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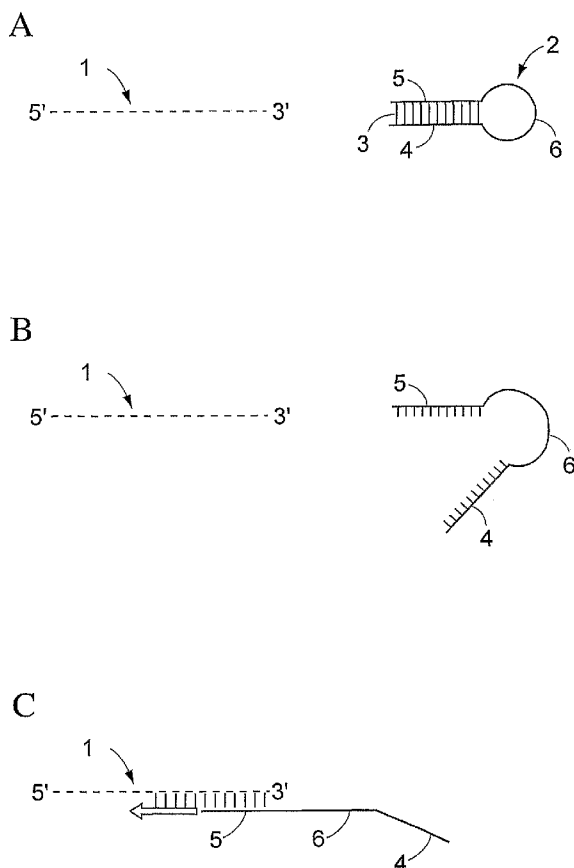
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[Continued on next page]

(54) Title: HOT START REVERSE TRANSCRIPTION BY PRIMER DESIGN



(57) Abstract: The present teachings provide methods, compositions, and kits for performing primer extension reactions. In some embodiments, a reverse transcription reaction is performed on a target polynucleotide with a hot start primer comprising a blunt-ended self-complementary stem, and a loop, and extension products form at high temperatures but reduce extension product formation at low temperatures.

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HOT START REVERSE TRANSCRIPTION BY PRIMER DESIGN

FIELD

The present teachings relate to methods of synthesizing nucleic acids by primer extension.

5 INTRODUCTION

The integrity of primer-mediated methods of synthesizing nucleic acids can be compromised by non-specific hybridization of primer to inappropriate target polynucleotides. The analysis of nucleic acids is benefited by approaches by approaches that minimize the generation of mis-extension products. For example, 'hot start' approaches have been employed in PCR, where inhibition of polymerase activity has been achieved. For example, U.S. Patent Number 5,338,671 describes the use of antibodies specific for a thermostable DNA polymerase to inhibit the DNA polymerase activity at low temperatures. Chemical treatment with citraconic anhydride is another way hot start PCR has been achieved (see U.S. Patent Number 15 5,773,258 and U.S. Patent Number 5,677,152). Hot start methods which use a heat labile material, such as wax, to separate or sequester reaction components are described in U.S. Patent Number 5,411,876. The application of such hot start approaches to reverse transcription have proven challenging. For example, many reverse transcriptases are not heat-stabile.

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SUMMARY

The present teachings provide a method for reducing the formation of primer extension products at a low temperature but allowing the formation of primer extension products at a high temperature comprising; forming a reaction mixture at the 25 low temperature below about 27C, wherein the reaction mixture comprises a target

polynucleotide, a primer extending enzyme, and a hot start primer, wherein the hot start primer comprises a loop and a self-complementary stem, wherein a target-specific region of the self-complementary stem comprises a sequence of at least six nucleotides that are complementary with the target polynucleotide, wherein the target-specific region of the self-complementary stem is substantially hybridized with a quencher region in the self-complementary stem when at the low temperature and wherein the self-complementary stem is substantially unable to hybridize with the target polynucleotide when at the low temperature; elevating the temperature of the reaction mixture to a high temperature between 35C-60C, wherein the target-specific region of the self-complementary stem is substantially unhybridized with the quencher region in the self-complementary stem at the high temperature, and wherein the target-specific region hybridizes to the target polynucleotide; extending the target-specific region of the self-complementary stem with the primer extending enzyme to form a primer extension product; and, generating a primer extension product at the high temperature but not at the low temperature.

In some embodiments, the present teachings provide a composition comprising a target polynucleotide and a hot start primer, wherein the hot start primer comprises a self-complementary stem, wherein the self-complementary stem comprises a target-specific region and a quencher region, wherein the target-specific region comprises at least six nucleotides, wherein the target-specific region is substantially unhybridized with the quencher region in the self-complementary stem, and wherein the target-specific region is hybridized with the target polynucleotide.

In some embodiments, the present teachings provide a kit for reducing the formation of primer extension products at a low temperature but allowing the formation of primer extension products at a high temperature comprising; a hot start primer, a

primer extending enzyme, a primer extending enzyme buffer, and dNTPs, wherein the hot start primer comprises a loop and a self-complementary stem, wherein the self-complementary stem comprises a target-specific region that is at least six nucleotides in length, wherein the target-specific region is complementary to target polynucleotide, and wherein the target-specific region is substantially hybridized to a quencher region when at a temperature of 27°C or lower.

These and other features of the present teachings are set forth herein.

DRAWINGS

Figure 1 depicts one illustrative embodiment according to the present teachings.

DESCRIPTION OF EXEMPLARY EMBODIMENTS

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not intended to limit the scope of the current teachings. In this application, the use of the singular includes the plural unless specifically stated otherwise. For example, "a forward primer" means that more than one forward primer can be present; for example, one or more copies of a particular forward primer species, as well as one or more different forward primer species. Also, the use of "comprise", "contain", and "include", or modifications of those root words, for example but not limited to, "comprises", "contained", and "including", are not intended to be limiting. The term and/or means that the terms before and after can be taken together or separately. For illustration purposes, but not as a limitation, "X and/or Y" can mean "X" or "Y" or "X and Y".

The section headings used herein are for organizational purposes only and are not to be construed as limiting the described subject matter in any way. All literature and similar materials cited in this application, including, patents, patent applications,

articles, books, treatises, and internet web pages are expressly incorporated by reference in their entirety for any purpose. In the event that one or more of the incorporated literature and similar defines or uses a term in such a way that it contradicts that term's definition in this application, this application controls. While the present teachings are described in conjunction with various embodiments, it is not intended that the present teachings be limited to such embodiments. On the contrary, the present teachings encompass various alternatives, modifications, and equivalents, as will be appreciated by those of skill in the art.

SOME DEFINITIONS

As used herein, the term "denaturing" refers to the melting of two complementary nucleic acid strands, and is typically achieved by elevating the temperature. In some embodiments, denaturing can be achieved by the addition of base (e.g.-NaOH) or other approaches for dissociating nucleic acids that are familiar to one of ordinary skill in the art of molecular biology.

As used herein, the term "complementary" refers to nucleic acid sequences that are capable of forming Watson-Crick base-pairs. For example, a self-complementary primer comprises a self-complementary stem which is capable of forming Watson-Crick base-pairs with itself at a low temperature. When at the low temperature, the strands of such a self-complementary stem are said to be hybridized to one another.

When at a high temperature, the strands of such a self-complementary stem are not hybridized to each other, and the target specific region of the self-complementary stem can be hybridized with a target. In this application, a statement that one sequence is complementary to another sequence encompasses situations in which the two sequences have slight mismatches. Here, the term "sequence"

encompasses, but is not limited to, nucleic acid sequences, polynucleotides,

oligonucleotides, probes, primers, primer-specific regions, and target-specific regions. Despite the mismatches, the two sequences should selectively hybridize to one another under appropriate conditions.

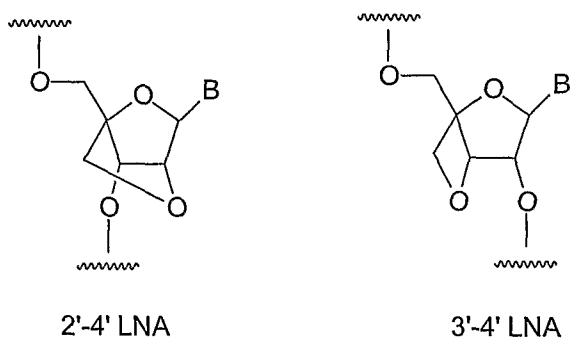
As used herein, the term "hot start primer" refers to a primer comprising a self-complementary stem and a loop, wherein the self-complementary stem comprises a target specific region and a quencher region. At low temperatures, the target-specific region is hybridized to the quencher region. At high temperatures, the target-specific region is not hybridized to the quencher region, and can hybridize to the corresponding target polynucleotide, thereby allowing for a hot start extension reaction. In some embodiments, the self-complementary stem is blunt-ended, such that there is not a nucleotide overlap at the 5' or 3' end of the self-complementary stem. In some embodiments, the mRNA primer comprises a nearly blunt-ended self-complementary stem, such as for example a single 3' nucleotide overhang. Generally, such 3' overhangs will be of minimal length to avoid undesired priming on targets prior to the melting of the self-complementary stem region by the high temperature. Overhangs on the 5' end are generally more tolerable, since extension does not proceed from the 5' of a sequence.

As used herein, the term "target polynucleotide" refers to a polynucleotide sequence that is sought to be reverse transcribed. The target polynucleotide can be obtained from any source, and can comprise any number of different compositional components. For example, the target can be nucleic acid (e.g. DNA or RNA), transfer RNA, siRNA, and can comprise nucleic acid analogs or other nucleic acid mimic, though typically the target will be messenger RNA (mRNA) and/or micro RNA (miRNA). The target can be methylated, non-methylated, or both. The target can be bisulfite-treated and non-methylated cytosines converted to uracil. Further, it will be

appreciated that "target polynucleotide" can refer to the target polynucleotide itself, as well as surrogates thereof, for example amplification products, and native sequences. In some embodiments, the target polynucleotide is a short DNA molecule derived from a degraded source, such as can be found in for example but not limited to forensics samples (see for example Butler, 2001, Forensic DNA Typing: Biology and Technology Behind STR Markers. The target polynucleotides of the present teachings can be derived from any of a number of sources, including without limitation, viruses, prokaryotes, eukaryotes, for example but not limited to plants, fungi, and animals. These sources may include, but are not limited to, whole blood, a tissue biopsy, lymph, bone marrow, amniotic fluid, hair, skin, semen, biowarfare agents, anal secretions, vaginal secretions, perspiration, saliva, buccal swabs, various environmental samples (for example, agricultural, water, and soil), research samples generally, purified samples generally, cultured cells, and lysed cells. It will be appreciated that target polynucleotides can be isolated from samples using any of a variety of procedures known in the art, for example the Applied Biosystems ABI Prism™ 6100 Nucleic Acid PrepStation, and the ABI Prism™ 6700 Automated Nucleic Acid Workstation, Boom et al., U.S. Patent 5,234,809., mirVana RNA isolation kit (Ambion), etc. It will be appreciated that target polynucleotides can be cut or sheared prior to analysis, including the use of such procedures as mechanical force, sonication, restriction endonuclease cleavage, or any method known in the art. In general, the target polynucleotides of the present teachings will be single stranded, though in some embodiments the target polynucleotide can be double stranded, and a single strand can result from denaturation.

The term "nucleotide", as used herein, refers to a compound comprising a nucleotide base linked to the C-1' carbon of a sugar, such as ribose, arabinose, xylose,

and pyranose, and sugar analogs thereof. The term nucleotide also encompasses nucleotide analogs. The sugar may be substituted or unsubstituted. Substituted ribose sugars include, but are not limited to, those riboses in which one or more of the carbon atoms, for example the 2'-carbon atom, is substituted with one or more of the same or different Cl, F, -R, -OR, -NR₂ or halogen groups, where each R is independently H, C₁-C₆ alkyl or C₅-C₁₄ aryl. Exemplary riboses include, but are not limited to, 2'-(C1 -C6)alkoxyribose, 2'-(C5 -C14)aryloxyribose, 2',3'-didehydroribose, 2'-deoxy-3'-haloribose, 2'-deoxy-3'-fluororibose, 2'-deoxy-3'-chlororibose, 2'-deoxy-3'-aminoribose, 2'-deoxy-3'-(C1 -C6)alkylribose, 2'-deoxy-3'-(C1 -C6)alkoxyribose and 2'-deoxy-3'-(C5 -C14)aryloxyribose, ribose, 2'-deoxyribose, 2',3'-dideoxyribose, 2'-haloribose, 2'-fluororibose, 2'-chlororibose, and 2'-alkylribose, e.g., 2'-O-methyl, 4'- α -anomeric nucleotides, 1'- α -anomeric nucleotides, 2'-4'- and 3'-4'-linked and other "locked" or "LNA", bicyclic sugar modifications (see, e.g., PCT published application nos. WO 98/22489, WO 98/39352, and WO 99/14226). Exemplary LNA sugar analogs within a polynucleotide include, but are not limited to, the structures:



where B is any nucleotide base.

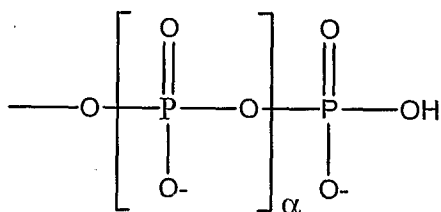
Modifications at the 2'- or 3'-position of ribose include, but are not limited to, hydrogen, hydroxy, methoxy, ethoxy, allyloxy, isopropoxy, butoxy, isobutoxy,

methoxyethyl, alkoxy, phenoxy, azido, amino, alkylamino, fluoro, chloro and bromo.

Nucleotides include, but are not limited to, the natural D optical isomer, as well as the

L optical isomer forms (see, e.g., Garbesi (1993) Nucl. Acids Res. 21:4159-65; Fujimori (1990) J. Amer. Chem. Soc. 112:7435; Urata, (1993) Nucleic Acids Symposium Ser. No. 29:69-70). When the nucleotide base is purine, e.g. A or G, the ribose sugar is attached to the N⁹-position of the nucleotide base. When the nucleotide base is pyrimidine, e.g. C, T or U, the pentose sugar is attached to the N¹-position of the nucleotide base, except for pseudouridines, in which the pentose sugar is attached to the C5 position of the uracil nucleotide base (see, e.g., Kornberg and Baker, (1992) *DNA Replication*, 2nd Ed., Freeman, San Francisco, CA).

One or more of the pentose carbons of a nucleotide may be substituted with a phosphate ester having the formula:



where α is an integer from 0 to 4. In certain embodiments, α is 2 and the phosphate ester is attached to the 3'- or 5'-carbon of the pentose. In certain embodiments, the nucleotides are those in which the nucleotide base is a purine, a 7-deazapurine, a pyrimidine, or an analog thereof. "Nucleotide 5'-triphosphate" refers to a nucleotide with a triphosphate ester group at the 5' position, and are sometimes denoted as "NTP", or "dNTP" and "ddNTP" to particularly point out the structural features of the ribose sugar. The triphosphate ester group may include sulfur substitutions for the various oxygens, e.g. α -thio-nucleotide 5'-triphosphates. For a review of nucleotide chemistry, see: Shabarova, Z. and Bogdanov, A. *Advanced Organic Chemistry of Nucleic Acids*, VCH, New York, 1994.

Some Specific Exemplary Embodiments

Figure 1 depicts an illustrative relationship between a hot start primer and a target polynucleotide according to some embodiments of the present teachings. In (A), a target polynucleotide (1) is shown with a hot start primer (2) in a reaction mixture at a low temperature (for example 25C, roughly room temperature). The hot start primer (2) comprises a self-complementary stem (3) comprising a target-specific region (4) and a quencher region (5). The hot start primer also comprises a loop (6). In (B), elevation to a high temperature is performed (for example 42C, an appropriate temperature for a reverse transcriptase), and the stem of the hot start primer melts, thereby liberating the target-specific region (5) of the self-complementary primer. In (C), the target-specific region (5) of the hot start primer can hybridize to the target polynucleotide (1), and extension of the primer by the reverse transcriptase can occur (bold arrow).

In some embodiments, the architecture of the hot start primer can differ from that depicted in Figure 1. For example, the loop (6) of the hot start primer shown in Figure 1 can itself form part of the target-specific region of the hot start primer. It will be appreciated that one of skill in the art would be able to make these and other minor modifications in the design of the hot start primer provided by the present teachings, and still be within their scope.

In some embodiments, the reverse transcription is set up at room temperature. In some embodiments, the reverse transcription is set up at 20C-27C. In some embodiments, the reverse transcription can be set up on ice. In some embodiments, the reverse transcription reaction can be set up at 4C-27C.

Certain methods of optimizing reverse transcription and amplification reactions are known to those skilled in the art. For example, it is known that PCR may be

optimized by altering times and temperatures for annealing, polymerization, and denaturing, as well as changing the buffers, salts, and other reagents in the reaction composition. Optimization may also be affected by the design of the amplification primers used. For example, the length of the primers, as well as the G-C:A-T ratio
5 may alter the efficiency of primer annealing, thus altering the amplification reaction.

Descriptions of amplification optimization can be found in, among other places, James G. Wetmur, "Nucleic Acid Hybrids, Formation and Structure," in Molecular Biology and Biotechnology, pp.605-8, (Robert A. Meyers ed., 1995); McPherson, particularly in Chapter 4; Rapley; and Protocols & Applications Guide, rev. 9/04, Promega.

10 In some embodiments, the present teachings contemplate single-tube RT-PCR approaches, and discussed for example in Mohamed et al., (2004) Journal of Clinical Virology, 30:150-156. In some embodiments, the reverse transcription products of the present teachings can be amplified in a multiplexed pre-amplifying PCR followed by a plurality of lower-plex decoding PCRs, as described for example in WO2004/051218
15 to Andersen and Ruff, U.S. Patent 6,605,451 to Xtrana, and U.S. Non-Provisional Application 11/090,830 to Andersen et al., and U.S. Non-Provisional Application 11,090,468 to Lao et al.,

Generally, the length of the stem of the hot start primer can vary according to the context of the application. For example, when the target-specific region of the hot
20 start primer is G:C rich, the length of the stem region can be shorter. Conversely, when the target-specific region of the hot start primer is A:T rich, the length of the stem region can be longer. Such procedures can be employed to adjust the length of the stem to correspond with a desired T_m , given a particular reaction context at hand. In some embodiments, the length of the stem is between 6-12 nucleotide base-pairs.

Generally, the length of the loop of the hot start primer will be between 8-24 nucleotides in length. Generally, short loops can have the beneficial effect of minimizing the likelihood of loop sequence displacing stem sequence at lower reaction temperatures. It will be appreciated by one of ordinary skill in the art that a variety of stem-loop configurations are available and within routine experimentation.

Illustrative molecular biology techniques of ready availability to one of skill in the art can be found in Sambrook et al., Molecular Cloning, 3rd Edition.

CERTAIN EXEMPLARY KITS

The instant teachings also provide kits designed to expedite performing certain of the disclosed methods. Kits may serve to expedite the performance of certain disclosed methods by assembling two or more components required for carrying out the methods. In certain embodiments, kits contain components in pre-measured unit amounts to minimize the need for measurements by end-users. In some embodiments, kits include instructions for performing one or more of the disclosed methods. Preferably, the kit components are optimized to operate in conjunction with one another.

Thus, in some embodiments the present teachings provide a kit for reducing primer extension products at a low temperature and allowing primer extension at a high temperature comprising; a hot start primer, a primer extending enzyme, a primer extending enzyme buffer, and dNTPs, wherein the hot start primer comprises a loop and a self-complementary stem, wherein the self-complementary stem comprises a target-specific region that is at least six nucleotides in length, wherein the target-specific region is complementary to target polynucleotide, wherein the target-specific region is substantially hybridized to a quencher region when at a temperature of 27°C or lower, and wherein the target-specific region when hybridized to the quencher

region forms a blunt end structure, a structure with a one nucleotide overlap, or a structure with a two nucleotide overlap. In some embodiments, the primer extending enzyme is a reverse transcriptase. In some embodiments, the loop of the hot start primer comprises 8-24 nucleotides. In some embodiments, the self-complementary stem of the hot start primer comprises 6-12 nucleotide base-pairs. In some
5 embodiments, the kit further comprises a forward primer and reagents for performing a PCR. In some embodiments, the reagents for performing the PCR are included in a vessel that is the same vessel that contains at least one of the hot start primer, the primer extending enzyme, the primer extending enzyme buffer, and the dNTPs. In
10 some embodiments, the target polynucleotide is selected from the group comprising messenger RNA, small non-coding RNA, and micro RNA.

The current teachings, having been described above, may be better understood by reference to examples. The following examples are intended for illustration purposes only, and should not be construed as limiting the scope of the teachings
15 herein in any way.

EXAMPLE

An illustrative experiment was performed comprising the primer and probe sequences found in Table 1 below directed to the ACTB messenger RNA. The results of different RT reactions using different RT primers, and reaction temperatures, were
20 quantitated using real-time PCR with a forward primer FP-ACTB (SEQ ID NO:1), a reverse primer RP-ACTB (SEQ ID NO:2), and a TaqMan 5' nuclease probe Taq-ACTB (SEQ ID NO:7). There were two reverse transcription temperature conditions: a low temperature RT at 20C, and a high temperature RT at 40C. There were six RT reactions compared for each of the two temperature conditions, using the following six
25 conditions.

- 1) RT linear primer (SEQ ID NO:2).
- 2) RT hot start primer with 8 base-pair stem (SEQ ID NO: 5).
- 3) RT hot start primer with 10 base-pair stem (SEQ ID NO:4).
- 4) RT hot start primer with 12 base-pair stem (SEQ ID NO:3).
- 5) buffer alone (no RT primer).
- 6) No template control.

SEQ ID	Oligo Name	Sequence
SEQ ID NO:1	FP-ACTB	CCCCGCGAGCACAGA
SEQ ID NO:2	RP-ACTB	CCACGATGGAGGGGAAGAC
SEQ ID NO:3	RP-ACTB-12	GTCTTCCCCTCCTTCCACGATGGAGGGGAAGAC
SEQ ID NO:4	RP-ACTB-10	GTCTTCCCCTTTCCACGATGGAGGGGAAGAC
SEQ ID NO:5	RP-ACTB-8	GTCTTCCCTTCCACGATGGAGGGGAAGAC
SEQ ID NO:6	RP-ACTB-6	GTCTTCTTCCACGATGGAGGGGAAGAC
SEQ ID NO:7	Taq-ACTB	(6-FAM) CTTTGCCGATCCGC (MGB)

The experiment was set up at room temperature. The RT reaction was done following manufacture's suggestion by using of Applied Biosystems High Capacity cDNA Archive Kit (CN: 4322171). In the low temperature condition, the reverse transcription reaction was performed at 20C for 30 minutes. In the high temperature condition, the reverse transcription was performed at 40C for 30 minutes.

Ct values derived from TaqMan [™] quantitative PCR using ABI TaqMan universal PCR master mix demonstrated a hot start effect for hot start primers comprising a self-complementary stem. Specifically, the fold reduction of RT product of linear primer to hot start primer at 20C was significantly greater than the fold reduction of RT product

of linear primer to hot start primer at 40C, thus illustrating inhibition of reverse transcription by the presence of the hot-start primer comprising the self-complementary stem. Further, hot start primers with longer self-complementary stems showed a stronger hot start effect than primers with shorter self-complementary stems. Thus, for example, the RT primer with a twelve nucleotide base-pair stem showed a stronger reduction in RT product formed at 20C compared to a linear RT primer, than did an eight nucleotide base-pair stem RT primer in the 20C RT reaction compared to the linear RT primer.

Although the disclosed teachings have been described with reference to various applications, methods, and kits, it will be appreciated that various changes and modifications may be made without departing from the teachings herein. The foregoing examples are provided to better illustrate the present teachings and are not intended to limit the scope of the teachings herein. Certain aspects of the present teachings may be further understood in light of the following claims.

WE CLAIM

1. A method of reducing primer extension products at a low temperature and allowing primer extension at a high temperature comprising;

forming a reaction mixture at the low temperature below about 27C, wherein

5 the reaction mixture comprises a target polynucleotide, a primer extending enzyme,

and a hot start primer, wherein the hot start primer comprises a loop and a self-

complementary stem, wherein a target-specific region of the self-complementary stem comprises at least six nucleotides that are complementary with the target

polynucleotide, wherein the target-specific region of the self-complementary stem is

10 hybridized with a quencher region in the self-complementary stem when at the low

temperature, and wherein the self-complementary stem is unable to hybridize with the target polynucleotide when at the low temperature;

elevating the temperature of the reaction mixture to a high temperature

between 35C-60C, wherein the target-specific region of the self-complementary stem

15 is unhybridized with the quencher region in the self-complementary stem at the high

temperature, and wherein the target-specific region hybridizes to the target

polynucleotide;

extending the target-specific region of the self-complementary stem with the primer extending enzyme to form a primer extension product; and,

20 generating a primer extension product at the high temperature but not at the low temperature.

2. The method according to claim 1 wherein the primer extending enzyme is a reverse transcriptase.

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3. The method according to claim 1 wherein the target polynucleotide is a messenger RNA.

4. The method according to claim 1 wherein the target polynucleotide is a small non-coding RNA.

5. The method according to claim 4 wherein the small non-coding RNA is a micro RNA.

6. The method according to claim 1 wherein the loop of the hot start primer comprises 8-24 nucleotides.

7. The method according to claim 1 wherein the self-complementary stem of the hot start primer comprises 6-12 nucleotide base-pairs.

8. The method according to claim 1 wherein the target-specific region of the hot start primer further comprises nucleotide s present in the loop of the hot start primer.

9. The method according to claim 1 wherein the target-specific region of the hot start primer, when hybridized to the quencher region of the hot start primer, forms a blunt-ended structure.

10. The method according to claim 1 wherein the high temperature between 35C-60C occurs for between 20 minutes and 40 minutes.

11. The method according to claim 1 wherein the primer extension reaction is a reverse transcription reaction, said method further comprising performing a polymerase chain reaction (PCR) on the primer extension product with a forward primer and a reverse primer, wherein the PCR comprises;

- 5 a denaturizing step performed after the extension reaction;
 an annealing step performed after the melting step; and,
 an extension step performed after the annealing step.

12. The method according to claim 11 wherein the reverse transcription reaction
10 comprises the forward primer.

13. A composition comprising a target polynucleotide and a hot start primer primer, wherein the hot start primer comprises a self-complementary stem, wherein the self-complementary stem comprises a target-specific region and a quencher region,
15 wherein the target-specific region comprises at least six nucleotide s, wherein the target-specific region is substantially unhybridized with the quencher region in the self-complementary stem, and wherein the target-specific region is hybridized with the target polynucleotide.

20 14. The composition according to claim 13 wherein the target polynucleotide is a messenger RNA.

15. The composition according to claim 13 wherein the target polynucleotide is a small non-coding RNA.

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16. The composition according to claim 15 wherein the small non-coding RNA is a micro RNA.

17. The composition according to claim 13 wherein the loop of the hot start primer
5 comprises 8-24 nucleotides.

18. The composition according to claim 18 wherein the self-complementary stem of the hot start primer comprises 6-12 nucleotide base-pairs.

10 19. The composition according to claim 13 wherein the target-specific region of the hot start primer further comprises at least one nucleotide present in the loop of the hot start primer.

20. The composition according to claim 13 wherein the target-specific region of the hot
15 start primer, when hybridized to the quencher region of the hot start primer, forms a blunt-ended structure.

21. A kit for reducing primer extension products at a low temperature and allowing primer extension at a high temperature comprising;

20 a hot start primer, a primer extending enzyme, a primer extending enzyme buffer, and dNTPs, wherein the hot start primer comprises a loop and a self-complementary stem, wherein the self-complementary stem comprises a target-specific region that is at least six nucleotide s in length, wherein the target-specific region is complementary to target polynucleotide, wherein the target-specific region is
25 substantially hybridized to a quencher region when at a temperature of 27C or lower,

and wherein the target-specific region when hybridized to the quencher region forms a blunt end structure, a structure with a one nucleotide overlap, or a structure with a two nucleotide overlap.

5 22. The kit according to claim 21 wherein the primer extending enzyme is a reverse transcriptase.

23. The kit according to claim 21 wherein the loop of the hot start primer comprises 8-24 nucleotides.

10

24. The kit according to claim 21 wherein the self-complementary stem of the hot start primer comprises 6-12 nucleotide base-pairs.

15 25. The kit according to claim 21 further comprising a forward primer and reagents for performing a PCR.

26. The kit according to claim 25 wherein the reagents for performing the PCR are included in a vessel that is the same vessel that contains at least one of the hot start primer, the primer extending enzyme, the primer extending enzyme buffer, and the
20 dNTPs.

27. The kit according to claim 21 wherein the target polynucleotide is selected from the group comprising messenger RNA, small non-coding RNA, and micro RNA.

FIG. 1A

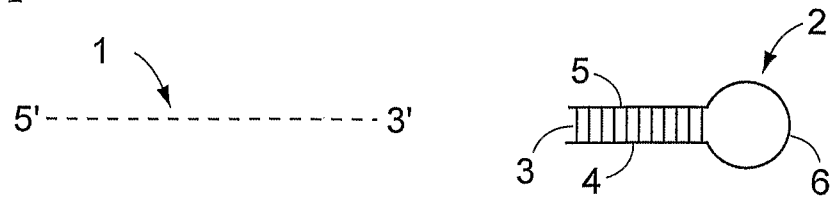


FIG. 1B

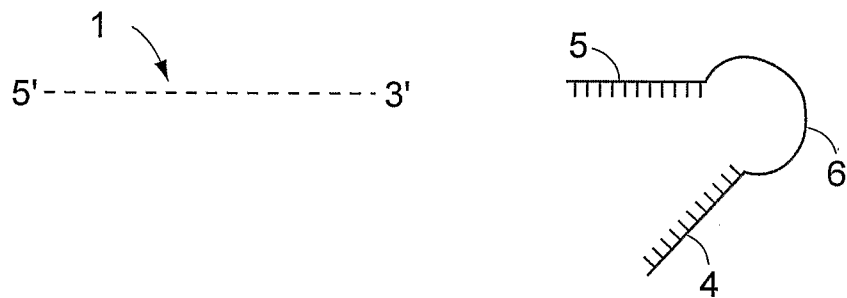


FIG. 1C

