary to the first stem nucleic acid sequence and wherein the 3′ terminal covalently linked moiety comprises a cyclic or polycyclic planar moiety that does not have a bulky portion, and wherein the 3′ terminus of the second stem region is non-extensible by a DNA polymerase, wherein the first condition-dependent stem region hybridizes to the second condition-dependent stem region in a temperature dependent manner to acquire a stem-loop hairpin conformation. In some embodiments, the 3′ terminal covalently linked moiety is non-identical to the 5′ terminal covalently linked moiety. In some embodiments, the loop nucleic acid sequence is a single nucleotide repeat sequence. In some embodiments, the single nucleotide repeat sequence is a poly-cytosine sequence. In some embodiments, the loop nucleic acid sequence is between 25 and 40 nucleotides in length. In some embodiments, the first condition-dependent stem region hybridizes to the second condition-dependent stem

region with a melting temperature of between 40 °C and 71 °C. In some embodiments, the first stem nucleic acid sequence and the second stem nucleic acid sequence are each 11 nucleotides in length.

In some embodiments, the at least one mispriming prevention reagents comprises a mispriming prevention reagent that comprises an oligonucleotide that has a 3' end and a stem-loop structure having a stem comprising a double-stranded region that has a length greater than six nucleotides and a terminus away from the loop comprising a 3' nucleotide and a 5' nucleotide, the stem having a calculated stem melting temperature (Tm) below 94° C, wherein (a) the 3' end is non-extensible by the DNA polymerase, (b) the oligonucleotide is not fluorescently labeled and does not contribute background fluorescence, and (c) the stem terminus is stabilized by means selected from the group consisting of non-fluorescent fluorophore- quenching moieties covalently attached to the 3' and 5' nucleotides of the stem terminus and pairs of non-natural nucleotides that bind more strongly than a natural DNA-DNA hybrid and that include each of the 3' and 5' nucleotides of the stem terminus.

In certain embodiments, provided herein are kits for the performance of the methods described herein and reaction solutions, primers and probes used in the methods provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows real-time amplification and melting analysis comparing the results of reverse transcription and amplification of a viroid synthetic RNA using primers with different melting temperatures. RT-LATE-PCR amplification was performed using low and high Tm antisense primers. Part A shows SYBR Green detection of double-stranded DNA during real-time amplification using an antisense primer OPV9-A32 for two-step RT-LATE-PCR. Inset shows the corresponding SYBR Green melt curve derivative. The melt peak at 85 °C corresponds to the viroid-specific amplification product. Peaks at lower temperatures correspond to non-specific amplification products (*e.g.*, primer dimers). Part B shows SYBR Green detection during amplification and melting (inset) in samples using antisense primer OPV9-A40 for two-step RT-LATE-PCR. Part C shows Cal Red fluorescence during the melt of the viroid-specific probe from the products shown in A. Inset shows the melt curve derivative with the peak at 62°C corresponding to the melting temperature of the probe. Part D shows Cal Red fluorescence during the melt of the viroid-specific probe from the products shown in B. Key: solid black line, 100 copies of synthetic viroid RNA; solid grey line, 1,000

copies of synthetic viroid RNA; dashed grey line, 1,000 copies of RNA but no Reverse Transcriptase; dashed black line, NTC.

Figure 2 shows real-time SYBR Green detection and specific probe melting analysis following pre-annealing, reverse transcription and amplification across the trans-spliced junction of nad5 transcripts from purified coconut palm RNA. Part A shows real-time SYBR Green fluorescence plots during one-step RT-PCR of *nad5* from different inputs of total coconut palm RNA. Part B shows *nad5*-specific probe fluorescence derivative from the post-PCR melt identifying the gene-specific product by the peak at 50 °C.

Figure 3 shows real-time SYBR Green detection and specific probe melting analysis following pre-annealing, reverse transcription and amplification across the trans-spliced junction of nad5 transcripts from purified coconut palm RNA and from coconut palm total nucleic acids extracted using PrimeStore™. RT-LATE-PCR amplification of coconut palm nad5 RNA in PrimeStore™ extracts. Part A shows Real-time SYBR Green fluorescence increase in PrimeStore™ extracts and purified RNA samples during RT-LATE-PCR without added PVP. Part B shows real-time SYBR Green fluorescence increase in PrimeStore™ extracts and purified RNA samples during RT-LATE-PCR with 1.5% PVP. Part C shows probe fluorescence derivative plots from post-PCR melting analysis of samples in Part A. Part D shows probe fluorescence derivative plots from post-PCR melting analysis of samples in Part B.

Figure 4 shows melting analysis using specific probes following pre-annealing, reverse transcription and co-amplification of the nad5 transcript and viroid synthetic RNA from coconut palm total nucleic acids extracted using PrimeStore™ containing different numbers of added viroid RNA. Combined probe fluorescence derivative plot from post-RT-LATE-PCR melting of co-amplified nad5 and viroid. All samples (except NTC controls) contain 100-10000 copies of synthetic viroid mixed with 50 ng of *cocos nucifera* total RNA.

Figure 5 shows a standard curve based on the results of pre-annealing, reverse transcription and co-amplification of nad5 and viroid RNAs. A standard curve for determining the concentration of viroid RNA in plant samples was generated using the ratio of the viroid probe fluorescent derivative peak height (at 65 °C) to the nad5 probe fluorescent derivative peak height (at 50 °C).

Figure 6 shows melting analysis with specific probes following pre-annealing at different temperatures prior to reverse transcription and co-amplification of nad5 and viroid from PrimeStore™ extracts. Probe fluorescence derivative plots from post RT-LATE-PCR

of nad5 and viroid sequences following pre-incubation steps (Part A) on ice, (Part B) at 65 °C, or (Part C) at 85 °C. Purified coconut palm RNA (50 ng RNA) was tested in the absence of synthetic viroid RNA. A 1/100 dilution of PrimeStore™ extract from coconut palm was mixed with 1,000 or 10,000 copies of synthetic viroid RNA. The nad5-probe melt peak at 50 °C is not significantly affected by the pre-incubation condition. Detection of the viroid probe melt peak improves with increasing pre-incubation temperature.

Figure 7 shows melting analysis with a viroid-specific probe following pre-annealing of purified oil palm RNA with a non- extensible opener, reverse transcription with random hexamers, and amplification. Results confirm that the oil palm was infected with Coconut Cadang Cadang Viroid (CCCVd). Probe fluorescence derivative plots from melting analysis following 2 step RT-LATE-PCR of viroid sequences using RT with a high-Tm 2'-O-methyl RNA opener and random hexamers. Four replicate samples with CCCVd-infected oil palm RNA had a melt peak at about 57 °C, the characteristic T_m of the probe used in this example with the amplified viroid sequence. One of four no RT samples also had a melt peak at that temperature. No probe melt peak was present in the NTC samples.

Figure 8 shows mean C_T values from real-time SYBR Green fluorescence increase as a function of starting RNA concentration following a pre-incubation of primers and RNA (closed circles) or no pre-incubation (open circles) prior to RT-PCR. Pre-incubation lowers the mean C_T value by more than 4 cycles at each step, reflecting much higher levels of cDNA that are generated during reverse transcription following that step.

Figure 9 shows melt derivative peaks for measuring relative CCCVd amplification in samples with different initial concentrations of the RNA target. Mean peak heights are compared for each RNA dilution in samples with ThermaStop-RT compared to samples without ThermaStop-RT.

DEFINITIONS

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

The articles "*a*" and "*an*" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

As used herein, the term "*amplicon*" refers to a nucleic acid generated using primer pairs in an amplification reaction (*e.g.*, PCR), such as those described herein. The amplicon

is typically single-stranded DNA (*e.g.*, the result of asymmetric amplification), however, it may be RNA or dsDNA.

The term "*amplifying*" or "*amplification*" in the context of nucleic acids refers to the production of multiple copies of a polynucleotide, or a portion of the polynucleotide, typically starting from a small amount of the polynucleotide (*e.g.*, a single polynucleotide molecule), where the amplification products or amplicons are generally detectable. Amplification of polynucleotides encompasses a variety of chemical and enzymatic processes. The generation of multiple DNA copies from one or a few copies of a target or template DNA molecule during a polymerase chain reaction (PCR) or a ligase chain reaction (LCR) are forms of amplification. In certain embodiments, the type of amplification is asymmetric PCR (*e.g.*, LATE-PCR) which is described in, for example, U.S. Pat. 7,198,897 and Sanchez et al., Proc. Natl. Acad. Sci. (USA), 101(7):1933-1938 and Pierce et al., Proc. Natl. Acad. Sci. (USA), 2005, 102(24):8609-8614, all of which are herein incorporated by reference in their entireties. In particular embodiments, LATE-PCR is employed using multiple end-point temperature detection (see, *e.g.*, U.S. Pat. Pub. 2006/0177841 and Sanchez et al., BMC Biotechnology, 2006, 6:44, pages 1-14, both of which are herein incorporated by reference).

As used herein, the terms "*complementary*" or "*complementarity*" are used in reference to polynucleotides (*i.e.*, a sequence of nucleotides) related by the base-pairing rules. For example, the sequence "5'-A-G-T-3'," is complementary to the sequence "3'-T-C-A-5'." Complementarity may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the rate, melting temperature, and stability of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods that depend upon binding between nucleic acids.

The terms *"hot-start"* and *"cold-stop"* describe the state of a reaction in which the DNA synthetic activity (as distinct from the exonuclease activity) of a DNA polymerase used in an amplification reaction is inhibited by an interaction with a temperature-dependent reagent, antibody and/or alkylating agent or some other means. Hot-start refers to activation of the polymerase by raising the temperature of the reaction above the annealing temperature for first time and holding the high temperature long enough to render the polymerase capable of DNA synthesis. Certain polymerase inhibitor reagents (*e.g.*, certain reagents described

herein) are able to be reactivated once the temperature of the reaction is reduced below the annealing temperature. Such reagents are referred to as "*cold-stop*" reagents.

As used herein, the term "*gene*" refers to a nucleic acid (*e.g.*, DNA) sequence that comprises coding sequences necessary for the production of a polypeptide or precursor. The term "gene" encompasses both cDNA and genomic forms of a gene.

The terms "*homology*," "*homologous*" and "*sequence identity*" refer to a degree of identity. There may be partial homology or complete homology. A partially homologous sequence is one that is less than 100% identical to another sequence. Determination of sequence identity is described in the following example: a primer 20 nucleobases in length which is otherwise identical to another 20 nucleobase primer but having two non-identical residues has 18 of 20 identical residues (18/20 = 0.9 or 90% sequence identity). In another example, a primer 15 nucleobases in length having all residues identical to a 15 nucleobase segment of a primer 20 nucleobases in length would have 15/20 = 0.75 or 75% sequence identity with the 20 nucleobase primer. Sequence identity may also encompass alternate or "modified" nucleobases that perform in a functionally similar manner to the regular nucleobases adenine, thymine, guanine and cytosine with respect to hybridization and primer extension in amplification reactions. In a non-limiting example, if the 5-propynyl pyrimidines propyne C and/or propyne T replace one or more C or T residues in one primer which is otherwise identical to another primer in sequence and length, the two primers will have 100% sequence identity with each other. In another non-limiting example, Inosine (I) may be used as a replacement for G or T and effectively hybridize to C, A or U (uracil). Thus, if inosine replaces one or more G or T residues in one primer which is otherwise identical to another primer in sequence and length, the two primers are considered to have 100% sequence identity with each other, in order to distinguish this type of hybridization from a destabilizing mismatch. Other such modified or universal bases may exist which would perform in a functionally similar manner for hybridization and amplification reactions and will be understood to fall within this definition of sequence identity.

As used herein, the term "*hybridization*" or "*hybridize*" is used in reference to the pairing of complementary nucleic acids. The thermodynamic stability of hybridization between two nucleic acid sequences is influenced by such factors as the degree of complementary between the nucleic acids, the temperature and salt concentrations of the solution, and the G:C ratio within the nucleic acids. The melting temperature of the hybrid is determined in part by that stability. A single molecule that contains pairing of

complementary nucleic acids within its full length is said to be "self-hybridized." An extensive guide to nucleic hybridization may be found in Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes, part I, chapter 2, "Overview of principles of hybridization and the strategy of nucleic acid probe assays," Elsevier (1993), which is incorporated by reference. As used herein, the phrase "hybridization sequence" is used is reference to a particular target sequence and a particular probe or primer, and it is the sequence in the target sequence that hybridizes to the particular probe or primer. The probe or primer may be fully or partially complementary to the target sequence over the length of the hybridization sequence.

As used herein, the term "*kit*" refers to any delivery system for delivering materials. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (*e.g.*, oligonucleotides, enzymes, etc. in the appropriate containers) and/or supporting materials (*e.g.*, buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (*e.g.*, boxes) containing the relevant reaction reagents and/or supporting materials. As used herein, the term "fragmented kit" refers to a delivery system comprising two or more separate containers that each contains a sub-portion of the total kit components. The term "kit" includes both fragmented and combined kits.

As used herein, the term "*Linear-After-The Exponential PCR*" or "*LATE-PCR*" refers to a non-symmetric PCR method that utilizes unequal concentrations of primers and yields single-stranded primer-extension products (referred to herein as amplification products or amplicons). LATE-PCR is described, for example, in U.S. Pat. No. 7,198,897 and 8,367,325, each of which is incorporated by reference in its entirety.

As used herein, the term "*nucleic acid molecule*" refers to any nucleic acid containing molecule, including but not limited to, DNA or RNA. The term encompasses sequences that include any of the known base analogs of DNA and RNA including, but not limited to, 4 acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxyl-methyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethyl-aminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudo-uracil, 1-methylguanine, 1-methylinosine, 2,2-dimethyl-guanine, 2-methyladenine, 2-methylguanine, 3-methyl-cytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxy-amino-methyl-2-thiouracil, beta-D-mannosylqueosine,

5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N- isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

As used herein, the term "*nucleobase*" is synonymous with other terms in use in the art including "nucleotide," "deoxynucleotide," "nucleotide residue," "deoxynucleotide residue," "nucleotide triphosphate (NTP)," or deoxynucleotide triphosphate (dNTP). As is used herein, a nucleobase includes natural and modified residues, as described herein.

An "*oligonucleotide*" refers to a nucleic acid that includes at least two nucleic acid monomer units (*e.g.*, nucleotides), typically more than three monomer units, and more typically greater than ten monomer units. The exact size of an oligonucleotide generally depends on various factors, including the ultimate function or use of the oligonucleotide. To further illustrate, oligonucleotides are typically less than 200 residues long (*e.g.*, between 15 and 100), however, as used herein, the term is also intended to encompass longer polynucleotide chains. Oligonucleotides are often referred to by their length. For example a 24 residue oligonucleotide is referred to as a "24-mer". Typically, the nucleoside monomers are linked by phosphodiester bonds or analogs thereof, including phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like, including associated counterions, *e.g.*, H^+, NH_4^+, Na^+, and the like, if such counterions are present. Further, oligonucleotides are typically single-stranded. Oligonucleotides are optionally prepared by any suitable method, including, but not limited to, isolation of an existing or natural sequence, DNA replication or amplification, reverse transcription, cloning and restriction digestion of appropriate sequences, or direct chemical synthesis by a method such as the phosphotriester method of Narang *et al.* (1979) *Meth Enzymol.* 68: 90-99; the phosphodiester method of Brown *et al.* (1979) *Meth Enzymol.* 68: 109-151; the diethylphosphoramidite method of Beaucage *et al.* (1981) *Tetrahedron Lett.* 22: 1859-1862; the triester method of Matteucci *et al.* (1981) *J Am Chem Soc.* 103:3185-3191; automated synthesis methods; or the solid support method of U.S. Pat. No. 4,458,066, entitled "PROCESS FOR PREPARING POLYNUCLEOTIDES," issued Jul. 3, 1984 to Caruthers *et al.*, or other methods known to those skilled in the art. All of these references are incorporated by reference.

The terms "*polynucleotide*" and "*nucleic acid*" are used herein interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, synthetic polynucleotides, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified, such as by conjugation with a labeling component.

As used herein, the term "*primer*" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, that is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product that is complementary to a nucleic acid strand is induced (*e.g.*, in the presence of nucleotides and an Inducing Agent such as a biocatalyst (*e.g.*, a DNA polymerase or the like) and at a suitable temperature and pH). The primer is typically single-stranded for maximum efficiency during amplification, but alternatively may be double-stranded at a particular temperature and condition. If double-stranded, the primer is generally first treated to separate its strands before being used to initiate extension for the generation of amplification products. In some embodiments, the primer is an oligodeoxyribonucleotide. The primer is sufficiently long to prime the synthesis of extension products in the presence of the Inducing Agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method. In certain embodiments, the primer is a capture primer.

As used herein, the term "*primer annealing temperature*" refers to the temperature used for primer binding during the majority of the thermal cycles in a PCR amplification reaction. This definition recognizes the possibility that the annealing temperature during certain thermal cycles, either at the beginning, soon after the beginning, during, or near the

end of an amplification reaction can be deliberately chosen to be above, or below, the annealing temperature chosen for the majority of thermal cycles.

The term "probe" as used herein refers to a material that may (i) provide a detectable signal, (ii) interact a first probe or a second probe to modify a detectable signal provided by the first or second probe, such as fluorescence resonance energy transfer (FRET).

As used herein, the term "*target specific*," when used in reference to an oligonucleotide reagent, as in, for example "*target-specific probe*" or "*target-specific primer*," refers to reagents designed and produced for hybridization to a specific target sequence (*e.g.*, for detection, characterization, or amplification of the target sequence). A target-specific reagent may be allele discriminating or mismatch tolerant. As used herein, the term "*reaction mix*" or "*reaction mixture*" refers to a combination of reagents (*e.g.*, nucleic acids, nucleic acid target molecules, mispriming prevention reagents ,nucleic acid polymerases, enzymes, fluorophores, buffers, salts, etc.) in solution in a single vessel (*e.g.*, microcentrifuge tube, PCR tube, well, microchannel, etc.).

As used herein a "*sample*" refers to anything capable of being analyzed by the methods provided herein. In some embodiments, the sample comprises or is suspected to comprise one or more nucleic acids capable of analysis by the methods. In some embodiments, the samples comprise nucleic acids (*e.g.*, DNA, RNA, cDNAs, etc.) from one or more pathogens or bioagents. Samples can include, for example, blood, saliva, urine, feces, anorectal swabs, vaginal swabs, cervical swabs, and the like. In some embodiments, the samples are "mixture" samples, which comprise nucleic acids from more than one subject or individual. In some embodiments, the methods provided herein comprise purifying the sample or purifying the nucleic acid(s) from the sample. In some embodiments, the sample is purified nucleic acid. In some embodiments, the sample comprises two or more strains or subtypes of the same microorganism.

"T_m," or "*melting temperature*," of an oligonucleotide describes the temperature (in degrees Celsius) at which 50% of the molecules in a population of a single-stranded oligonucleotide are hybridized to their complementary sequence and 50% of the molecules in the population are not-hybridized to said complementary sequence. The T_m of a primer or probe can be determined empirically by means of a melting curve. In some cases it can also be calculated. For the design of symmetric PCR primer pairs, balanced T_m's are generally calculated by one of the three methods discussed earlier, that is, the "% GC", or the "2(A+T) plus 4 (G+C)", or "Nearest Neighbor" formula at some chosen set of conditions of

monovalent salt concentration and primer concentration. The use of Nearest Neighbor calculations the T_m's of both primers is more accurate, and is particularly important in the case of asymmetric PCR, as T_m's depend on the concentrations chosen for use in calculation or measurement. The following equation is an example of a Nearest Neighbor formula, $T_m=\Delta H/(\Delta S+R \ln(C/2))-273.15+12 \log [M]$. This formula is based on the published formula (Le Novere, N. (2001), "MELTING, Computing the Melting Temperature of Nucleic Acid Duplex," Bioinformatics 17: 1226 7). ΔH is the enthalpy and ΔS is the entropy (both ΔH and ΔS calculations are based on Allawi and SantaLucia, 1997), C is the concentration of the oligonucleotide (10^{-6}M), R is the universal gas constant, and [M] is the molar concentration of monovalent cations (*e.g.*, 0.05). According to this formula the nucleotide base composition of the oligonucleotide (contained in the terms ΔH and ΔS), the monovalent salt concentration, and the concentration of the oligonucleotide (contained in the term C) influence the T_m. In general, for oligonucleotides of the same length, the T_m increases as the percentage of guanine and cytosine bases of the oligonucleotide increases but the T_m decreases as the concentration of the oligonucleotides decrease. The concentration of divalent cations such as magnesium, which are present in most amplification reactions, have a strong effect on T_m, but are typically not included in most of the commonly used formulas, including the nearest neighbor equation above. Even so, the equation is useful for estimating relative T_m's of different primers. In preferred embodiments, T_m is calculated using formulas that include factors for the effect of magnesium. The T_m values presented in this application were obtained using Visual OMP computer software (DNA Software),which utilizes a Nearest Neighbor formula plus proprietary factors for estimating the effects of magnesium and particular nucleotide mismatches. In the case of a primer with nucleotides other than A, T, C and G or with covalent modification, T_m is measured empirically by hybridization melting analysis as known in the art. The T_m depends on the concentration of both strands. However, when one strand is in much higher concentration than the other, as is typically the case for PCR, the T_m depends primarily on the concentration of the most abundant molecule (*i.e.*, the primer). The initial hybridization of the primer may be to a target oligonucleotide that is only partially complementary. In that case, the T_m will be lower than that to a fully complementary target. Targets with partial complementary to the primer (*i.e.*, the presence of mismatched nucleotide pairs in the hybrid) will often still hybridize and be extended by polymerases. Once an amplicon is generated by the extension of a pair of primers with standard nucleotides, the amplicon contains sequences that are fully complementary to the

primers and the T_m may increase. When designing primers, it is important to consider such changes. The T_m for a primer a partially complementary target (including multiple mismatches) can be calculated using Visual OMP. Tm can be determined empirically or calculated as described in Santa Lucia, J. PNAS (USA) 95:1460-1465 (1998), which is hereby incorporated by reference.

T_m^A means the melting temperature of an amplicon, either a double-stranded amplicon or a single-stranded amplicon hybridized to its complement. The melting point of an amplicon, or T_m^A can be calculated by the following % GC formula: T_m^A=81.5+0.41(% G+% C)-500/L+16.6 log [M]/(1+0.7 [M]), where L is the length in nucleotides and [M] is the molar concentration of monovalent cations. In some embodiments, T_m^A is calculated using Visual OMP, which utilizes a factor for magnesium concentration not included in the %GC formula. T_m^A can also be determined empirically following amplification using a double-stranded DNA-binding dye such as SYBR Green in combination with melting analysis as is well known by those skilled in the art.

T_m^P refers to the concentration-adjusted melting temperature of the probe to its target, or the portion of probe that actually is complementary to the target sequence (*e.g.*, the loop sequence of a molecular beacon probe). In the case of most linear probes, T_m^P is calculated using the Nearest Neighbor formula given above or using Visual OMP, as for primer T_m, or preferably is measured empirically. In the case of molecular beacons, a rough estimate of T_m^P can be calculated using commercially available computer programs that utilize the % GC method, see Marras, S.A. et al. (1999) "Multiplex Detection of Single-Nucleotide Variations Using Molecular Beacons," Genet. Anal. 14:151 156, or using the Nearest Neighbor formula, or preferably is measured empirically. In the case of probes having non-conventional bases and for double-stranded probes, T_m^P is determined empirically.

C_T means threshold cycle and signifies the cycle of a real-time PCR amplification assay in which signal from a reporter indicative of amplicons generation first becomes detectable above background. Because empirically measured background levels can be slightly variable, it is standard practice to measure the C_T at the point in the reaction when the signal reaches 10 standard deviations above the background level averaged over the 5-10 thermal cycles preceding fluorescence increase. Software provided with many thermal cyclers uses default parameters for determining C_T values.

DETAILED DESCRIPTION

General

Intramolecular base pairing of single-stranded CCCVd nucleic acids result in a secondary structure that may slow or prevent the hybridization of primers and probes to the CCCVd target. Increasing temperature, decreasing salt concentrations, or including additives such as DMSO can decrease the stability of intramolecular base pairing and thereby reduce secondary structure, but these changes also decrease the stability and/or melting temperature of the primer to target or probe to target hybrids. Also, any method used to increase the hybridization of primers to their targets must also be compatible with reverse transcription if the target is RNA, and with amplification methods such as symmetric PCR, asymmetric PCR, or LATE-PCR whether the target is RNA or DNA. In most cases this limits the range of salt concentrations, incubation temperatures, and primer T_m's. In the case of reverse transcription of RNA, reverse transcriptases are typically used at temperatures from 30 °C to 55 °C. At those temperatures, CCCVd RNAs will have extensive secondary structure. Using temperatures in the 60 °C to 70 °C range is possible with some engineered enzymes, but often still results in very short half-life of enzyme activity, or requires increasing the concentration of additives such as DTT, which can interfere with DNA polymerase activity or with fluorescent probe detection.

Widely used protocols, including those included for the use of commercial products describe heating RNA solutions to about 65 °C to 70 °C for up to 5 minutes, then rapid cooling prior to the addition of reverse transcription reagents. While this may reduce some base pairing between different RNA molecules, it is unlikely to cause a long-term change in the intramolecular base pairing which can rapidly reform as the temperature is lowered – much more rapidly than a reaction involving two separate molecules.

In some aspects, provided herein are methods of amplifying and or detecting CCCVd nucleic acid. In some embodiments, amplification methods include a forming a reaction mixture in the absence of a nucleic acid polymerase and incubating the mixture at one or more temperatures disclosed herein for one or more (*e.g.*, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten or more) periods of time. To avoid the inefficiencies of primer hybridization to the primer that hybridizes with the RNA (usually referred to as the antisense primer) methods are described herein that include a pre-RT incubation step, or just "pre-incubation" that includes the antisense primer and the RNA and at temperatures of 50 °C or higher (*e.g.*, at 50 °C or

higher, 55 °C or higher, 60 °C or higher, 65 °C or higher, 70 °C or higher, 80 °C or higher , or 85 °C or higher) with salt concentrations and melting temperatures that enable hybridization during that step. The pre-incubation can be done prior to either one-step (both reactions done without processing between them), or two-step RT-PCR. The amplification can be symmetric PCR , asymmetric PCR, LATE-PCR, or other amplification methods. Methods have been previously described for pre-annealing of primers to RNA targets with low- to medium-levels of secondary structure, but where the salt concentrations were low and hybridization takes place at room temperature (Pierce, K.E. et al, 2010. Design and optimization of a novel reverse transcription linear-after-the-exponential PCR for the detection of foot-and-mouth disease virus. J Appl Microbiol 109: 180-9; Pierce, K.E. and Wangh, L.W., 2013. Rapid detection and identification of hepatitis C virus (HCV) sequences using mismatch-tolerant hybridization probes: A general method for analysis of sequence variation. Biotechniques 55: 125-32).

CCCVd Nucleic Acid Amplification

In certain aspects, provided herein are methods of amplifying CCCVd nucleic acids by forming a reaction mixture in without a nucleic acid polymerase, and pre-incubating the reaction mixture at one or more temperatures for one or more (*e.g.*, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten or more) periods of time. In some embodiments, a nucleic acids polymerase (*e.g.*, reverse transcriptase or DNA polymerase) is added to the reaction mixture after the pre-incubation step. In some embodiments, the reaction mixture includes a CCCVd nucleic acid target molecule with a nucleic acid target sequence (*i.e.,* a CCCVd RNA sequence) and one or more primers that hybridize to the CCCVd nucleic acid target sequence. In some embodiments, the reaction mixture is incubated at a temperature between 50 °C and the melting temperature of the one or more primers hybridized to CCCVd target sequence. The reaction mixture may be incubated at a temperature of at least 50°C, at least 51°C, at least 52°C, at least 53°C, at least 54°C, at least 55°C, at least 56°C, at least 57°C, at least 58 °C, at least 59°C, at least 60 °C, at least 61°C, at least 62°C, at least 63°C, at least 64°C, at least 65°C, at least 66°C, at least 67°C, at least 68°C, at least 69 °C, at least 70°C, at least 71°C, at least 72°C, at least 73°C, at least 74°C, at least 75°C, at least 76°C, at least 77°C, at least 78°C, at least 79°C, at least 80°C, at least 81°C, at least 82 °C, at least 83°C, at least 84°C, at least 85°C, at least 86°C, at least 87°C, at least 88°C, at least 89°C, at least 90°C, at least 91°C, at least 92°C, at least 93°C, at least 94°C, at least 95°C, at least 96°C, at least 97°C, at

least 98°C, at least 99°C, or at least 100°C. In some embodiments, the reaction mixture may be incubated at two or more temperatures for one or more (*e.g.*, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten or more) periods of time. The period of time may be at least 1 second, at least 2 seconds, at least 3 seconds, at least 4 seconds, at least 5 seconds, at least 6 seconds, at least 7 seconds, at least 8 seconds, at least 9 seconds, at least 10 seconds, at least 15 seconds, at least 20 seconds, at least 25 seconds, at least 30 seconds, at least 45 seconds, at least 60 seconds, at least 5 minutes, at least 10 minutes, at least 15 minutes, at least 20 minutes, at least 30 minutes. In some embodiments, the method further comprises lowering the temperature of the reaction mixture to at a temperature of no more than 15°C, no more than 16°C, no more than 17°C, no more than 18°C, no more than 19°C, no more than 20°C, no more than 21°C, no more than 22°C, no more than 23°C, no more than 24°C, no more than 25°C, no more than 26°C, no more than 27°C, no more than 28°C, no more than 29°C, no more than 30°C, no more than 31°C, no more than 32°C, no more than 33°C, no more than 34°C, or no more than 35°C). The primers may be diluted at least 5 fold, at least 6 fold, at least 7 fold, at least 8 fold, at least 9 fold, at least 10 fold.

In some embodiments, a nucleic acid polymerase is then added to the reaction mixture, followed by incubating the reaction mixture under conditions such that the one or more nucleic acid primers is extended by the nucleic acid polymerase to create an amplification product comprising the CCCVd nucleic acid target sequence or a complement thereof. In some embodiments, more than one nucleic acid polymerase is added to the reaction mixture. The nucleic acid polymerase may be reverse transcriptase, an RNA polymerase, and/or DNA polymerase. In some embodiments, the nucleic acid polymerases are added to the reaction mixture at the same time. In other embodiments, the nucleic acid polymerases are added to the reaction mixture at different times. For example, reverse transcriptase may be added to the reaction mixture, and the reaction mixture may then be incubated a one or more temperatures to allow reverse transcription of a CCCVd RNA target sequence, followed by addition of DNA polymerase to amplify the resulting nucleic acid sequence.

In some embodiments, the primers, when hybridized to a CCCVd target sequence, have a melting temperature of at least 80 °C, at least 81°C, at least 82 °C, at least 83°C, at least 84°C, at least 85°C, at least 86°C, at least 87 °C, at least 88°C, at least 89°C, at least

90°C, at least 91°C, at least 92 °C, at least 93°C, at least 94°C, at least 95°C, at least 96°C, at least 97 °C, at least 98°C, at least 99°C, or at least 100°C.

In some embodiments, a mispriming prevention agent disclosed herein is added to the reaction mixture.

Primers specific for CCCVd nucleic acids (*e.g.*, nucleic acid primers or extensible nucleic acid primers) disclosed herein may comprise a random sequence of nucleotides or primers may be sequence specific primers. In some embodiments, the melting temperature of the primer to the RNA or DNA target is equal to or higher than the pre-incubation temperature. In some embodiments, the RNA or DNA target has predicted intramolecular hybridization at the pre-incubation temperature that includes nucleotides targeted by the primer. In some embodiments, the T_m of primer to target is at least 5 degrees higher, at least 6 degrees higher, at least 7 degrees higher, at least 8 degrees higher, at least 9 degrees higher, at least 10 degrees higher, at least 11 degrees higher, at least 12 degrees higher, at least 13 degrees higher than the pre-incubation temperature. In other embodiments, the T_m of the primer to target during pre-incubation is at least 75 °C. In other embodiments, the incubation temperature is 70 °C or higher. In some embodiments, the target is a CCCVd target sequence. Exemplary primer sequences can be found in Table 1. In the embodiments, RT-PCR is one-step. The pre-incubation step may be brief (*e.g.* at least 5 seconds, at least 10 seconds, or at least 30 seconds) or may be several minutes in duration, and may include multiple steps over a range of temperatures at which the primer(s) should hybridize the RNA or DNA target(s) (*e.g.* between the T_m and 10 degrees below the T_m), or a slow (*e.g.* 1°C per second, 0.1°C per second, 0.01°C per second), continuous change over that range of temperatures. Prior to this pre-incubation step, the method may include a step at a temperature above the T_m of the primer and target hybrid in order to reduce secondary structure of the target. In some RT reactions or amplification reactions (PCR or other methods), primers having very high T_m with the target can be used directly without a pre-incubation step. In such cases, the T_m is often far above the RT incubation temperature or the PCR annealing temperature and care must be taken to avoid non-specific amplification. Therefore, in some embodiments the T_m is over 75 °C and reverse transcription and/or PCR is done in the presence of additives or solutions disclosed herein, and/or a mispriming reagents disclosed herein. Exemplary mispriming prevention reagents are shown in Table 2, The reaction mixtures disclosed herein may also have Single Strand Binding Protein, which increase the specificity of the reactions. In other embodiments, the T_m is over 75 °C, but the

concentration of that primer is 100 nM or lower during RT, or is diluted to that concentration for amplification, resulting in a lower T_m during that step. In some embodiments, primers having a T_m with the target that is above 75 °C at a concentration of at least 500 nM in the pre-incubation or RT reactions and are diluted to a concentration of 100 nM or lower for LATE-PCR amplification.

TABLE 2: ThermaStop

Black Hole Quencher 2 - 5'GAATAATATAGCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCTATATTATTC 3' - Biosearch Blue
ThermaGo Top Strand: Spacer of three carbons - 5' GAGCAGACTCGCACTGAGGTA 3' - Biosearch Blue
ThermaGo Bottom Strand: Black Hole Quencher 2 - 5' TACCTCAGTGCGAGTCTGCTC 3' – Biosearch Blue

The T_m of hairpins formed on a single-stranded RNA or DNA molecule may be very high (*e.g.*, at least 50 °C, at least 55 °C, at least 60 °C, at least 65 °C, at least 70 °C, or at least 80 °C) at typical RT or PCR temperatures or conditions. Base pairing on viroid circular RNA molecules can be even higher melting temperatures (*e.g.*, 85 °C). However, hairpin formation is dynamic and these structures are melting and reforming during the course of incubation. Although an intramolecular T_m above the annealing temperature may slow hybridization due to a smaller fraction of targets that are available at any given time, the primer molecules will hybridize to available targets over time if the pre-incubation temperature reasonably close to that T_m (*e.g.*, within 10 °C, or preferably within 5 °C) and the T_m of the primer – target hybrid is at or above the melting temperature, preferably at least 5 °C above that temperature. Since the thermodynamic stability is in generally higher for that intermolecular hybrid compared to the alternative intramolecular hybrid, a large percentage of the primer will be hybridized to the target if the reaction is allowed to reach equilibrium, but the time necessary to reach that point may be longer with targets having higher secondary structure. In some embodiments, a pre-incubation time between 5 seconds and 60 seconds may be sufficient for targets with minimal secondary structure at the incubation temperature. In some embodiments, the pre-incubation time is 1 to 3 minutes. In other

embodiments, in which the target has predicted intramolecular base pairing at the pre-incubation temperature, the pre-incubation time is at least 3 minutes, preferably at least 5 minutes, or at least 10 minutes.

Since primer – target T_m increases with increasing primer concentration, it is useful to have high concentrations of primer during pre-annealing or before amplification, and then dilute that solution into an RT reagent mix or RT-PCR reagent mix. Therefore, in some embodiments the concentration of the primer is at least 1 μM during pre-annealing. In more embodiments, the concentration of the primer is at least 2 μM during pre-annealing. In some embodiments, the concentration of the primer is at least 1 μM, at least 2 μM, at least 3 μM, at least 4 μM, at least 5 μM, at least 6 μM, at least 7 μM, at least 8 μM, at least 9 μM, or at least 10 μM during pre-annealing.

Non- extensible oligonucleotides that hybridize with the CCCVd target and have a high T_m can be used to reduce secondary structure and enable hybridization of a nucleic acid primer during the same pre-incubation or during the subsequent RT or PCR. Alternatively, they can be added directly to the RT mix or the PCR mix, to enable primers to bind to RNA, or to DNA, respectively. The non- extensible opener, typically modified on the 3' end to prevent extension by a polymerase, is designed to hybridize with the nucleotides on the target that might otherwise have base pairing with nucleotides targeted by a primer. Thus, the non- extensible oligonucleotide serves as an "opener" for the primer. One advantage of using this method is that the opener can be designed with very high T_m without the risk of increasing non-specific product during subsequent amplification. In some embodiments, the opener or non-extensible oligonucleotide has a chemical modification. In some embodiments, the chemical modification is one or more 2'-O-methyl nucleosides. The non-extensible oligonucleotide can include non-conventional nucleotides, such as 2'-O-methyl RNA, PNAs, or LNAs that increase the T_m. In some embodiments, the opener (*i.e.*, the non-extensible nucleotide) has a T_m that is at least 10 degrees higher than the pre-annealing temperature, or the RT incubation temperature if the target is RNA and pre-annealing is not used, or the annealing temperature during PCR if the target is DNA. In some embodiments, the opener has a T_m that is higher than the Tm of any predicted intramolecular base-pairing that includes nucleotides targeted by the opener or the primer. In some embodiments, the primers comprise a random sequence of nucleotides (*e.g.*, the primer is a random hexamer). In some embodiments, random hexamers are used as primers in a two-step RT-PCR following pre-incubation of RNA with the opener. In some preferred embodiments, gene-

specific (sequence-specific) primers are used for 1-step RT-PCR following pre-incubation of RNA with the opener. In some preferred embodiments, the primer includes non-conventional bases that increase the T_m relative to DNA oligonucleotides. In some embodiments, the target of the opener is CCCVd RNA .. In some embodiments, provided herein are methods for amplifying a nucleic acid (*e.g.*, a CCCVd RNA) by forming a reaction mixture comprising a CCCVd nucleic acid target molecule comprising a CCCVd nucleic acid target sequence, a non-extendible oligonucleotide (*i.e.*, an opener), and an extensible nucleic acid primer. In some embodiments, the reaction mixture is then incubated at one or more temperatures disclosed herein for a period of time disclosed herein to allow the non-extensible oligonucleotide (*e.g.*, a non- extensible oligonucleotide of Table 3) and the extendible nucleic acid primer (*e.g.*, a primer of Table 1) to hybridize with the CCCVd nucleic acid target sequence. In some embodiments, the extendible nucleic acid primer and the non-extendible oligonucleotides hybridize to a regions of the CCCVd nucleic acid target sequence that are non-overlapping, and the non-extensible oligonucleotide hybridized to the CCCVd nucleic acid target sequence has a predicted melting temperature that is at least 10 °C higher than the predicted melting temperature of the extensible nucleic acid primer hybridized to the CCCVd nucleic acid target sequence. In some embodiments, the predicted melting temperature is at least 1°C higher, at least 2°C higher, at least 3°C higher, at least 4°C higher, at least 5°C higher, at least 6°C higher, at least 7°C higher, at least 8°C higher, at least 9°C higher, at least 10°C higher, at least 11°C higher, at least 12°C higher, at least 13°C higher, at least 14°C higher, at least 15°C higher, at least 16°C higher, at least 17°C higher, at least 18°C higher, at least 19°C higher, or at least 20°C higher. In some embodiments, non-extensible oligonucleotide hybridized to the CCCVd nucleic acid target sequence has a predicted melting temperature of at least 80 °C, at least 81°C, at least 82 °C, at least 83°C, at least 84°C, at least 85°C, at least 86°C, at least 87 °C, at least 88°C, at least 89°C, at least 90°C, at least 91°C, at least 92 °C, at least 93°C, at least 94°C, at least 95°C, at least 96°C, at least 97 °C, at least 98°C, at least 99°C, or at least 100°C.

An Exemplary Opener Sequence can be found below in Table 3.

Table 3: Exemplary CCCVd Opener or Non-Extensible Oligonucleotide

Number	Sequence (5'to 3')
1	GGCCGGGCGUCGAAGCUACGAAGGAGUC

In some embodiments, a nucleic acid polymerase is then added to the reaction mixture after the pre-incubation step. The reaction mixture may then be incubated under conditions such that the one or more nucleic acid primers are extended by the nucleic acid polymerase to create an amplification product comprising the CCCVd nucleic acid target sequence or a complement thereof.

In some aspects, provided herein are methods of amplifying a CCCVd nucleic acid by forming a reaction mixture with a CCCVd nucleic acid target molecule comprising a CCCVd nucleic acid target sequence, one or more primers that hybridize to the CCCVd nucleic acid target sequence, and incubating the reaction mixture under conditions such that the one or more nucleic acid primers is extended by the nucleic acid polymerase to create an amplification product comprising the CCCVd nucleic acid target sequence or a complement thereof. In some embodiments, the one or more primers comprise a primer that has a predicted melting temperature of at least 80 °C, at least 81°C, at least 82°C, at least 83°C, at least 84°C, at least 85°, at least 86°C, at least 87°C, at least 88°C, at least 89°C, at least 90 °C, at least 91°C, at least 92°C, at least 93°C, at least 94°C, or at least 95 °C with the nucleic acid target sequence and a nucleic acid polymerase.

Provided herein are methods of CCCVd nucleic acid amplification comprising forming a reaction mixture and adding one or more nucleic acid polymerases to the reaction mixture. The nucleic acid polymerase provided in any one of methods disclosed herein may be reverse transcriptase and/or DNA polymerase. In some embodiments, the nucleic acid polymerases are added to the reaction mixture at the same time (*e.g.*, one step PCR or RT-PCR). In other embodiments, the nucleic acid polymerases are added to the reaction mixture at different times (*e.g.*, two step PCR). In some embodiments, the reaction mixture comprises a DNA polymerase (*e.g.,* Taq DNA polymerase, Tfi DNA polymerase, Pfu DNA polymerase, Bst DNA polymerase, $Vent_R$ DNA polymerase Deep $Vent_R$ DNA polymerase, KlearKall polymerase from LGC Biosearch, and Taq polymerase from Hain Lifescience). In some embodiments, the reaction mixture comprises dNTPs (*e.g.,* dATP, dCTP, dGTP, dTTP, and/or dUTP). In some embodiments, the reaction mixture comprises a reverse transcriptase. Some methods which generate the target sequence or complement thereof are LATE-PCR amplification of DNA sequences or RNA sequences (RT-LATE-PCR). LATE-PCR amplifications and amplification assays are described in, for example, European patent EP 1,468,114 and corresponding United States patent 7,198,897; published European patent application EP 1805199 A2; Sanchez et al. (2004) Proc. Nat. Acad. Sci. (USA) 101: 1933-

1938; and Pierce et al. (2005) Proc. Natl. Acad. Sci. (USA) 102: 8609-8614. All of these references are hereby incorporated by reference in their entireties. LATE-PCR is a non-symmetric DNA amplification method employing the polymerase chain reaction (PCR) process utilizing one oligonucleotide primer (the "Excess Primer") in at least five-fold excess with respect to the other primer (the "Limiting Primer"), which itself is utilized at low concentration, up to 200 nM, so as to be exhausted in roughly sufficient PCR cycles to produce fluorescently detectable double-stranded amplicon. After the Limiting Primer is exhausted, amplification continues for a desired number of cycles to produce single-stranded product using only the Excess Primer, referred to herein as the Excess Primer strand. LATE-PCR takes into account the concentration-adjusted melting temperature of the Limiting Primer at the start of amplification, $Tm_{[0]}^{L}$, the concentration-adjusted melting temperature of the Excess Primer at the start of amplification, $Tm_{[0]}^{X}$, and the melting temperature of the single-stranded amplification product ("amplicon"), Tm_A. For LATE-PCR primers, $Tm_{[0]}$ can be determined empirically, as is necessary when non-natural nucleotides are used, or calculated according to the "nearest neighbor" method (Santa Lucia, J. (1998), PNAS (USA) 95: 1460-1465; and Allawi, H.T. and Santa Lucia, J. (1997), Biochem. 36: 10581-10594) using a salt concentration adjustment, which in our amplifications is generally 0.07 M monovalent cation concentration. For LATE-PCR the melting temperature of the amplicon is calculated utilizing the formula: Tm = 81.5 + 0.41 (%G+%C) – 500/L + 16.6 log [M]/(1 + 0.7 [M]), where L is the length in nucleotides and [M] is the molar concentration of monovalent cations. Melting temperatures of linear, or random-coil, probes can be calculated as for primers. Melting temperatures of structured probes, for example molecular beacon probes, can be determined empirically or can be approximated as the Tm of the portion (the loop or the loop plus a portion of the stem) that hybridizes to the amplicon. In a LATE-PCR amplification reaction $Tm_{[0]}^{L}$ is preferably not more than 5 °C below $Tm_{[0]}^{X}$, more preferably at least as high and even more preferably 3-10 °C higher, and Tm^{A} is preferably not more than 25 °C higher than $Tm_{[0]}^{X}$, and for some preferred embodiments preferably not more than about 18 °C higher.

LATE-PCR is a non-symmetric PCR amplification that, among other advantages, provides a large "temperature space" in which actions may be taken. See WO 03/054233 and Sanchez et al. (2004), cited above. Certain embodiments of LATE-PCR amplifications include the use of hybridization probes, in this case sets of signaling and quencher probes, whose Tm's are below, more preferably at least 5 °C below, the mean primer annealing

temperature during exponential amplification after the first few cycles. Sets of signaling and quencher probes are included in LATE-PCR amplification mixtures prior to the start of amplification. A DNA dye, if used, can also be incorporated into the reaction mixture prior to the start of amplification.

Amplification and detection methods provided herein enable single-tube, homogeneous assays to detect variants of a particular variable sequence, for example, a viroid RNA. In some embodiments of the method described herein, the reaction mixture further comprises a detection reagent for detecting the formation of the amplification product. In some embodiments, the detection reagent comprises a dsDNA fluorescent dye (*e.g.,* SYBR Green, PicoGreen). In some embodiments, the detection reagent comprises a detectably labeled probe (*e.g.,* a molecular beacon, a TaqMan probe, a scorpion probe). In some embodiments, the detection reagent comprises a Lights-On probe and a Lights-Off probe. In some embodiments, the detection reagent comprises a Lights-Off Only probe and a dsDNA fluorescent dye.

In nucleic acid samples which contain both RNA and DNA and detection of the RNA is desired, a practice well known in the art is to use primers that target different exons of a gene. The RNA transcripts will lack a large number of nucleotides, often several thousand that would be present in the amplification product from the DNA. In some cases, amplification conditions can be adjusted to minimize amplification of the DNA (*e.g.*, by keeping the extension step duration short), or the products from the DNA can be distinguished from products amplified from mRNA by using gel electrophoresis or by measuring the melting temperature of the amplification products using a DNA binding dye such as SYBR Green. However, these steps may not always be possible or convenient. To insure that only the RNA will be amplified from a mixture of plant nucleic acids, we have chosen to amplify plant mitochondrial nad5 mRNA using primers to exons 2 and 4. These exons are separated by more than 33,000 nucleotides on the DNA and are transcribed separately, then joined together in "trans splicing" events that also include a transcript from exon 3 that is even more distant on the chromosome and encoded on the opposite DNA strand. Another advantage of choice of the nad5 mitochondrial gene is its high degree of conservation that will enable tests on a wide range of plants using the same primers or slight modifications of the primers described herein. Other genes with introns tens of thousands of bases in length or genes known to have trans-splicing could be similarly used. Although

trans splicing has not been identified in animals, it is possible that such genes do exist and these principals could be extended to animals as well.

Preparation of CCCVd Nucleic Acid Samples

In some aspects, provided herein are methods of amplifying CCCVd nucleic acids that have been prepared in a solution prior to the amplification steps provided herein. In some embodiments, provided herein are methods of amplifying CCCVd RNA by first lysing cells in a solution comprising a chaotrope, a reducing agent, a detergent, a chelator and a buffer (*e.g.*, a PrimeStore™ solution) and application of mechanical disruption to form a nucleic acid solution comprising a nucleic acid target molecule, and then forming a reaction mixture comprising one or more primers that hybridize to the CCCVd target molecule and a nucleic acid polymerase, and incubating the reaction mixture under conditions such that the one or more nucleic acid primers is extended by the nucleic acid polymerase to create an amplification product comprising the CCCVd nucleic acid target sequence or a complement thereof.

In some embodiments, the nucleic acid solution is diluted without performing a nucleic acid purification step to form a reaction mixture comprising the CCCVd nucleic acid target molecule. In some embodiments, the nucleic acid solution is diluted in step by an amount sufficient to reduce the concentration of the chaotrope, the reducing agent, the detergent, the chelator and the buffer to a level whereby the nucleic acid polymerase has at least 10%, at least 20%, at least 30% at least 40%, at least 50%, at least 60%, or at least 70%, of the activity it has in a reaction mixture that does not comprise the chaotrope, the reducing agent, the detergent, the chelator and the buffer. Dilution of the nucleic acid solution may be done in one step, two steps, or more steps. The nucleic acid solution disclosed herein may be diluted by a factor of at least 5 fold, at least 10 fold, at least 15 fold, at least 20 fold, at least 25 fold, at least 30 fold, at least 35 fold, at least 40 fold, at least 45 fold, at least 50 fold, at least 55 fold, at least 60 fold, at least 65 fold, at least 70 fold, or at least 75 fold. In some embodiments, the first dilution step is carried out in a buffer that contains at least one (*e.g.*, at least two, at least three, at least four, or at least five) DNA oligonucleotide primer(s) and wherein said first-dilution step is followed by heating to at least 80 °C, at least 81 °C, at least 82 °C, at least 83 °C, at least 84 °C, at least 85 °C, at least 86 °C, at least 87 °C, at least 88 °C, at least 89 °C, at least 90 °C, at least 91 °C, at least 92 °C, at least 93 °C, at least 94 °C, or at least 95 °C followed by gradual cooling, prior to a second dilution step. In some embodiments, the methods further comprise co-amplification of a non-viroid target sequence

by incubating the reaction mixture under conditions such that the one or more nucleic acid primers is extended by a nucleic acid polymerase to create an amplification product comprising the non-viroid target sequence (*e.g.*, transcripts of nad5) or a complement thereof.

In some aspects, provided herein are compositions and solutions, as well as methods of employing them, that may advantageously improve conventional collection, lysis, transport and storage methods for the preparation of CCCVd nucleic acids from one or more biological sources. The solutions and methods provided herein may provide a collection and preservation formulation to inactivate and lyse a biological specimen containing nucleic acids, and preserve nucleic acids (*e.g.*, RNA and/or DNA) within the biological specimen, preferably all in a single reaction vessel, such that the integrity of the nucleic acids is at least substantially or fully maintained, so that a portion of the nucleic acids are readily available for analysis. The methods and compositions provide herein may also enable isolated nucleic acids to remain at least substantially stable, without requiring consistent and constant cooler temperatures, such as refrigeration or freezing.

In some embodiments, provided herein is a composition that includes: a) one or more chaotropes (*e.g.*, present in the composition an amount from about 0.5 M to about 6 M); b) one or more detergents (*e.g.*, present in the composition an amount from about 0.1% to about 1%); c) one or more chelators (*e.g.*, present in the composition in an amount from about 0.01 mM to about 1 mM); d) one or more reducing agents (*e.g.*, present in the composition in an amount from about 0.05 M to about 0.3 M); and e) one or more defoaming agents (*e.g.*, present in the composition in an amount from about 0.0001% to about 0.3%).

Exemplary chaotropes include, without limitation, guanidine thiocyanate (GuSCN), guanidine hydrochloride (GuHCl), guanidine isothionate, potassium thiocyanate (KSCN), sodium iodide, sodium perchlorate, urea, or any combination thereof. Descriptions of additional exemplary chaotropes and chaotropic salts can be found in, inter alia, U.S. Pat. No. 5,234,809 (specifically incorporated herein in its entirety by express reference thereto).

Exemplary detergents include, without limitation, sodium dodecyl sulfate (SDS), lithium dodecyl sulfate (LDS), sodium taurodeoxycholate (NaTDC), sodium taurocholate (NaTC), sodium glycocholate (NaGC), sodium deoxycholate (NaDC), sodium cholate, sodium alkylbenzene sulfonate (NaABS), N-lauroyl sarcosine (NLS), salts of carboxylic acids (*i.e.*, soaps), salts of sulfonic acids, salts of sulfuric acid, phosphoric and polyphosphoric acid esters, alkylphosphates, monoalkyl phosphate (MAP), and salts of

perfluorocarboxylic acids, anionic detergents including those described in U.S. Pat. No. 5,691,299 (specifically incorporated herein in its entirety by express reference thereto), or any combination thereof.

Exemplary reducing agents include, without limitation, 2-mercaptoethanol (.beta.-ME), tris(2-carboxyethyl)phosphine (TCEP), dithiothreitol (DTT), formamide, dimethylsulfoxide (DMSO), or any combination thereof. In a preferred embodiment, the reducing agent includes or is TCEP.

Exemplary chelators include, without limitation, ethylene glycol tetraacetic acid (EGTA), hydroxyethylethylenediaminetriacetic acid (HEDTA), diethylene triamine pentaacetic acid (DTPA), N,N-bis(carboxymethyl)glycine (NTA), ethylenediaminetetraacetic (EDTA), citrate anhydrous, sodium citrate, calcium citrate, ammonium citrate, ammonium bicitrate, citric acid, diammonium citrate, potassium citrate, magnesium citrate, ferric ammonium citrate, lithium citrate, or any combination thereof. In preferred embodiments, the chelator includes EDTA, a citrate, or a combination thereof. In a more preferred embodiment, the chelator includes EDT. In some embodiments, the compositions disclosed herein may further include one or more buffers (*e.g.,* present in the final composition in an amount from about 1 mM to about 1 M). Exemplary buffers include, without limitation, tris(hydroxymethyl)aminomethane (Tris), citrate, 2-(N-morpholino)ethanesulfonic acid (MES), N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic Acid (BES), 1,3-bis(tris(hydroxymethyl)methylamino)propane (Bis-Tris), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-(N-morpholino)propanesulfonic acid (MOPS), N,N-bis(2-hydroxyethyl)glycine (Bicine), N-[tris(hydroxymethyl)methyl]glycine (Tricine), N-2-acetamido-2-iminodiacetic acid (ADA), N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES), piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), bicarbonate, phosphate, or any combination thereof. In a preferred embodiment, the buffer includes a citrate. The inclusion of one or more buffers is desirable to control the pH of the formulations, since it has been found that nucleic acid extraction is optimal in a pH range of about 5 to 7. Preferably, the one or more buffers employed in the disclosed compositions are chosen to provide a significant buffering capacity in the range from a pH of about 6 to a pH of about 8, more preferably within a pH range of about 6 to about 7, and more preferably still, within a pH range of about 6.2 to about 6.8.

The compositions disclosed herein can further include a defoaming agent to prevent the formation of bubbles that typically result from the presence of detergents in the

formulation. Defoaming agents facilitate pipetting and handling of the disclosed compositions. Exemplary surfactants/defoaming agents include, without limitation, cocoamidopropyl hydroxysultaine, alkylaminopropionic acids, imidazoline carboxylates, betaines, sulfobetaines, sultaines, alkylphenol ethoxylates, alcohol ethoxylates, polyoxyethylenated polyoxypropylene glycols, polyoxyethylenated mercaptans, long-chain carboxylic acid esters, alkonolamides, tertiary acetylenic glycols, polyoxyethylenated silicones, N-alkylpyrrolidones, alkylpolyglycosidases, silicone polymers such as Antifoam A.RTM., or polysorbates such as Tween.RTM., or any combination thereof. In a preferred embodiment, a defoaming agent includes a silicone polymer.

The compositions disclosed herein may also further optionally include one or more short-chain (preferably from 1- to 6-carbon [*i.e.*, C.sub.1-C.sub.6] alcohols)alkanols (*e.g.*, present in the composition in an amount from about 1% to about 25%, although higher percentages of the alcohols may be employed if desired). Exemplary short-chain alkanols include linear and branched-chain alcohols, such as, without limitation, methanol, ethanol, propanol, butanol, pentanol, hexanol, or any combination thereof.

Provided herein are methods for obtaining a population of polynucleotides from a sample suspected of containing CCCVd nucleic acids. The method generally involves associating the sample with an amount of one of the disclosed compositions, under conditions effective to obtain a population of polynucleotides from the sample. Such sample may be of any origin, including, without limitation, a clinical or veterinary sample; an environmental or ecological sample, a forensic or crime scene sample, or such like, and may contain one or more nucleic acids that are of viral, microbial, animal, or plant origin, or any combination thereof. A sample may comprise plant cells, animal cells, fungus cells, bacterial cells or parasite cells.

Commercial detection assays may utilize rapid nucleic acid purification techniques. These often provide more challenges than using purified RNA or DNA. The methods described herein are shown to work well with PrimeStore™, a reagent containing a reducing agent and a chaotropic compound. In some embodiments, the source of the nucleic acid for pre-incubation with a primer or opener contains a chaotropic compound and that incubation is done at 60 °C or higher. In preferred embodiments, the incubation is done at 70 °C or higher. In other preferred embodiments, two or more antisense primers target different RNA molecules or different genes, or exons with a gene. In other preferred embodiments, one antisense primer targets an internal control RNA or gene. Additional information about

nucleic acid solutions disclosed herein can be found in U.S. Patent 9,212,399, which is incorporated herein in its entirety.

Mispriming Prevention Reagents

Provided herein are methods of amplifying CCCVd nucleic acids by forming a reaction mixture comprising one or more mispriming reagent disclosed herein. The mispriming prevention reagent may be a single- or a multi-stranded mispriming prevention reagent.

Single-Stranded Mispriming Prevention Reagents

In certain aspects, provided herein are single-stranded mispriming prevention reagents. In some embodiments, the reagents described here fall into a class of reagents that, when added to a primer-based amplification reaction, such as PCR assays or other primer-dependent DNA amplification reactions at a functional temperature-dependent concentration relative to the concentration of DNA polymerase in the reaction, is effective in preventing at least one manifestation of mispriming, including amplification of primer-dimers, increasing polymerase selectivity against 3' terminal mismatches, reducing scatter among replicates, and lower than maximal yield of amplification of one or more reaction products.

In certain embodiments, mispriming prevention reagents described herein are capable of preventing or inhibit one or more manifestations of mispriming in at least some PCR amplification reactions and/or reverse transcription reactions. As used herein, "prevent a manifestation of mispriming" refers to the elimination or the reduction of the formation of one or more products of mispriming in a nucleic acid amplification reaction containing a reagent described herein compared to in an otherwise identical nucleic acid amplification reaction in which the reagent was omitted.

In certain embodiments, the reagents described herein comprise a single-stranded oligonucleotide that can be in an open configuration or a closed-hairpin configuration depending on whether six or more complementary nucleotides at or near the 3′ terminus and the 5′ terminus of the oligonucleotide are hybridized to each other in a temperature-dependent manner. The reagent is active (*i.e.*, inhibits mispriming) in the closed stem-loop hairpin conformation. In this conformation it binds to and increases the specificity of the DNA polymerase, including by greatly reducing the rate of DNA synthesis.

Thus, in certain embodiments the mispriming prevention reagents described herein reduce or prevent Type 1 and/or Type 2 mispriming. In some embodiments, the mispriming prevention reagent provided herein reversibly acquires a principally stem-loop hairpin

conformation at a first temperature but not at a second, higher temperature. In some embodiments, the first temperature is a temperature that is below an annealing temperature of an amplification reaction and the second temperature is a temperature that is above the annealing temperature of an amplification reaction. In certain embodiments, the stem-loop hairpin confirmation of the mispriming prevention reagent reduces the activity of a thermostable DNA polymerase (*e.g.,* Taq polymerase). Thus, in some embodiments, the mispriming prevention reagent is able to act as both a "hot-start" reagent and a "cold-stop" reagent during the performance of a primer-based nucleic acid amplification process.

As described herein, the melting temperature, Tm, of a hairpin reagent having a stem of fixed sequence can be adjusted by increasing or decreasing the number of cytosine nucleotides in the loop. However, while hairpin Tm decreases as a function of increasing loop length, the relationship between loop length and hairpin Tm is not linear. Moreover, the empirically observed hairpin Tm differs from the *in silico* calculated Tm due the presence of the chemical moieties linked to the 3′ and 5′ ends of the stem. In general, paired identical moieties stabilize the closed stem structure to a greater extent than paired non-identical moieties. In some embodiment, the reagent described herein comprises non-identical 3′ and 5′ paired moieties.

In some embodiments, the mispriming prevention reagent oligonucleotide described herein comprises, in 5′ to 3′ order, a first condition-dependent "stem" region, a condition-dependent "loop" region and a second condition-dependent "stem" region, wherein the first stem region hybridizes to the second stem region in a temperature dependent manner to acquire a stem-loop hairpin conformation (*e.g.,* a stem-loop hairpin with a 3′ or 5′ overhang or a blunt-ended stem-loop hairpin). In some embodiments, the first stem region is linked to a first moiety and the second stem region is linked to a second, non-identical moiety. In some embodiments, the first moiety and the second moiety are cyclic or polycyclic planar moieties that do not have a bulky portion (*e.g.,* a dabcyl moiety, a Black Hole Quencher moiety, such as a Black Hole Quencher 3 moiety or a coumarin moiety).

In some embodiments, the first stem region comprises a first stem nucleic acid sequence (*e.g.,* a nucleic acid sequence of at least 6, 7, 8, 9 or 10 nucleotides in length). In some embodiments, the first stem nucleic acid sequence is no more than 20, 19, 18, 17, 16, 15, 14, 12 or 11 nucleotides in length. In some embodiments, the first stem nucleic acid sequence is 10 nucleotides in length. In some embodiments, the first stem region comprises a 5′ terminal moiety. In some embodiments, the 5′ terminal moiety is linked (either directly or

indirectly) to the most 5′ nucleotide of the first stem region. In some embodiments, the 5′ terminal moiety is linked (either directly or indirectly) to one of the 2, 3, 4, or 5 most 5′ nucleotides of the first stem region. In some embodiments, the 5′ terminal moiety comprises a cyclic or polycyclic planar moiety that does not have a bulky portion (not including the linker, if present). In some embodiments, the 5′ terminal moiety is a dabcyl moiety. In some embodiments, the 5′ terminal moiety is a coumarin moiety (*e.g.,* Coumarin 39, Coumarin 47 or Biosearch Blue).

In some embodiments, the loop region comprises a loop nucleic acid sequence (*e.g.,* a nucleic acid sequence of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 nucleotides in length). In some embodiments, the loop nucleic acid sequence is between 25 and 40 nucleotides in length. In some embodiments, the loop nucleic acid sequence is a single nucleotide repeat sequence (*e.g.,* a poly-cytosine, a poly-guanine, a poly-thymine, a poly-adenine or a poly-uracil sequence). Use of a single nucleotide sequence, particularly cytosines, for the loop reduces the possibility of the loop sequence base-pairing within the loop or with naturally occurring nucleic acid sequences that may be present in an amplification reaction. In some embodiments, the single nucleotide repeat sequence is a poly-cytosine sequence.

In some embodiments, the second stem region comprises a second stem nucleic acid sequence (*e.g.,* a nucleic acid sequence of at least 6, 7 or 8 nucleotides in length). In some embodiments, the second stem nucleic acid sequence is no more than 20, 19, 18, 17, 16, 15, 14, 12 or 11 nucleotides in length. In some embodiments, the second stem nucleic acid sequence is 10 nucleotides in length. In some embodiments, the second stem nucleic acid sequence is complementary to the second stem nucleic acid sequence. In some embodiments, the second stem region comprises a 3′ terminal moiety. In some embodiments, the 3′ terminal moiety is linked (either directly or indirectly) to the most 3′ nucleotide of the second stem region. In some embodiments, the 3′ terminal moiety is linked (either directly or indirectly) to one of the 2, 3, 4, or 5 most 3′ nucleotides of the second stem region. In some embodiments, the 3′ terminal moiety comprises a cyclic or polycyclic planar moiety that does not have a bulky portion (not including the linker, if present). In some embodiments, the 3′ terminal moiety is a dabcyl moiety. In some embodiments, the 3′ terminal moiety is a coumarin moiety (*e.g.,* Coumarin 39, Coumarin 47 or Biosearch Blue). In some embodiments, the 3′ terminal moiety is non-identical to the 5′ terminal moiety. In some

embodiments, the 3′ terminus of the second stem region is non-extensible by a DNA polymerase.

In some embodiments, the first stem region hybridizes to the second stem region in a temperature dependent manner to acquire a stem-loop hairpin conformation. In some embodiments, the stem-loop conformation comprises a 3′ or 5′ overhang of 0, 1, 2, 3, 4 or 5 nucleotides. In some embodiments, the first stem region hybridizes to the second stem region with a melting temperature that is at least 30 °C, 31 °C, 32 °C, 33 °C, 34 °C, 35 °C, 36 °C, 37 °C, 38 °C, 39 °C, 40 °C, 41 °C, 42 °C, 43 °C, 44 °C or 45 °C. In some embodiments, the first stem region hybridizes to the second stem region with a melting temperature that is no greater than 71 °C, 70 °C, 69 °C, 68 °C, 67 °C, 66 °C, 65 °C, 64 °C, 63 °C, 62 °C, 61 °C, 60 °C, 59 °C, 58 °C, 57 °C, 56 °C, 55 °C, 54 °C, 53 °C, 52 °C, 51 °C or 50 °C. In some embodiments, the first stem region hybridizes to the second stem region with a melting temperature that is between 40 °C and 71 °C, between 40 °C and 55 °C or between 45 °C and 55 °C. In some embodiments, the first stem region hybridizes to the second stem region with a melting temperature that is less than the annealing temperature of a nucleic acid amplification reaction (*e.g.*, between 0 and 10 °C less than the annealing temperature, between 0 and 9 °C less than the annealing temperature, between 0 and 8 °C less than the annealing temperature, between 0 and 7 °C less than the annealing temperature, between 0 and 6 °C less than the annealing temperature or between 0 and 5 °C less than the annealing temperature).

In some embodiments, the mispriming prevention reagents described herein include a G/C clamp at one or both ends of the stem regions. In some embodiments, the most 3′ nucleic acid of the first stem nucleic acid sequence is cytosine and the most 5′ nucleic acid of the second stem nucleic acid sequence is guanine. In some embodiments, the most 3′ nucleic acid of the first stem nucleic acid sequence is guanine and the most 5′ nucleic acid of the second stem nucleic acid sequence is a cytosine. In some embodiments, the most 5′ nucleic acid of the first stem nucleic acid sequence is cytosine and the most 3′ nucleic acid of the second stem nucleic acid sequence is guanine. In some embodiments, the most 5′ nucleic acid of the first stem nucleic acid sequence is guanine and the most 3′ nucleic acid of the second stem nucleic acid sequence is a cytosine.

In some embodiments, the reagent does not fluoresce when present in an amplification reaction. In some embodiments, the reagent does not fluoresce because is not stimulated with an appropriate excitation wavelength. In some embodiments, the reagent

does not fluoresce because it does not comprise a fluorescent moiety. In some embodiments, the 3′ terminal moiety and/or the 5′ terminal moiety is a quencher of electromagnetic energy, including fluorescent light released from a fluorescent DNA-binding dye, such as SYBR Green, that intercalates into the stem of the closed-hairpin.

Multi-Stranded Mispriming Prevention Reagents

In certain aspects, provided herein is a multi-stranded mispriming prevention reagent comprising at least two non-identical 5′ or 3′ terminal moieties. In some embodiments, the multi-stranded mispriming prevention reagent is a double-stranded mispriming prevention reagent.

In some embodiments, the multi-stranded mispriming prevention reagent comprises a first nucleic acid strand of and a second nucleic acid strand. In some embodiments, the first and/or second nucleic acid strand of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23,24, 25,26, 27, 28, 29, 30, 31,32, 33, 34, 35, 36, 37, 38, 39 or 40 nucleotides in length. In some embodiments, the first and/or second nucleic acid strand is between 18 and 24 nucleotides in length. In some embodiments, the first and/or second nucleic acid strand is between 20 and 22 nucleotides in length. In some embodiments, the first and/or second nucleic acid strand is 21nucleotides in length. In some embodiments, the first and second strand are the same length. In some embodiments, the first and second strand are different lengths. In some embodiments, the first nucleic acid strand hybridizes to the second nucleic acid strand with a melting temperature that is no less than 25 °C, 30 °C, 32 °C, 35 °C, 40 °C, 41 °C, 42 °C, 43 °C, 44 °C, 45 °C, 46 °C, 47 °C, 48 °C, 49 °C or 50 °C. In some embodiments, the first nucleic acid strand hybridizes to the second nucleic acid strand with a melting temperature that is no greater than 77 °C, 76 °C, 75 °C, 74 °C, 73 °C, 72 °C, 71 °C, 70 °C, 69 °C, 68 °C, 67 °C, 66 °C, 65 °C, 64 °C, 63 °C, 62 °C, 61 °C or 60 °C.

In some embodiments, first and second nucleic acid strand collectively comprise at least two non-identical 5′ or 3′ terminal moieties (*e.g.,* 2, 3 or 4 terminal moieties). In some embodiments, the at least two non-identical moieties are selected from dabcyl moieties, Black Hole Quencher moieties and coumarin moieties. In some embodiments, the at least two non-identical moieties comprise a dabcyl moiety and a coumarin moiety (*e.g., C*oumarin 39, Coumarin 47 and Biosearch Blue). In some embodiments, one of the non-identical moieties is located at the 5′ terminus of the first nucleic acid strand and one of the non-identical moieties is located at the 3′ terminus of the second nucleic acid strand. In some

embodiments, one of the non-identical moieties is located at the 3′ terminus of the first nucleic acid strand and one of the non-identical moieties is located at the 5′ terminus of the second nucleic acid strand. In some embodiments, a dabcyl moiety is located at the 5′ terminus of the first nucleic acid strand and a Biosearch Blue moiety is located at the 3′ terminus of the second nucleic acid strand. In some embodiments, a Biosearch Blue moiety is located at the 5′ terminus of the first nucleic acid strand and a dabcyl moiety is located at the 3′ terminus of the second nucleic acid strand. In some embodiments, a coumarin moiety is located at the 3′ terminus of the first strand and the 3′ terminus of the second strand and a Biosearch Blue moiety is located at the 5′ terminus of the second strand. In some embodiments, a carbon spacer is located at the 5′ terminus of the first strand. In some embodiments, the non-identical terminal moieties are linked (either directly or indirectly) to the most 3′ or the most 5′ nucleotide of the first or second nucleic acid strand. In some embodiments, the non-identical terminal moieties are linked (either directly or indirectly) to one of the 2, 3, 4, or 5 most 3′ or most 5′ nucleotides of the first or second nucleic acid strand.

In some embodiments, the mispriming prevention reagent is an oligonucleotide that has a 3' end and a stem-loop structure having a stem comprising a double-stranded region that has a length greater than six nucleotides and a terminus away from the loop comprising a 3' nucleotide and a 5' nucleotide, the stem having a calculated stem melting temperature (Tm) below 94° C. In some embodiments, the 3' end is non-extensible by the DNA polymerase. In some embodiments,
the oligonucleotide is not fluorescently labeled and does not contribute background fluorescence. In some embodiments, the stem terminus is stabilized by means selected from the group consisting of non-fluorescent fluorophore- quenching moieties covalently attached to the 3' and 5' nucleotides of the stem terminus and pairs of non-natural nucleotides that bind more strongly than a natural DNA-DNA hybrid and that include each of the 3' and 5' nucleotides of the stem terminus.

Information on mispriming prevention agents disclosed herein can be found in U.S. Patent 7,517,922 and PCT Published Application WO 2016/100335, each of which are incorporated in their entirety.

In some embodiments, PrimeSafe or ThermaStop are added to reverse transcription reactions and PCR in order to inhibit enzyme activity until the temperature is increased to the desired reaction temperature. In more preferred embodiments, versions of ThermaStop for

reverse transcriptase include RNA nucleotides or RNA analogs, such as 2'-O-methyl RNA which are likely to have higher affinity for reverse transcriptase (an RNA-dependent DNA polymerase). A two-step RT-PCR may also include a ThermaStop version with only DNA nucleotides that will have higher affinity to the DNA-dependent DNA polymerase (*e.g.* Taq polymerase). In the most preferred embodiments, both types of ThermaStop are included in a one-step RT-PCR. In such cases, it is necessary that both enzymes be inactive at temperatures below that used for the reverse transcription step, and that the ThermaStop inhibiting reverse transcriptase at low temperatures is no longer inhibitory (or at least only partially inhibitory) at the temperature of the reverse transcription step (*e.g.* 40 °C, or 45 °C, or 50 °C). This can be accomplished using a first version of ThermaStop that includes RNA nucleotides in a stem-loop structure with a T_m below that temperature. At the reverse transcription temperature, the DNA polymerase should remain inhibited by a second version of ThermaStop with DNA nucleotides in a stem-loop structure with a T_m at least 5 degrees higher, preferably at least 10 degrees higher than that of the first ThermaStop. Once the reverse transcription step is completed and the temperature is increased for PCR, the second ThermaStop no longer inhibits the DNA polymerase. Partial inhibition of reverse transcriptase by the second ThermaStop during the reverse transcription step is possible, but desired cDNA production can be accomplished by increasing the duration of the reverse transcription step and/or by adjusting the concentrations of both ThermaStops.

EXAMPLES

Example 1: The use of primers with very high melting temperature (T_m) improves two-step RT-LATE-PCR amplification of a synthetic viroid RNA.

A synthetic double-stranded DNA having the following positive strand sequence was custom synthesized by Integrated DNA technologies (Coralville, Iowa, USA). The sequence shown below is comprised of a T7 promoter (underlined) followed by a DNA sequence analogous to CCCVd RNA.

5'-

TAATACGACTCACTATAGGGGAAACCTCAAGCGAATCTGGGAAGGGAGCGTACC
TGGGTCGATCGTGCGCGTTGGAGGAGACTCCTTCGTAGCTTCGACGCCCGGCCG
CCCCTCCTCGACCGCTTGGGAGACTACCCGGTGGATACAACTCACGCGGCTCTTA
CCTGTTGTTAGTAAAAAAAGGTGTCCCTTTGTAGCCCCTCTGGGGAAATCTACAG
GGCACCCCAAAAACTACTGCAGGAGAGGCCGCTTGAGGGATCC-3'

An RNA transcript was generated from the synthetic DNA using HiScribe™ T7 Quick High Yield RNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. RNA was treated with DNase, purified using RNAzol (Sigma-Adrich, St. Louis, MO, USA), recovered using isopropanol precipitation and resuspended in RNase-free water using standard methods. The concentration of the purified RNA transcript was measured using Nanodrop instrument (ThermoFisher Scientific, Waltham, MA, USA) and aliquots were stored at -80 °C.

Reverse transcription (RT) was done using one of the following antisense primers:
OPV9-A32 (Lim) 5'-**TT**CTGCAGTAGTTTTTGGGGTGCCCTGTAGAT-3' or
OPV9-A40 (Lim) 5'-**TT**GGCCTCTCCTGCAGTAGTTTTTGGGGTGCCCTGTAGAT-3'
The 30 nucleotides at the 3' end of these primers are identical, but the A40 primer has additional nucleotides at the 5' end. Each primer is complementary to the CCCVd sequence, except for the two T nucleotides at the 5' end (shown in bold) which were included to improve LATE-PCR amplification. The predicted T_m of the shorter (A32) primer to the RNA target is 86.3°C at a primer concentration of 1 µM (the concentration during the pre-incubation) and is 75.8°C to the fully complementary DNA sequence at a primer concentration of 50 nM (the concentration used during LATE-PCR). The predicted T_m of the longer (A40) primer to the RNA target is 91.0 °C and 80.2°C to the fully complementary DNA. All other conditions of the RT and LATE-PCR steps are identical, so differences in results between samples with the different primers are likely due to differences in the ability of the antisense primers to hybridize with the highly folded RNA target during the RT step. All primers and probes described in the examples were custom synthesized by BioSearch Technologies (Petaluma, CA, USA).

Synthetic viroid RNA was pre-incubated with 1 µM antisense primers and 0.2 U/µL RNase Inhibitor (Clontech laboratories, Mountain View, CA, USA) in 1X PrimeScript buffer (75 mM potassium chloride, 3 mM magnesium chloride, 50 mM TRIS, pH 8.3. (Clontech Laboratories) at 85 °C for 3 minutes, then 60 °C for 10 minutes to enhance hybridization. The pre-incubation mixes then were cooled to 25 °C and kept on ice until diluted with an equal volume of reverse transcription mix to achieve concentrations of 500 nM antisense primer, 400nM dNTPs, 1 µM Reagent 1, 1 U/µL RNase Inhibitor (Clontech laboratories), 1 U/µL PrimeScript Reverse Transcriptase (Clontech Laboratories) and 1X PrimeScript buffer. Control samples without Reverse Transcriptase were run in parallel to monitor for

amplification from any residual synthetic viroid DNA. All samples were incubated at 50 °C for 5 minutes, 85 °C for 5', then cooled to 25 °C.

Two µL of the RT sample was diluted with 18 µL of a LATE-PCR reagent mix to obtain the final concentrations of 50 nM antisense primer, 1 µM sense primer OPV191-S24 (5'-TTGGGAGACTACCCGGTGGATACA-3'), 250 nM hybridization probe OPV-nt197-A20 (5'-CalRed610-ATGTAAGAGCCGCGTGAGAT-Black Hole Quencher2 (BHQ2)-3', 400nM dNTPs, 0.25X SYBR Green 1 µM Reagent 1, 0.06 U/µL Invitrogen Taq DNA Polymerase (ThermoFisher Scientific), 3mM $MgCl_2$, and 1X Reaction Buffer (ThermoFisher Scientific). Each condition was tested using 4 replicate samples. Thermal cycling and fluorescence detection were done using a Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA, USA). An initial denaturation step of 95 °C for 2 minutes was followed by 60 cycles of 95 °C for 10 seconds, 68°C for 10 seconds, and 72°C for 35 seconds with detection for SYBR Green. The temperature was then lowered gradually (approximately 2°C per minute) and held at 45 °C for 2 minutes to allow hybridization of the Cal Red-labeled probe to the single-stranded amplification product. Temperature was increased in 0.5 °C steps from 45 °C to 95 °C, measuring SYBR Green and Cal Red fluorescence at each step. Real-time SYBR Green fluorescence data was analyzed using the adaptive baseline setting of the Stratagene software. Cal Red fluorescence data was exported to Microsoft Excel and was normalized using the fluorescence at 75 °C, a temperature at which there is no detectable hybridization of probe and viroid amplification product.

Real-time detection of double-stranded DNA by SYBR Green in samples containing the lower-T_m antisense primer, OPV9-A32, are shown in Figure 1, Part A. Samples containing 1,000 copies of synthetic viroid RNA had a mean C_T value of 32.2 ± 0.4 (standard deviation). Samples containing 100 copies of synthetic viroid RNA had a mean C_T value of 33.3 ± 3.2. The wide range of C_T values in that group (30.7 to 37.2) did not reflect a range of specific product amplification, but reflects a high level of non-specific amplification. Each of the 4 replicate samples at both RNA concentrations had a SYBR Green melt peak at approximately 85 °C, corresponding to the viroid-specific amplicon (inset in Figure 1, Part A), but 2 samples in the 100 copy group had a relatively small peak at 85 °C and a much larger non-specific product peak at approximately 80 °C. One of four samples processed without reverse transcriptase ("no RT") and two of four samples without RNA (NTC) showed SYBR Green fluorescence increase with C_T values in the 35 to 37 cycle range, but SYBR Green melt analysis showed only non-specific product peaks around 80 °C.

Real-time detection of double-stranded DNA by SYBR Green in samples containing the higher-T_m antisense primer, OPV9-A40, are shown in Figure 1, Part B. Samples containing 1,000 copies of synthetic viroid RNA had a mean C_T value of 28.2 ± 0.2. Samples containing 100 copies of synthetic viroid RNA had a mean C_T value of 30.6 ± 0.2. The replicate reactions in samples with this primer were more consistent, as indicated by the low standard deviations. These C_T values are lower than those obtained using the shorter primer with lower T_m, indicating that the higher T_m primer increased the efficiency of reverse transcription of RNA targets into cDNA. The C_T differences between the 1,000 copy samples with the different antisense primers suggest that there may be as much as a 10-fold increase in the number of cDNA copies generated. Interestingly, none of the no RT or no RNA samples showed increased fluorescence, even though the higher-Tm primer was used at the same annealing temperature and might be expected to be more likely to mis-prime under such non-stringent conditions.

The viroid sequence-specific probe fluorescence during the post-PCR melt confirmed the reverse transcription and amplification of the synthetic viroid RNA target. Samples with the lower-Tm antisense primer showed fluorescence signal above background in all samples with RNA and reverse transcription, but 3 of the 4 samples with 100 RNA copies showed relatively low fluorescence signal (Figure 1, Part C). In contrast, all samples with RNA targets and reverse transcriptase and the higher-Tm antisense primer generated strong fluorescence signal (Figure 1, Part D). The expected T_m of the probe – amplicon hybrid of about 62°C was confirmed in the derivative plots (insets). None of the RT or NTC samples generated detectable probe fluorescence above background.

Example 2. Plant nad5 trans-spliced mitochondrial gene provides an RNA-specific internal control that could be used in RT-PCR tests for the detection of plant pathogens.

Verifying adequate RNA isolation, reverse transcription, and amplification is required in RNA pathogen (*e.g.*, virus and viroid) detection tests to insure against false negative results. Typically, a host species gene is amplified to confirm the quality of these steps. It is customary that such control genes include a region that includes an intron, a transcribed segment that is removed from the final messenger RNA. However, it is important to make sure that the PCR conditions do not enable amplification of the longer genomic DNA target, or that detection methods are able to distinguish mRNA amplification from longer genomic DNA amplification product. This example demonstrates amplification across "trans-splicing" junctions of the plant mitochondrial gene, *nad5*. The sense primer is

complementary to a site in exon 2 and the antisense primer is complementary to a site in exon 4. These exons are separated by more than 33,000 nucleotides in the date palm mitochondrial genome (GenBank Accession number NC016740). (The size and organization of this gene is presumed to be similar in other palms, as the gene is highly conserved among plants.) Exon 3 is only 21 nucleotides in length and is encoded on the opposite DNA strand over 70,000 nucleotides away from exon 2. Generating the final mRNA requires joining separate transcripts from those regions. The primers used in this example produce an amplicon of about 150 nucleotides from the mRNA, but cannot amplify the gene from DNA using typical PCR protocols because of the large distance between primer hybridization sites.

Small pieces (about 20 mm^2) of young leaves from a coconut palm (*Cocos nucifera*) were disrupted in the presence of Plant RNA Isolation Aid (ThermoFisher Scientific, Waltham, MA, USA). Tissue was lysed and total RNA was recovered using the *mir*Vana™ miRNA Isolation Kit (ThermoFisher Scientific) according to the manufacturer's instructions. RNA concentrations were measured using the Nanodrop instrument.

Total coconut palm RNA was incubated with 1 μM antisense primer, NAD5x4-63-A38 (5'-TTAGGTATTAGTTTTGTAATGGTTGGAGCAGCAAACTC-3') and 0.2U/μL RNase Inhibitor in 1X PrimeScript buffer at 85 °C for 3 minutes, then 60 °C for 10 minutes, then cooled to 25 °C and diluted 10 fold with an RT-LATE-PCR reagent mix to obtain the final concentrations of 100 nM antisense primer, 1 μM sense primer NAD5X2-1213-S25, (5'-CTCGGGAGTCTCTTTGTAGGATACT-3'), 250 nM probe NAD5x4-24-A16 (CR) (5'-CalRed610-TTGGTAGTACGAAGAA-BHQ2-3'), 400 nM dNTPs, 0.25X SYBR Green, 1 μM Reagent 1, 2 U/μL PrimeScript Reverse Transcriptase, 0.06 U/μL Taq DNA Polymerase (Invitrogen), 3 mM $MgCl_2$, 1.5% polyvinylpyrrolidone (PVP) and 1X Reaction Buffer (Invitrogen) in a sample volume of 20 μL. Total palm RNA was tested over the range of 0.5 to 500 nanograms per sample.

Samples were incubated at 50 °C for 10 minutes for reverse transcription, then 95° for 5 minutes, followed immediately by thermal cycling and post-PCR melt analysis using the steps described in example 1.

The real-time amplification results from the one-step RT-LATE-PCR are shown in Figure 2, Part A. SYBR Green fluorescence increase gave mean C_T values that were inversely proportional to the log of the total RNA input. The difference of about 3.5 cycles per 10-fold difference in RNA concentration indicates that amplification of the cDNA was efficient over this range of total palm RNA. Figure 2, Part B shows a large melt peak at 50

°C in the fluorescence derivative from the probe, confirming the *nad5* sequence of the amplified product. Those peak heights as well as the normalized signal above background (not shown) were similar at all RNA inputs, probably because enough amplification product had been made to saturate all of the probe molecules in all samples. A smaller peak at 41°C was also present and may be due to a splicing variant (the probe hybridizes near the 5' end of the exon 4 sequence), nucleotide modification of the transcript (*e.g.*, C to U RNA editing), or to an artifact of PCR amplification. The lack of any probe signal from samples without reverse transcriptase demonstrates the absence of any DNA contamination in the palm RNA sample and/or the inability of the primers to generate the extremely long product using this PCR protocol.

Thus, this control could be useful not only for verifying the quality of a test for detecting infectious agents, but also for quantifying relative levels of that agent by comparing real-time C_T values in separate reactions, or by comparing the real-time SYBR signal of the control to the quantitative probe signal from the infectious agent. It should be possible to obtain quantitative nad5 probe signals at end point if the probe concentration is increased, the number of cycles decreased, and/or the efficiency of the nad5 amplification reduced (*e.g.*, by reducing the concentration and/or T_m of one or both primers).

Example 3. RT-LATE-PCR of nad5 following rapid nucleic acid preparation using PrimeStore™.

One hundred mg of coconut palm leaf tissue was excised, rinsed in RNase-free water, and immersed in 300 µL PrimeStore™ (a molecular transport medium that includes a chaotrope, a reducing agent, a detergent, a chelator and a buffer, Longhorn Vaccines and Diagnostics, Bethesda, MD) supplemented with 2.5% PVP. Tissues were disrupted for 1 hour in a 2 mL tube containing 2 stainless steel beads (5 mm) in a TissueLyzer (Qiagen, Hilden, Germany). Homogenate was clarified by centrifugation for 5 minutes at 14,000g, transferred to a clean RNase-free tube and stored at -80 °C.

Extracts were diluted by a factor of 10, 100, or 1,000 in 10 mM TRIS, pH 8.3, and added to a pre-incubation mix containing antisense primer NAD5x4-63-A38, 0.2U/µL RNase Inhibitor in 1X PrimeScript buffer. Samples were heated at 85 °C for 3 minutes, then 60 °C for 10 minutes, then cooled to 25 °C and diluted 10 fold with an RT-LATE-PCR reagent mix to obtain the final reagent concentrations as the in the previous example, except that some samples did not contain PVP in the RT-LATE-PCR reagent mix. The RT

incubation, denaturation, thermal cycling, and post-PCR melting were done as described in that experiment.

Figures 3A and 3B show the real-time SYBR Green fluorescence plots of nad5 amplification from the PrimeStore-prepared plant nucleic acid and from 50 ng of total coconut palm RNA purified as described in the previous example. Samples in (A) have no added PVP during RT-PCR; samples in (B) contain 1.5% PVP. Final fluorescence was reduced and C_T values were higher than expected in the PrimeStore™ 1/10 dilution samples relative to other dilutions, although the fluorescence reduction was less in samples with PVP. The mean C_T values for PrimeStore™ 1/100 dilution samples was 3.5 cycles lower than the PrimeStore™ 1/1,000 dilution samples; 3.6 cycles lower in the corresponding samples with PVP (Table 4, below), within the range expected for a 10-fold dilution. SYBR Green fluorescence plateaus in those PrimeStore™ samples were at similar or higher levels compared to those obtained from purified RNA. Interestingly, all 4 NTC replicates without PVP showed fluorescence increase (mean C_T value of 34.4), but there was no fluorescence increase in the 4 NTC replicates with PVP. All "no RT" samples had SYBR Green increase (due to non-specific amplification, see below), but the mean C_T value was several cycles higher in samples with PVP. The results with those control samples indicate that the presence of PVP reduces non-specific amplification due to primer dimer formation and from mis-priming on the DNA in the PrimeStore™ samples. Those knowledgeable in the Art understand that such mis-priming events can interfere with amplification from the intended target and may reduce sensitivity to intended targets at low concentrations.

Figure 3, Parts C and D show the nad5 probe fluorescence derivative plots for the same samples without PVP (C) and with PVP (D). Replicates of the PrimeStore™ 1/10 dilution without PVP had relatively low fluorescence from the nad5-specific probe, confirming the inhibition of specific product amplification and/or fluorescence detection. All other PrimeStore™ and purified RNA samples without PVP had strong fluorescence from the nad5-specific probe. Replicates of the PrimeStore™ 1/10 dilution with PVP had fluorescence from the nad5-specific probe at nearly the level observed with higher PrimeStore™ dilutions and purified RNA. Taken together, these results indicate that inhibition of specific product amplification or detection occurs at high concentrations of the PrimeStore™ extract, either due to components from the plant or from the PrimeStore™ itself, but that the presence of 1.5% PVP can at least partially overcome that inhibition. No nad5-specific probe fluorescence was observed above background in control samples

without reverse transcriptase or without RNA, confirming that all SYBR Green fluorescence increase in those samples was due to non-specific amplification. That result also confirms the inability of the nad5 primers to amplify coconut palm DNA, which is present in the PrimeStore™ samples.

TABLE 4. SYBR Green C_T values from RT-LATE-PCR samples with and without PVP.

	C_T values (mean ± std dev)	
Sample	without PVP	with PVP
50 ng RNA	18.4 ± 0.1	18.4 ± 0.1
PrimeStore 1/10	23.6 ± 0.3	22.4 ± 0.2
PrimeStore 1/100	24.9 ± 0.1	24.4 ± 0.2
PrimeStore 1/1000	28.5 ± 0.1	27.9 ± 0.2
no RT	35.8 ± 1.0	38.5 ± 4.0
NTC	33.4 ± 3.3	no C_T

Example 4. One-step RT-LATE-PCR for combined amplification and detection of CCCVd and nad5 RNAs

The synthetic viroid RNA, coconut palm RNA, and the primers and probes described in the previous examples can be combined in a single tube for RT-LATE-PCR. This example shows that the plant gene can serve as a control for reverse transcription and amplification in a detection assay for viroid RNAs. The relative probe signal levels for the viroid and control gene provide a means to quantify the level of viroid RNA in the plant.

Fifty ng of purified coconut palm RNA and 100, or 1,000, or 10,000 copies of synthetic viroid RNA were mixed with 1 µM antisense primer NAD5x4-63-A38 and 1 µM antisense primer OPV9-A40, 0.2 U/µL RNase Inhibitor, and 500 ng Extreme Thermostable Single Strand Binding Protein (New England Biolabs) in 1X PrimeScript buffer. Pre-incubation, 1-step RT-LATE-PCR, and the post-PCR melt program were as described in the previous example with the viroid sense primer and probe from example 1 included in the reagent mix.

Figure 4 shows the probe fluorescence derivative plot averages for each replicate group from the post-PCR melt. The nad5 probe melt peak at 50 °C and the viroid probe melt peak at 65 °C were detected in all samples with both RNAs. The mean height of the nad5

probe melt peak was similar for each replicate group, including those without synthetic viroid RNA, indicating that co-amplification of the viroid sequence did not interfere with nad5 amplification. The mean height of the viroid probe peak (above the mean NTC background) was related to the number of RNA transcripts in the sample. Figure 5 shows a quantification curve based on the ratio of nad5 peak height to viroid peak height in individual samples. Those knowledgeable in the art would recognize that the area under these peaks could also be used for estimating RNA copy number. Thus, it should be possible to estimate the relative levels of viroid infection in different plants or in different regions of an individual plant.

Example 5. High pre-incubation temperatures improve RT-LATE-PCR detection of synthetic viroid RNA in an assay with plant nad5 internal control.

Some RNA molecules, including ribosomal RNA, GC-rich mRNAs, RNA virus non-coding regions, viroids and other circular RNAs have stable secondary structure due to the high stability of RNA to RNA nucleotide interactions. This secondary structure, although dynamic, can slow or prevent the hybridization of the antisense primer at temperatures typically used with reverse transcriptase. This experiment tests the effects of pre-incubating the RNA and antisense primer at different temperatures prior to the reverse transcription step.

Purified coconut palm RNA (without viroid RNA), or a 1/100 dilution of PrimeStore™ preparation of coconut palm nucleic acid plus either 1,000 or 10,000 copies of synthetic viroid RNA were mixed with 1 μM antisense primer NAD5x4-63-A38, 1 μM antisense primer OPV9-A40, 0.2 U/μL RNase Inhibitor, and 500 ng Extreme Thermostable Single Strand Binding Protein (New England Biolabs) in 1X PrimeScript buffer. Three aliquots were prepared for each RNA mixture. One set of aliquots was kept on ice and not heated prior to the addition of the RT-PCR mix. A second set of aliquots was heated to 65 °C for 3 minutes, then 60 °C for 10 minutes. The third set of aliquots was heated to 85 °C for 3 minutes, then 60 °C for 10 minutes. The RT-LATE-PCR reagent mix, the RT-LATE-PCR incubations and cycling, and the post-PCR melt programs were as described in that example.

Figure 6 shows the combined nad5 probe and viroid probe fluorescence derivative plot from post-PCR melting analysis following pre-incubation on ice (A), at 65 °C (B), or 85 °C (C). The nad5 probe melt peak height from purified plant RNA, or from the PrimeStore™ samples was largely unaffected by differences in the pre-incubation temperature. In contrast, detection of the viroid probe melt peak at 65 °C varied depending on the pre-incubation

temperature. Following pre-incubation on ice there was no viroid probe melt peak in any of the samples containing synthetic viroid. Following pre-incubation at 65 °C, a viroid probe melt peak was detected in 2 of 3 replicates containing 10,000 copies of the viroid RNA, but none of the samples containing 1,000 copies of the viroid RNA. Following pre-incubation at 85 °C, a viroid melt peak was detected in all 3 replicates containing 10,000 copies of the viroid RNA and 2 of 3 samples containing 1,000 copies of the viroid RNA.

The results demonstrate that the high degree of secondary structure in the viroid RNA prevents the antisense primer from hybridizing to the probe. The 50 °C temperature of the reverse transcription step is not sufficiently high to enable primer hybridization, for samples pre-incubated on ice. A temperature of 65 °C enables hybridization of the primer to at least some of the viroid RNA molecules. Some fraction of the viroid RNA molecules may be single-stranded at the site of primer hybridization at that temperature. Since intramolecular hybridization (secondary structure formation) is a dynamic process, increasing the duration of the incubation at 65 °C is likely to result in additional primer hybridization and improved detection in subsequent RT-PCR results. Increasing the temperature to 85 °C improved sensitivity of viroid detection, indicating that the percentage of viroid molecules hybridized with the primer is higher at that temperature. Those temperatures are not compatible with most reverse transcriptases. However, as this experiment shows, the primer – viroid hybrid is maintained after the temperature of the sample is lowered, presumably due to the higher thermodynamic stability of that duplex compared to that of the viroid RNA secondary structure. Once the reagents for reverse transcription are added and incubation is continued at a temperature appropriate for that enzyme, the cDNA molecules can be generated.

Example 6. Pre-incubation and RT-LATE-PCR of CCCVd using a 2'O-methyl RNA opener and random hexamers.

RNA was isolated from the leaves of oil palm plants that had symptoms of infection by CCCVd. The RNA was extracted using a combination of TRIzol reagent (ThermoFisher Scientific) and Chloroform, followed by purification with miRNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. The precipitated RNA was resuspended in RNase-free water and stored at -80 °C. Upon initial thaw, the sample was diluted 10 fold in The RNA Storage Solution (ThermoFisher Scientific), containing 1 U/µL RNase inhibitor.

A pre-incubation mix of oil palm RNA (1 µL per 10 µL final volume), 4 µM (non-extendible) 2'O-methyl RNA opener (5'-GGCCGGGCGUCGAAGCUACGAAGGAGUC-(C3spacer)-3' with all 2'-O-methyl nucleosides and a predicted T_m of 89.6°C at 1 µM), 5 µM

random hexamer primers (Clontech Laboratories), and 1 U/μL RNase Inhibitor in PrimeScript buffer. The opener is fully complementary to nucleotides 124 through 151 of the viroid. Those viroid nucleotides are partially complementary with nucleotides 121 through 94 in the viroid and are hybridized with those nucleotides in predicted secondary structures. Hybridization of the opener to the viroid RNA should allow primers to access to that latter segment of RNA nucleotides. The pre-incubation mix was heated at 75 °C for 3 minutes, 70 °C for 3 minutes, 65 °C for 5 minutes, then 60 °C for 30 minutes, cooled to room temperature, then placed on ice. The pre-incubation mix was combined with an equal volume of a 2X RT reagent mix to obtain final concentrations of 200 nM dNTPs, 1 U/μL RNase Inhibitor, and 5 U/μL PrimeScript reverse transcriptase in 1X PrimeScript buffer. Reverse transcription incubation was at 30 °C for 10 minutes, 42°C for 10 minutes, 85 °C for 5 minutes (to inactivate the reverse transcriptase), cooled to 25 °C, then placed on ice.

Each LATE-PCR sample included 2 μL of the reverse transcription sample diluted into 18 μL of a reagent mix to obtain final concentrations of 50 nM antisense primer OPV91-A25 (5'-TTCGCACGATCGACCCAGGTACGCT-3'), 1 μM sense primer OPV16-S30t (5'-TCTTTGTAGCCTCTCTGGGGAAATCTACAG-3), 50 nM probe OPV-nt31-A26 (5'-Quasar670-AAGCCTCTCCTGCAGTAGTTTTTGTT-BHQ2-3'), 400 nM dNTPs, 0.24X SYBR Green, and 0.06 U/μL Platinum Tfi DNA polymerase (exo-) (ThermoFisher Scientific) in 1X Platinum Tfi buffer. A two minute incubation at 95 °C was followed by 60 cycles of 95 °C for 10 second and 68°C for 45 seconds with fluorescence detection, then cooled from 68°C to 45 °C at approximately 2°C per minute and held at 45 °C for 2 minutes. Temperature was then increased in 0.5 °C steps from 45 °C to 95 °C, measuring SYBR Green and Quasar fluorescence at each step.

All four replicate samples with the coconut palm RNA had SYBR Green fluorescence increase with a mean C_T value of 37.0 and a product melt peak of 87.7°C (plots not shown). The Quasar labeled probe used in this experiment had a melt derivative peak at the expected temperature of about 57°C (Figure 7), confirming the presence of the viroid RNA infection in the plant. One of the no reverse transcription controls with the plant RNA sample had a higher C_T value of 40.7, a (SYBR Green) product melt peak at 87.7°C, and a probe melt peak at 57°C. That result might be due to a low-level of viroid DNA in the RNA sample, or to contamination of the no RT sample with a cDNA molecule (from the RNA samples) prior to the LATE-PCR step. The 3 other no RT samples and 4 NTC samples

showed either no amplification or amplification of non-specific products that did not generate a probe signal.

Example 7. High temperature pre-incubation of primers and RNA improves two-step RT-LATE-PCR results for CCCVd RNA in the presence of ThermaStop-RT.

This example demonstrates the improvements obtained using a pre-incubation step with primers and RNA template over a wide range of RNA target concentrations. This ability in enhanced by using ThermaStop-RT, a modified oligonucleotide that includes 2'-O-methyl RNA nucleotides at each end of the molecule (shown as mA, mC, mG, and mU in the sequences below). ThermaStop-RT greatly reduces activity of reverse transcriptase at room temperature, thereby providing a hot-start for the RT step and greatly reducing non-specific amplification in the subsequent PCR.

RNA with the CCCVd sequence was transcribed in vitro as described above and stored at -80 °C. An aliquot of the RNA was thawed and serially diluted using 10 mM TRIS, pH 8.0 containing 1 unit/microliter RNase Inhibitor (Takara). Samples of RNA at each dilution were mixed with 500 nM primer OPV9-A40, 50 ng/microliter Extreme Thermostable Single-Stranded DNA Binding Protein (ET-SSB, New England Biolabs), and 2 units/microliter RNase Inhibitor in 1X First Strand Buffer (SuperScript III First-Strand Synthesis System, ThermoFisher Scientific) and were incubated 3 minutes at 85 °C, 10 minutes at 65 °C, then cooled to 25 °C. The pre-incubation mixes were then diluted with an equal volume of an RT reagent mixture to obtain final concentrations of 250 nM primer OPV9-A20, 5,000 nM primer OPV-184 S24, 25 ng/microliter ET-SSB, 1 unit/microliter RNase Inhibitor, 0.4 mM each dNTP, 4 micromolar ThermaStop-RT (5'- Black Hole Quencher 1 - mUmAmAmUmAmGmUmGmUmACCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC CmUmGmUmAmUmUmAmUmUmA-Biosearch Blue - 3'), and 2.5 units/microliter SuperScript III reverse transcriptase in 1X First Strand Buffer. Other samples of the serially diluted CCCVd RNA were not pre-incubated with primer, but were mixed directly with the above components at the same final concentrations. Aliquots were removed from the RT mixtures prior to the addition of reverse transcriptase in order to provide negative controls. All RT samples were incubated 10 minutes at 50 °C, 2 minutes at 95 °C, and cooled to 25 °C. RT samples were then diluted 1:4 with a PCR reagent mixture containing 0.4 mM dNTP, 0.3X SYBR Green, 1.25 micromolar ThermaStop DBB (5'- Dabcyl - GAATAATATAGCCCCCCCCCCCCCCCCCCCCCCCCCCCCCTATATTATTC -

Biosearch Blue -3'), 625 nM probe OPV nt197-A20 Cal Red, 3 mM magnesium chloride, and 0.075 units/microliter Invitrogen Taq Polymerase in 1X Invitrogen PCR buffer. Four PCR replicate samples of 20 microliters were prepared from each RT sample. PCR cycling and post-PCR melting were done using a Stratagene Mx3005P. The thermal profile included 95 °C for 2 minutes, followed by 50 cycles of 95 °C for 10 seconds, 68°C for 10 seconds, and 72°C for 35 seconds with fluorescence detection during the 72°C step. Samples were cooled at a rate of approximately 4°C per minute to 30 °C, held for 2 minutes at 30 °C, and melted in 0.5 °C increments to a final temperature of 95 °C. Each melt step was 35 seconds in duration to enable 3 fluorescent endpoint detections for SYBR Green and Cal Red. Real-time SYBR Green fluorescence was analyzed using the adaptive baseline settings of the Mx3005P software.

Real-time SYBR Green fluorescence showed that pre-incubation of RNA with primers lowered C_T values by an average of 4.4 cycles relative to those of samples without pre-incubation. The plots of mean C_T values vs RNA dilution for seven orders of magnitude is shown in Figure 8. One of the 10^{-11} RNA dilution samples without pre-incubation failed to generate fluorescence increase. SYBR Green melt fluorescence detected a single product peak at the expected melting temperature for the CCCVd amplicon in all samples (not shown). The results indicate that pre-incubation of primer and RNA target increases the amount of CCCVd-specific cDNA more than 10-fold, improving both efficiency and sensitivity of RT-PCR. The 10 minute RT step used in this experiment is shorter than that typically used and is likely to decrease the transcription of non-specific products relative to that observed with a longer RT step.

Example 8. ThermaStop-RT reduces non-specific amplification and improves duplex RT-LATE-PCR of CCCVd and nad5 transcripts from coconut palm RNA.

Reverse transcription of non-specific products is a potential problem for RT-PCR from cellular RNA and can reduce the efficiency and sensitivity for the desired RNA targets. Use of multiple primer pairs can increase that problem further. The reagent ThermaStop-RT described in the previous example is a variation of ThermaStop, a reagent designed to interact with DNA polymerase and thereby provide a hot-start for PCR. Similarly, ThermaStop-RT is designed to have a higher affinity with reverse transcriptase, greatly reducing the activity of the enzyme at low temperatures (*e.g.*, 0 °C, 4°C, 18°C, 25 °C), but not at temperatures used for reverse transcription (*e.g.*, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C), thereby providing a hot-start for RT-PCR. This experiment demonstrates the improvement in

RT-LATE-PCR results using a hot start for reverse transcription. It also demonstrates that two distinct RNA targets can be quantified following a duplex reverse transcription followed by LATE-PCR, even when those targets vary in concentration by several orders of magnitude.

The reverse transcription procedure was similar to that used in the previous example with the following modifications. Primer nad5x4-63-A38 and primer nad5x2-1213-S25 were included in the RT mixtures at concentrations of 250 nM and 5,000 nM, respectively. Two RT mixtures were prepared, one containing 4 micromolar ThermaStop-RT and the other without that reagent. RNA isolated from coconut palm (using the same 10^{-2} dilution for all samples) and serial dilutions of synthetic CCCVd RNA (prepared by in vitro transcription) were mixed directly with the RT mixtures without a pre-incubation step and were incubated at 50 °C for 30 minutes, then 95 °C for 2 minutes, then cooled to 25 °C. The PCR reagent mixture was similar to that used in the previous example, but also included 625 nM probe nad5x4-24-A16-Cal Red. It should be noted that all samples included ThermaStop DBB during PCR to provide hot start for DNA polymerase. Thermal cycling and melt profiles were identical to those of the previous example.

SYBR Green dye binds double-stranded DNA from both of the specific products and from non-specific amplification and therefore could not be used to quantify individual targets. RT-LATE-PCR generates single-stranded DNA from each of the targeted RNA sequences and the relative quantities of a product can be measured using sequence-specific probes during post-PCR melting analysis. Both probes used in this experiment are labeled with a Cal Red fluorophore, but can be distinguished by melting temperature; the CCCVd probe melting at 61°C, and the nad5 probe melting at 50 °C. All samples had the same initial concentration of coconut palm RNA and generated a nad5 probe melt peak at 50 °C. The mean peak height (relative to a baseline from samples without reverse transcriptase) was 342 units with a standard deviation of 36 units for the 28 samples with ThermaStop. The mean peak height was 283 units with a standard deviation of 39 units for the 28 samples without ThermaStop-RT. The difference is extremely statistically significant ($P < 0.0001$) and the lower mean quantity of the nad5 amplicon in samples without ThermaStop-RT is likely due to an increased synthesis of non-specific cDNA formed during the RT step.

Figure 9 shows the mean derivative peak height of the CCCVd probe for samples either with or without ThermaStop-RT and CCCVd RNA at dilutions of 10^{-6} to 10^{-11}. The peak heights were roughly proportional to the logarithm of the initial concentration of

CCCVd RNA. Samples with dilutions of 10^{-7} to 10^{-10} showed higher mean peak values for samples with ThermaStop-RT compared to samples without ThermaStop-RT. The difference became greater as the initial RNA concentration decreased. At a dilution of 10^{-11}, only 2 of 4 samples with ThermaStop and none of the 4 samples without ThermaStop had a detectable melt peak above baseline. Dilutions of 10^{-12} were also tested, but none had a detectable CCCVd probe melt peak. These results also support the conclusion that higher levels of non-specific cDNA are formed during reverse transcription in the absence of ThermaStop-RT and can reduce specific amplification from the intended RNA targets.

The results also demonstrate that RT-LATE-PCR can quantify the initial concentrations of multiple targets even when that concentration may differ by several orders of magnitude. This is not possible using with symmetric RT-PCR (primer pairs at the same concentration), as amplification of the more abundant target generates large quantities of double-stranded DNA that inhibit further amplification and interfere with the detection of the less abundant target.

Incorporation by Reference

All publications, patents, and patent applications mentioned herein are hereby incorporated by reference in their entirety as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We claim:

1. A method of amplifying a CCCVd nucleic acid, comprising

(a) forming a reaction mixture comprising a CCCVd nucleic acid target molecule comprising a CCCVd nucleic acid target sequence and one or more primers that hybridize to the CCCVd nucleic acid target sequence, wherein the reaction mixture does not include a nucleic acid polymerase;

(b) incubating the reaction mixture at a temperature between 50 °C and the melting temperature of the one or more primers hybridized to the target sequence;

(c) lowering the temperature of the reaction mixture;

(d) adding a nucleic acid polymerase to the reaction mixture; and

(e) incubating the reaction mixture under conditions such that the one or more nucleic acid primers is extended by the nucleic acid polymerase to create an amplification product comprising the CCCVd nucleic acid target sequence or a complement thereof.

2. The method of claim 1, wherein the CCCVd nucleic acid target molecule is isolated from a sample and prepared in a solution comprising a chaotrope, a reducing agent, a detergent, a chelator and a buffer prior to step (a).

3. The method of claim 2, wherein the reducing agent is 2 mercaptoethanol, tris(2-carboxyethyl)phosphine, dithiothreitol, dimethylsulfoxide, or any combination thereof.

4. The method of claim 2 or claim 3, wherein the chaotrope is guanidine thiocyanate, guanidine isocyanate, guanidine hydrochloride, or any combination thereof.

5. The method of any one of claims 2 to 4, wherein the detergent is sodium dodecyl sulfate, lithium dodecyl sulfate, sodium taurodeoxycholate, sodium taurocholate, sodium glycocholate, sodium deoxycholate, sodium cholate, sodium alkylbenzene sulfonate, N-lauroyl sarcosine, or any combination thereof.

6. The method of any one of claims 2 to 5, wherein the chelator is ethylene glycol tetraacetic acid, hydroxyethylethylenediaminetriacetic acid, diethylene triamine pentaacetic acid, N,N-bis(carboxymethyl)glycine, ethylenediaminetetraacetic, citrate anhydrous, sodium citrate, calcium citrate, ammonium citrate, ammonium bicitrate, citric acid, diammonium citrate, ferric ammonium citrate, lithium citrate, or any combination thereof.

7. The method of any one of claims 2 to 6, the wherein the buffer is tris(hydroxymethyl)aminomethane, citrate, 2-(N-morpholino)ethanesulfonic acid, N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 1,3-bis(tris(hydroxymethyl)methyl

amino)propane, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 3-(N-morpholino) propanesulfonic acid, bicarbonate, phosphate, or any combination thereof.

8. The method of claim 1, wherein the one or more nucleic acid primers are diluted at least 5-fold prior step (e)

9. The method of any one of claims 1 to 8, wherein the reaction mixture is incubated in step (b) at a temperature of at least 60 °C.

10. The method of any one of claims 1 to 8, wherein the reaction mixture is incubated in step (b) at a temperature of at least 65 °C.

11. The method of any one of claims 1 to 8, wherein the reaction mixture is incubated in step (b) at a temperature of at least 70 °C.

12. The method of any one of claims 1 to 8, wherein the reaction mixture is incubated in step (b) at a temperature of at least 75 °C.

13. The method of any one of claims 1 to 8, wherein the reaction mixture is incubated in step (b) at a temperature of at least 80 °C.

14. The method of any one of claims 1 to 8, wherein the reaction mixture is incubated in step (b) at a temperature of at least 85 °C.

15. The method of any one of claims 1 to 14, wherein the reaction mixture is lowered to a temperature of no more than 30 °C in step (c).

16. The method of any one of claims 1 to 14, wherein the reaction mixture is lowered to a temperature of no more than 25 °C in step (c).

17. The method of any one of claims 1 to 14, wherein the reaction mixture is lowered to a temperature of no more than 20 °C in step (c).

18. The method of any one of claims 1 to 17, further comprising sequentially incubating the reaction mixture at two or more temperatures in step (b), wherein each temperature is between 50 °C and the melting temperature of the one or more primers hybridized to target sequence.

19. The method of any one of claims 1 to 18, wherein the reaction mixture is incubated in step (b) for at least 5 seconds.

20. The method of any one of claims 1 to 18, wherein the reaction mixture is incubated in step (b) for at least 10 seconds.

21. The method of any one of claims 1 to 18, wherein the reaction mixture is incubated in step (b) for at least 30 seconds.

22. The method of any one of claims 1 to 21, wherein the melting temperature of the one or more primers hybridized to target sequence is at least 85 °C.

23. The method of any one of claims 1 to 21, wherein the melting temperature of the one or more primers hybridized to target sequence is at least 90 °C.

24. The method of any one of claims 1 to 21, wherein the melting temperature of the one or more primers hybridized to target sequence is at least 95 °C.

25. The method of any one of claims 1 to 24, wherein the one or more primers comprise a primer comprising the sequence 5'TTCTGCAGTAGTTTTTGGGGTGCCCTGTAGAT3'.

26. The method of any one of claims 1 to 24, wherein the one or more primers comprise a primer comprising the sequence e 5'-TTGGCCTCTCCTGCAGTAGTTTTTGGGGTGCCCTGTAGAT3'.

27. The method of any one of claims 1 to 24, wherein the one or more primers comprise a primer comprising the sequence 5'TTGGGAGACTACCCGGTGGATACA3'.

28. The method of any one of claims 1 to 24, wherein the one or more primers comprise a primer comprising the sequence 5'TTCGCACGATCGACCCAGGTACGCT-3'.

29. The method of any one of claims 1 to 24, wherein the one or more primers comprise a primer comprising the sequence 5'TCTTTGTAGCCTCTCTGGGGAAATCTACAG3'.

30. The method of any one of claims 1 to 29, further comprising adding a probe to the reaction mixture in step (d) or step (e).

31. The method of claim 30, wherein the probe comprises the sequence 5' 5'-TTGGTAGTACGAAGAA-3'.

32. The method of claim 30, wherein the probe comprises the sequence 5'-ATGTAAGAGCCGCGTGAGAT3'.

33. The method of claim 30, wherein the probe comprises the sequence 5' AAGCCTCTCCTGCAGTAGTTTTTGTT-3'.

34. The method of any one of claims 30 to 33, wherein the probe comprises a fluorophore.

35. The method of any one of claims 1 to 35, wherein the nucleic acid is an RNA.

36. The method of any one of claims 1 to 35, wherein the nucleic acid polymerase is reverse transcriptase.

37. The method of any one of claims 1 to 36, further comprising adding a second nucleic acid polymerase to the reaction mixture in step (c).

38. The method of claim 37, wherein the second nucleic acid polymerase is DNA polymerase.

39. The method of any one of claims 1 to 38, wherein at least one mispriming prevention reagent is added to the reaction mixture.

40. The method of claim 39, wherein the mispriming prevention reagent comprises a nucleic acid molecule comprising, in 5′ to 3′ order:

(i) a first condition-dependent stem region comprising a 5′ terminal covalently linked moiety and a first stem nucleic acid sequence, wherein the first stem nucleic acid sequence is at least 6 nucleotides in length and wherein the 5′ terminal covalently linked moiety comprises a cyclic or polycyclic planar moiety that does not have a bulky portion;

(ii) a condition-dependent loop region comprising a loop nucleic acid sequence of at least 3 nucleotides in length; and

(iii) a second condition-dependent stem region comprising a second stem nucleic acid sequence and a 3′ terminal covalently linked moiety, wherein the second stem nucleic acid sequence is at least 6 nucleotides in length and is complementary to the first stem nucleic acid sequence and wherein the 3′ terminal covalently linked moiety comprises a cyclic or polycyclic planar moiety that does not have a bulky portion, and wherein the 3′ terminus of the second stem region is non-extendable by a DNA polymerase,

wherein the first condition-dependent stem region hybridizes to the second condition-dependent stem region in a temperature dependent manner to acquire a stem-loop hairpin conformation.

41. The method of claim 40, wherein the loop nucleic acid sequence is a single nucleotide repeat sequence.

42. The method of claim 40 or 41, wherein the single nucleotide repeat sequence is a poly-cytosine sequence.

43. The method of any one of claims 40 to 42, wherein the loop nucleic acid sequence is between 25 and 40 nucleotides in length.

44. The method of any one of claims 40 to 43, wherein the first condition-dependent stem region hybridizes to the second condition-dependent stem region with a melting temperature of between 40 °C and 71 °C.

45. The method of any one of claims 40 to 44, wherein the first stem nucleic acid sequence and the second stem nucleic acid sequence are each 11 nucleotides in length.

46. The method of claim 39, wherein the mispriming prevention reagent is an oligonucleotide that has a 3' end and a stem-loop structure having a stem comprising a double stranded region that has a length greater than six nucleotides and a terminus away from the loop comprising a 3' nucleotide and a 5' nucleotide, the stem having a calculated stem melting temperature (Tm) below 94° C, wherein

a) the 3' end is non-extendable by a DNA polymerase,

b) the oligonucleotide is not fluorescently labeled and does not contribute background fluorescence, and

c) the stem terminus is stabilized by means selected from the group consisting of non-fluorescent fluorophore quenching moieties covalently attached to the 3' and 5' nucleotides of the stem terminus and pairs of non-natural nucleotides that bind more strongly than a natural DNA-DNA hybrid and that include each of the 3' and 5' nucleotides of the stem terminus.

47. A method for amplifying CCCVd nucleic acid from a sample comprising:

(a) forming a reaction mixture comprising:

(i) a CCCVd nucleic acid molecule comprising an CCCvd nucleic acid target sequence;

(ii) a non-extendable oligonucleotide; and

(iii) an extendable nucleic acid primer; and

(b) incubating the reaction mixture at one or more temperatures for a period of time to hybridize the non-extendable oligonucleotide and the extendable nucleic acid primer to the CCCvd nucleic acid target sequence;

wherein the extendable nucleic acid primer and the non- extendable oligonucleotide hybridize to a regions of the nucleic acid target sequence that are non-overlapping, and the non-extendable oligonucleotide hybridized to the CCCvd nucleic acid target sequence has a predicted melting temperature that is at least 10 °C higher than the predicted melting temperature of the extendable nucleic acid primer hybridized to the CCCvd nucleic acid target sequence.

48. The method of claim 47, wherein the CCCvd nucleic acid is prepared in a solution comprising a chaotrope, a reducing agent, a detergent, a chelator and a buffer prior to step (a).

49. The method of claim 48, wherein the reducing agent is 2 mercaptoethanol, tris(2-carboxyethyl)phosphine, dithiothreitol, dimethylsulfoxide, or any combination thereof.

50. The method of claim 48 or 49, wherein the chaotrope is guanidine thiocyanate, guanidine isocyanate, guanidine hydrochloride, or any combination thereof.

51. The method of any one of claims 48 to 50, wherein the detergent is sodium dodecyl sulfate, lithium dodecyl sulfate, sodium taurodeoxycholate, sodium taurocholate, sodium glycocholate, sodium deoxycholate, sodium cholate, sodium alkylbenzene sulfonate, N-lauroyl sarcosine, or any combination thereof.

52. The method of any one of claims 48 to 51, wherein the chelator is ethylene glycol tetraacetic acid, hydroxyethylethylenediaminetriacetic acid, diethylene triamine pentaacetic acid, N,N-bis(carboxymethyl)glycine, ethylenediaminetetraacetic, citrate anhydrous, sodium citrate, calcium citrate, ammonium citrate, ammonium bicitrate, citric acid, diammonium citrate, ferric ammonium citrate, lithium citrate, or any combination thereof.

53. The method of any one of claims 48 to 52, the wherein the buffer is tris(hydroxymethyl)aminomethane, citrate, 2-(N-morpholino)ethanesulfonic acid, N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 1,3-bis(tris(hydroxymethyl)methyl amino)propane, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 3-(N-morpholino) propanesulfonic acid, bicarbonate, phosphate, or any combination thereof.

54. The method of any one of claims 47 to 53, wherein the non-extendable oligonucleotide, hybridized to the CCCVd nucleic acid target sequence has a predicted melting temperature of at least 85 °C .

55. The method of any one of claims 47 to 53, wherein the non-extendable oligonucleotide hybridized to the CCCVd nucleic acid target sequence has a predicted melting temperature of at least 90 °C .

56. The method of any one of claims 47 to 53, wherein the non-extendable oligonucleotide hybridized to the CCCVd nucleic acid target sequence has a predicted melting temperature of at least 95 °C.

57. The method of any one of claims 47 to 56, wherein the non-extendable oligonucleotide comprises the sequence 5'GGCCGGGCGUCGAAGCUACGAAGGAGUC-3'.

58. The method of any one of claims 47 to 57, wherein the extendable nucleic acid primer comprises the sequence 5'TTCTGCAGTAGTTTTGGGGTGCCCTGTAGAT3'.

59. The method of any one of claims 47 to 57, wherein the extendable nucleic acid primer comprises the sequence 5'-TTGGCCTCTCCTGCAGTAGTTTTGGGGTGCCCTGTAGAT3'.

60. The method of any one of claims 47 to 57, whereinthe extendable nucleic acid primer comprises the sequence 5'TTGGGAGACTACCCGGTGGATACA3'.

61. The method of any one of claims 47 to 57, wherein the extendable nucleic acid primer comprises the sequence 5'TTCGCACGATCGACCCAGGTACGCT-3'.

62. The method of any one of claims 47 to 57, wherein the extendable nucleic acid primer comprises the sequence 5'TCTTTGTAGCCTCTCTGGGGAAATCTACAG3'.

63. The method of any one of claims 47 to 62, further comprising adding a probe to the reaction mixture.

64. The method of claim 63, wherein the probe comprises the sequence 5' TTGGTAGTACGAAGAA-3'.

65. The method of claim 63, wherein the probe comprises the sequence 5'-ATGTAAGAGCCGCGTGAGAT3',

66. The method of claim 63, wherein the probe comprises the sequence 5' AAGCCTCTCCTGCAGTAGTTTTTGTT-3'.

67. The method of any one of claims 63 to 66, wherein the probe comprises a fluorophore.

68. The method of any one of claims 47 to 67, wherein the CCCVd target nucleic acid molecule is RNA. .

69. The method of any one of claims 47 to 68, wherein the non-extendable oligonucleotide comprises a chemical modification.

70. The method of claim 69, wherein 3′ terminus on the non-extendable oligonucleotide comprises a 2'-O-methyl nucleoside.

71. The method of any one of claims 47 to 70, wherein the extendable nucleic acid primer comprises a random sequence of nucleotides.

72. The method of any one of claims 47 to 70, wherein the extendable nucleic acid primer is a sequence specific primer.

73. The method of any one of claims 47 to 72, wherein the one or more temperatures comprises a temperature of at least 85 °C.

74. The method of any one of claims 47 to 72, wherein the one or more temperatures comprises a temperature of at least 80 °C.

75. The method of any one of claims 47 to 72, wherein the one or more temperatures comprises a temperature of at least 75 °C.

76. The method of any one of claims 47 to 72, wherein the one or more temperatures comprises a temperature of at least 70 °C.

77. The method of any one of claims 47 to 72, wherein the one or more temperatures comprises a temperature of at least 65 °C.

78. The method of any one of claim 47 to 77, wherein the period of time is one minute.

79. The method of any one of claim 47 to 77, wherein the period of time is thirty minutes.

80. The method of any one of claims 47 to 79, the method further comprises adding a nucleic acid polymerase to the reaction mixture and incubating the reaction mixture under conditions such that the extendable nucleic acid primer is extended by the nucleic acid polymerase to create an amplification product comprising the target nucleic acid sequence or a complement thereof.

81. The method of claim 80, wherein a mispriming prevention reagent is added to the reaction mixture.

82. The method of claim 81, wherein the mispriming prevention reagent comprises a nucleic acid molecule comprising, in 5′ to 3′ order:

(i) a first condition-dependent stem region comprising a 5′ terminal covalently linked moiety and a first stem nucleic acid sequence, wherein the first stem nucleic acid sequence is at least 6 nucleotides in length and wherein the 5′ terminal covalently linked moiety comprises a cyclic or polycyclic planar moiety that does not have a bulky portion;

(ii) a condition-dependent loop region comprising a loop nucleic acid sequence of at least 3 nucleotides in length; and

(iii) a second condition-dependent stem region comprising a second stem nucleic acid sequence and a 3′ terminal covalently linked moiety, wherein the second stem nucleic acid sequence is at least 6 nucleotides in length and is complementary to the first stem nucleic acid sequence and wherein the 3′ terminal covalently linked moiety comprises a cyclic or polycyclic planar moiety that does not have a bulky portion, and wherein the 3′ terminus of the second stem region is non-extendable by a DNA polymerase,

wherein the first condition-dependent stem region hybridizes to the second condition-dependent stem region in a temperature dependent manner to acquire a stem-loop hairpin conformation.

83. The method of claim 82, wherein the loop nucleic acid sequence is a single nucleotide repeat sequence.

84. The method of claim 82, wherein the single nucleotide repeat sequence is a poly-cytosine sequence.

85. The method of claim 82, wherein the loop nucleic acid sequence is between 25 and 40 nucleotides in length.

86. The method of any one of claims 82 to 85, wherein the first condition-dependent stem region hybridizes to the second condition-dependent stem region with a melting temperature of between 40 °C and 71 °C.

87. The method of any one of claims 82 to 86, wherein the first stem nucleic acid sequence and the second stem nucleic acid sequence are each 11 nucleotides in length.

88. The method of claim 81, wherein the mispriming prevention reagent is an oligonucleotide that has a 3' end and a stem-loop structure having a stem comprising a doublestranded region that has a length is greater than six nucleotides and a terminus away from the loop comprising a 3' nucleotide and a 5' nucleotide, the stem having a calculated stem melting temperature (Tm) below 94° C, wherein

a) the 3' end is non-extendable by the DNA polymerase,

b) the oligonucleotide is not fluorescently labeled and does not contribute background fluorescence, and

c) the stem terminus is stabilized by means selected from the group consisting of non-fluorescent fluorophore quenching moieties covalently attached to the 3' and 5' nucleotides of the stem terminus and pairs of nonnatural nucleotides that bind more strongly than a natural DNA-DNA hybrid and that include each of the 3' and 5' nucleotides of the stem terminus.

89. A method of amplifying CCCVd nucleic acid, comprising

(a) forming a reaction mixture comprising an CCCVd nucleic acid target molecule comprising an CCCVd nucleic acid target sequence, one or more primers that hybridize to the CCCVd nucleic acid target sequence, wherein the one or more primers comprise a primer that has a predicted melting temperature of at least 85°C with the nucleic acid target sequence and a nucleic acid polymerase; and

(b) incubating the reaction mixture under conditions such that the one or more nucleic acid primers is extended by the nucleic acid polymerase to create an amplification product comprising the nucleic acid target sequence or a complement thereof.

90. The method of claim 89, wherein the CCCVd nucleic acid target molecule is RNA.

91. The method of claim 90, wherein the nucleic acid polymerase is reverse transcriptase.

92. The method of claim 91, wherein the reaction mixture further comprises a DNA polymerase.

93. A method of amplifying a CCCVd RNA, comprising

(a) lysing cells in a solution comprising a chaotrope, a reducing agent, a detergent, a chelator and a buffer and application of mechanical disruption to form a nucleic acid solution comprising a CCCVd RNAtarget molecule;

(b) diluting the nucleic acid solution without performing a nucleic acid purification step to form a reaction mixture comprising the CCCVd RNA target molecule, one or more primers that hybridize to the nucleic acid target molecule and a nucleic acid polymerase; and

(c) incubating the reaction mixture under conditions such that the one or more nucleic acid primers is extended by the nucleic acid polymerase to create an amplification product comprising a CCCVd nucleic acid target sequence in the CCCVd RNA target molecule or a complement thereof.

94. The method of claim 93, wherein the reducing agent is 2 mercaptoethanol, tris(2-carboxyethyl)phosphine, dithiothreitol, dimethylsulfoxide, or any combination thereof.

95. The method of claim 93 or claim 94, wherein the chaotrope is guanidine thiocyanate, guanidine isocyanate, guanidine hydrochloride, or any combination thereof.

96. The method of any one of claims 93 to 95, wherein the detergent is sodium dodecyl sulfate, lithium dodecyl sulfate, sodium taurodeoxycholate, sodium taurocholate, sodium glycocholate, sodium deoxycholate, sodium cholate, sodium alkylbenzene sulfonate, N-lauroyl sarcosine, or any combination thereof.

97. The method of any one of claims 93 to 96, wherein the chelator is ethylene glycol tetraacetic acid, hydroxyethylethylenediaminetriacetic acid, diethylene triamine pentaacetic acid, N,N-bis(carboxymethyl)glycine, ethylenediaminetetraacetic, citrate anhydrous, sodium citrate, calcium citrate, ammonium citrate, ammonium bicitrate, citric acid, diammonium citrate, ferric ammonium citrate, lithium citrate, or any combination thereof.

98. The method of any one of claims 93 to 97, the wherein the buffer is tris(hydroxymethyl)aminomethane, citrate, 2-(N-morpholino)ethanesulfonic acid, N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 1,3-bis(tris(hydroxymethyl)methyl amino)propane, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 3-(N-morpholino) propanesulfonic acid, bicarbonate, phosphate, or any combination thereof.

99. The method of any one of claims 93 to 98, wherein the nucleic acid solution is diluted in step (b) by an amount sufficient to reduce the concentration of the chaotrope, the

reducing agent, the detergent, the chelator and the buffer to a level whereby the nucleic acid polymerase has at least 20% of the activity it has in a reaction mixture that does not comprise the chaotrope, the reducing agent, the detergent, the chelator and the buffer.

100. The method of any one of claims 93 to 99, wherein the diluting in step (b) is performed in two or more steps.

101. The method of claim 100, wherein the first dilution step is carried out in a buffer that contains at least one DNA oligonucleotide primer and wherein said first-dilution step is followed by heating to at least 85 °C followed by gradual cooling, prior to a second dilution step.

102. The method of any one of claims 93 to 101, wherein the nucleic acid polymerase is reverse transcriptase.

103. The method of any one of claims 93 to 102, wherein the nucleic acid solution is diluted in step (b) by a factor of at least 10-fold.

104. The method of any one of claims 93 to 102, wherein the nucleic acid solution is diluted in step (b) by a factor of at least 50-fold.

105. The method of any one of claims 93 to 104, wherein the cells comprise plant cells, animal cells, fungus cells, bacterial cells or parasite cells.

106. The method of any one of claims 93 to 105, further comprising co-amplification of a non-viroid target sequence by incubating the reaction mixture under conditions such that the one or more nucleic acid primers is extended by a nucleic acid polymerase to create an amplification product comprising the non-viroid target sequence or a complement thereof.

107. The method of claim 106, wherein the non-viroid target sequence comprises transcripts of nad5.

108. The method of claim 106, wherein the one or more nucleic acid primers comprise a primer comprising the sequence 5'-TTAGGTATTAGTTTTGTAATGGTTGGAGCAGCAAACTC3'.

109. The method of claim 106, wherein the one or more nucleic acid primers comprise a primer comprising the sequence 5'CTCGGGAGTCTCTTTGTAGGATACT3'

Figure 1

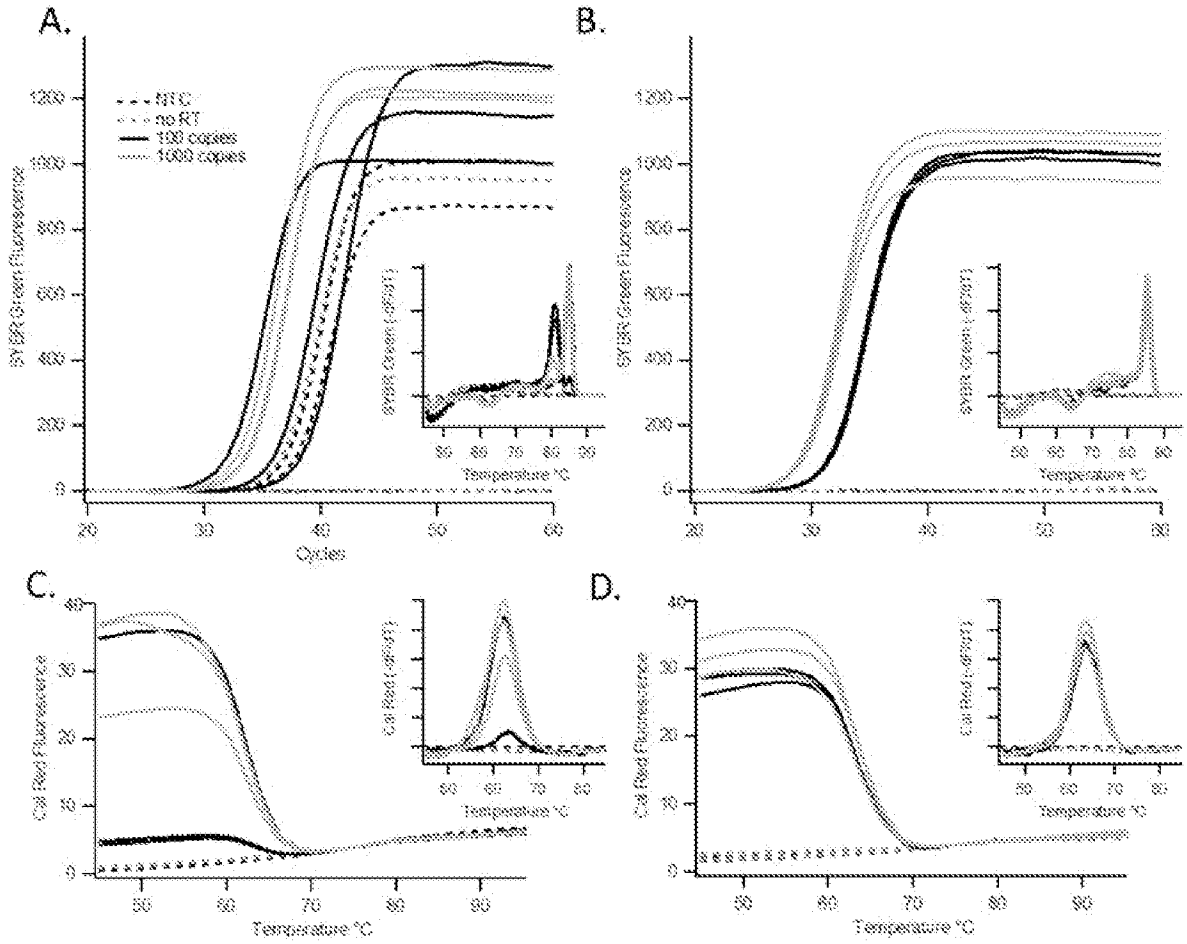

A.
B.
C.
D.
NTC
no RT
100 copies
1000 copies
SYBR Green Fluorescence
Cycles
Temperature °C

Figure 2

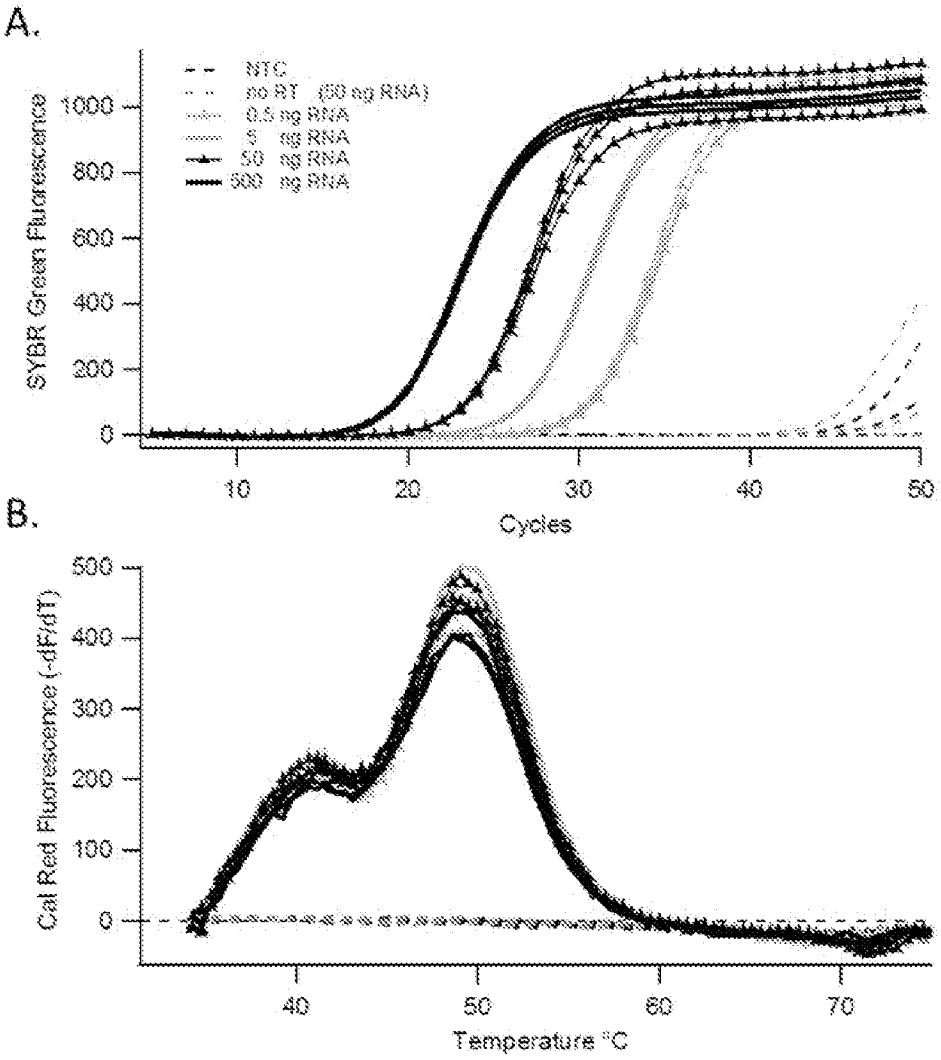

A.
NTC
no RT (50 ng RNA)
0.5 ng RNA
5 ng RNA
50 ng RNA
500 ng RNA
SYBR Green Fluorescence
1000
800
600
400
200
0
10
20
30
40
50
Cycles
B.
Cal Red Fluorescence (-dF/dT)
500
400
300
200
100
0
40
50
60
70
Temperature °C

Figure 3

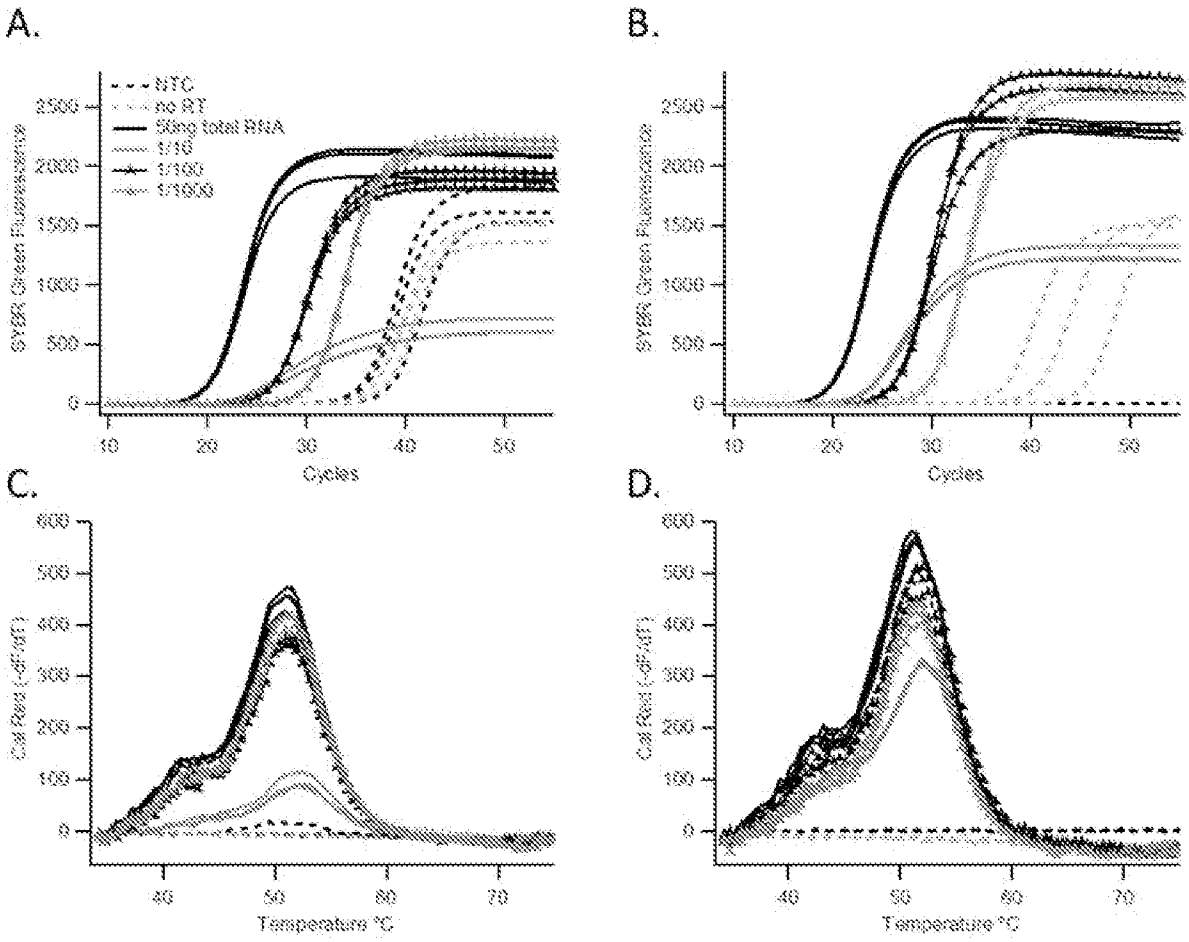

A.
NTC
no RT
50ng total RNA
1/10
1/100
1/1000
SYBR Green Fluorescence
Cycles
B.
SYBR Green Fluorescence
Cycles
C.
Cal Red (-dF/dT)
Temperature °C
D.
Cal Red (-dF/dT)
Temperature °C

Figure 4

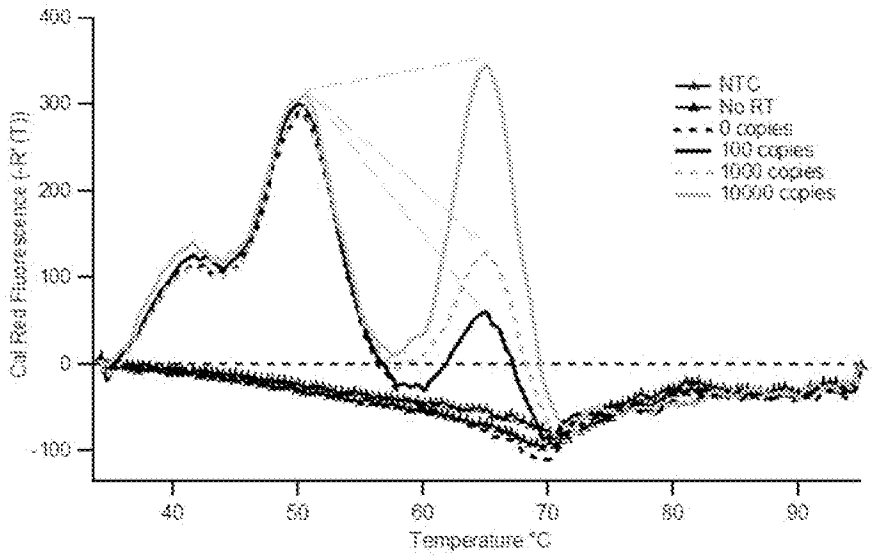

400
300
200
100
0
-100
Cal Red Fluorescence (-R'(T))
40
50
60
70
80
90
Temperature °C
NTC
No RT
0 copies
100 copies
1000 copies
10000 copies

Figure 5

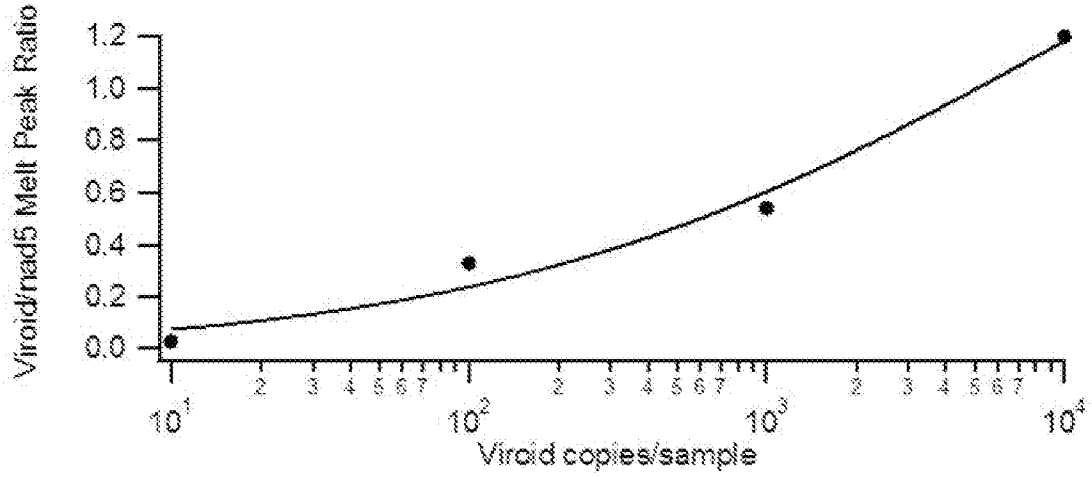

Viroid/rnd5 Melt Peak Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10^1
10^2
10^3
10^4
Viroid copies/sample

Figure 6

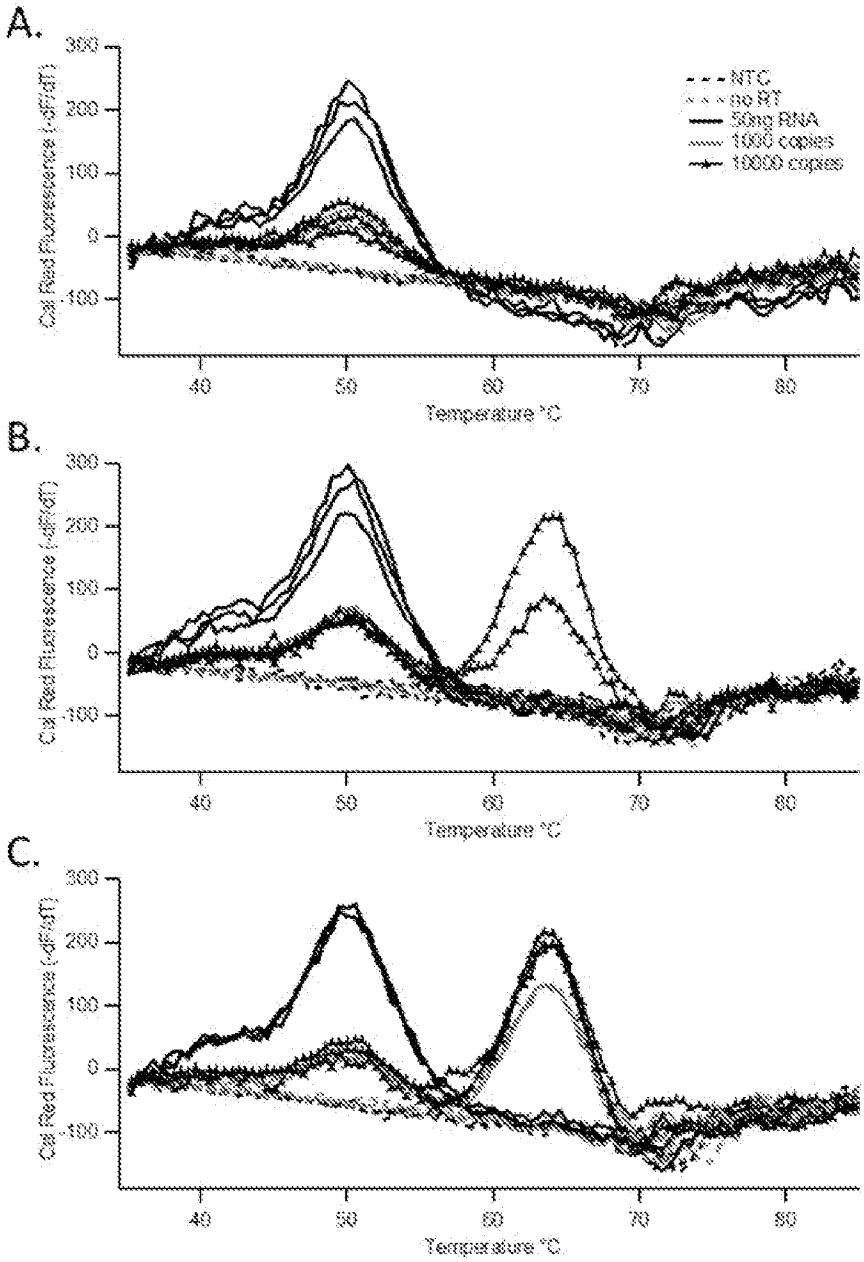

A.
B.
C.
NTC
no RT
50ng RNA
1000 copies
10000 copies
Cal Red Fluorescence (-dF/dT)
Temperature °C
300
200
100
0
-100
40
50
60
70
80

Figure 7

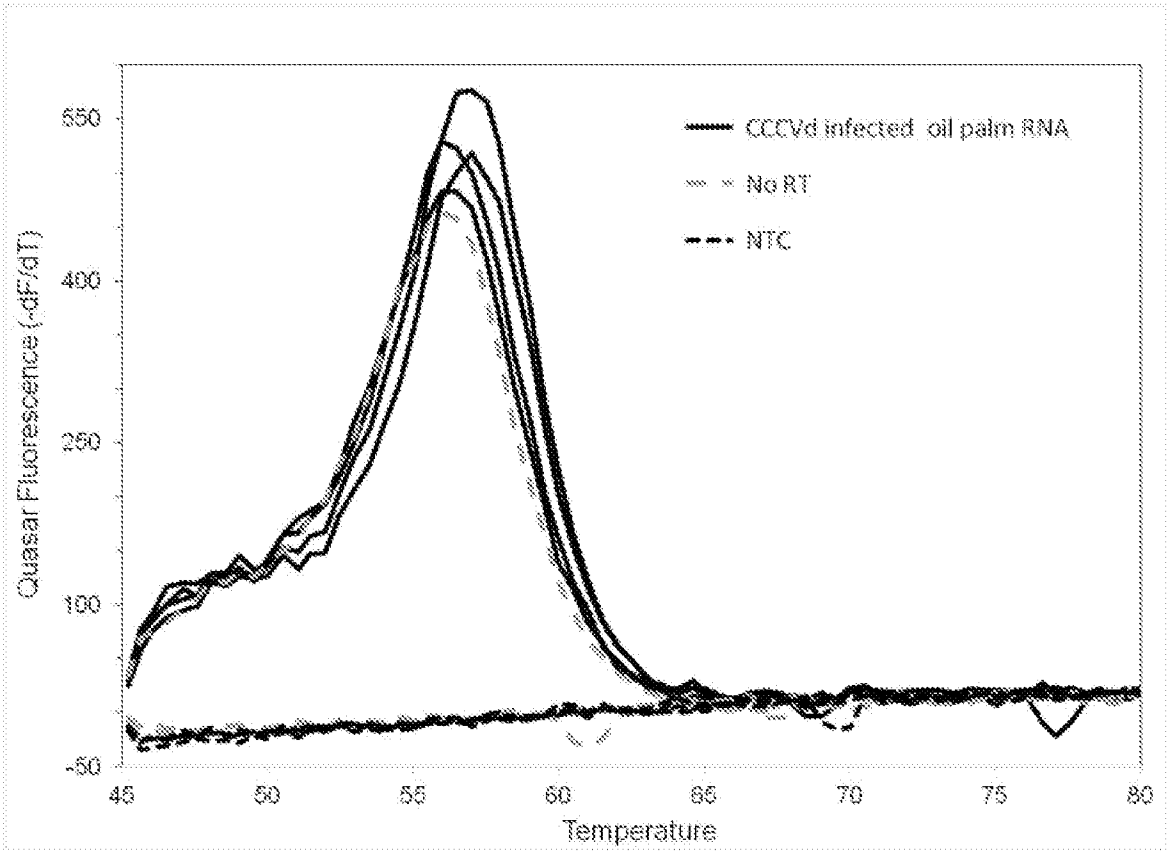

CCCVd infected oil palm RNA
No RT
NTC
Quasar Fluorescence (-dF/dT)
550
400
250
100
-50
45
50
55
60
65
70
75
80
Temperature

Figure 8

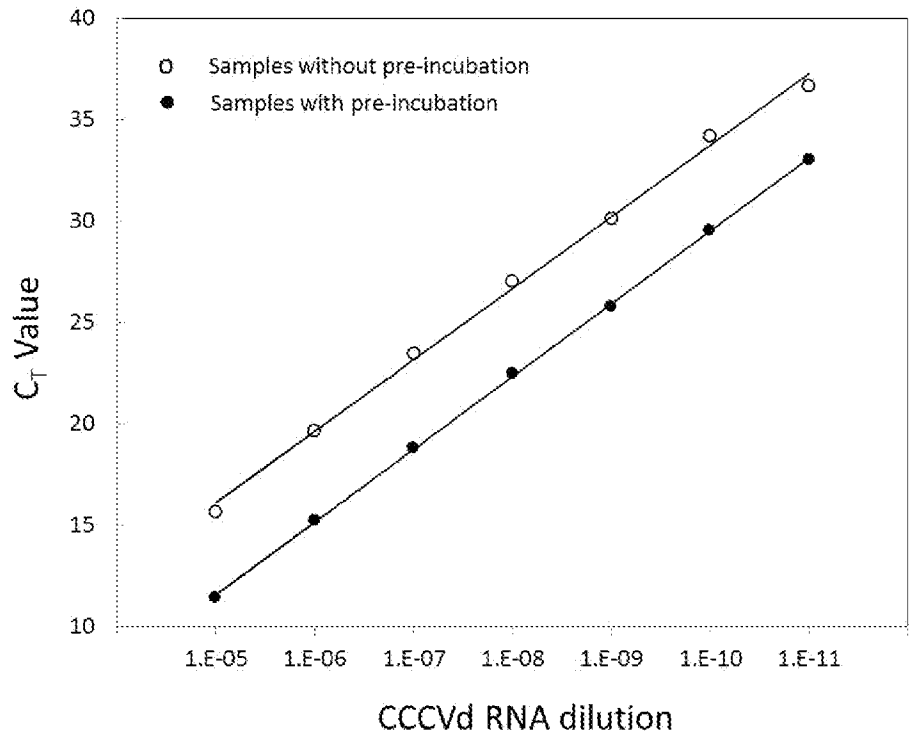
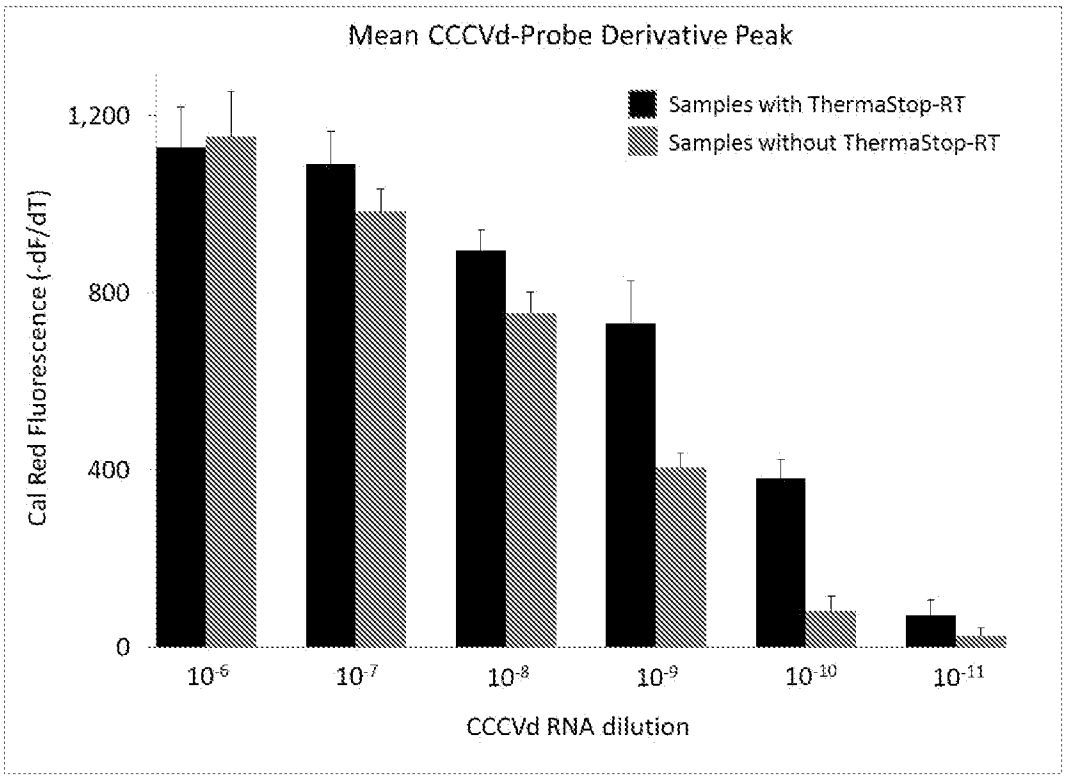

40
35
30
25
20
15
10
C_T Value
Samples without pre-incubation
Samples with pre-incubation
1.E-05
1.E-06
1.E-07
1.E-08
1.E-09
1.E-10
1.E-11
CCCVd RNA dilution

Figure 9


Mean CCCVd-Probe Derivative Peak
Samples with ThermaStop-RT
Samples without ThermaStop-RT
Cal Red Fluorescence (-dF/dT)
1,200
800
400
0
10-6
10-7
10-8
10-9
10-10
10-11
CCCVd RNA dilution

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/063059

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/70 C12Q1/68
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, Sequence Search, 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.
X	SATHIS SRI THANARAJOO ET AL: "Detection of Coconut cadang-cadang viroid (CCCVd) in oil palm by reverse transcription loop-mediated isothermal amplification (RT-LAMP)", JOURNAL OF VIROLOGICAL METHODS, vol. 202, 1 June 2014 (2014-06-01), pages 19-23, XP055350814, NL ISSN: 0166-0934, DOI: 10.1016/j.jviromet.2014.02.024 pages 19-20 ----- -/--	1-92

[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
2 March 2017	03/05/2017

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	Dolce, Luca

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Virology ET AL: "774 PHYTOPATHOLOGY Specific Identification of Coconut Tinangaja Viroid for Differential Field Diagnosis of Viroids in Coconut Palm", , 1 August 1998 (1998-08-01), XP055350815, Retrieved from the Internet: URL:http://apsjournals.apsnet.org/doi/pdf/10.1094/PHYTO.1998.88.8.774 [retrieved on 2017-03-01] figure 5	1-92
X	K.E. PIERCE ET AL: "Design and optimization of a novel reverse transcription linear-after-the-exponential PCR for the detection of foot-and-mouth disease virus", JOURNAL OF APPLIED MICROBIOLOGY, 1 December 2009 (2009-12-01), pages no-no, XP055100866, ISSN: 1364-5072, DOI: 10.1111/j.1365-2672.2009.04640.x table 3	1-92
A	CN 1 600 865 A (JIN CHANGHE TECH DEV BEIJING [CN]) 30 March 2005 (2005-03-30) the whole document	1-92
A	US 6 210 931 B1 (FELDSTEIN PAUL A [US] ET AL) 3 April 2001 (2001-04-03) the whole document	1-92

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/063059

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

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

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

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

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

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

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

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

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

1-92

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-92

   a RT-LATE PCR method for amplifying CCCVd RNA comprising a high-temperature pre-incubation step before the RT reaction

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2. claims: 93-109

   a method for amplifying CCCVd RNA comprising the steps of lysing cells and without purification, diluting the sample and subjecting it to an amplification reaction

   ---

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2016/063059

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 1600865 A	30-03-2005	NONE	
US 6210931 B1	03-04-2001	NONE	