US 20040209276A1

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2004/0209276 A1**

Smith et al. (43) **Pub. Date: Oct. 21, 2004**

(54) **THERMOSTABLE REVERSE TRANSCRIPTASES AND USES THEREOF**

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(21) Appl. No.: **10/661,819**

(22) Filed: **Sep. 15, 2003**

Related U.S. Application Data

(60) Provisional application No. 60/410,283, filed on Sep. 13, 2002.

Publication Classification

(51) **Int. Cl.**[7] **C12Q 1/68**; C07H 21/04; C12P 19/34; C12N 9/22; C12N 15/74

(52) **U.S. Cl.** **435/6**; 435/69.1; 435/91.2; 435/199; 435/252.3; 435/320.1; 536/23.2

(57) **ABSTRACT**

The present invention is in the fields of molecular and cellular biology. The invention is generally related to reverse transcriptase enzymes and methods for the reverse transcription of nucleic acid molecules, especially messenger RNA molecules. Specifically, the invention relates to reverse transcriptase enzymes which have been mutated or modified to increase thermostability, decrease terminal deoxynucleotidyl transferase activity, and/or increase fidelity, and to methods of producing, amplifying or sequencing nucleic acid molecules (particularly cDNA molecules) using these reverse transcriptase enzymes or compositions. The invention also relates to nucleic acid molecules produced by these methods and to the use of such nucleic acid molecules to produce desired polypeptides. The invention also concerns kits comprising such enzymes or compositions.

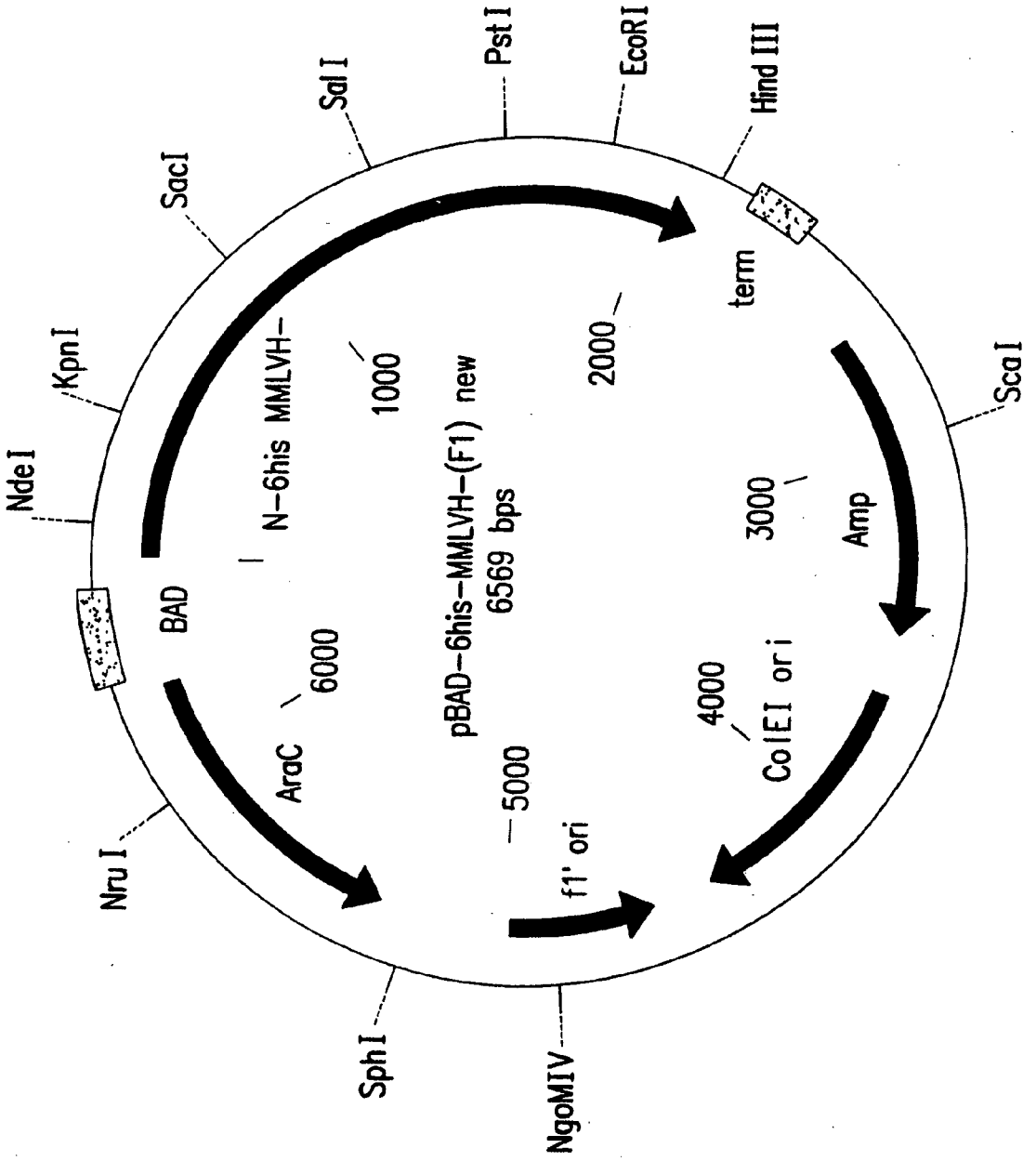
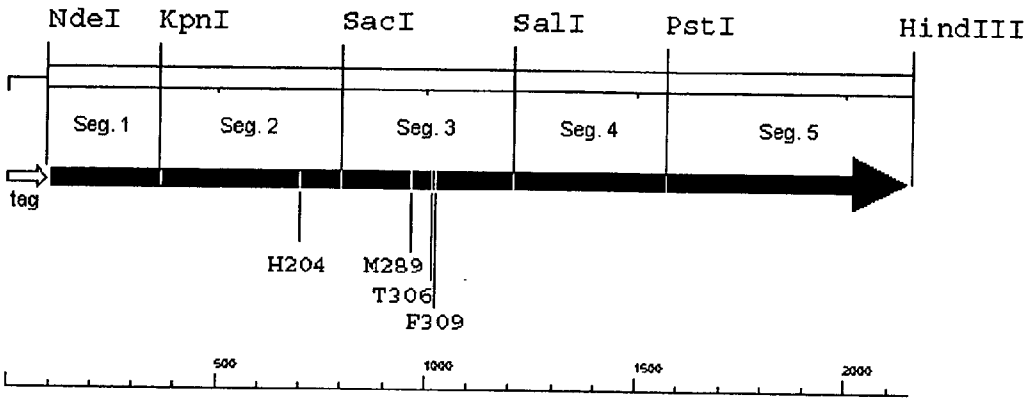

Sal I
Pst I
EcoR I
Hind III
Sac I
Kpn I
Nde I
Sca I
Nru I
Sph I
NgoMIV
BAD
N-6his MMLVH-
1000
2000
term
pBAD-6his-MMLVH-(F1) new
6569 bps
3000
Amp
AraC
6000
5000
4000
ColEI ori
f1' ori

FIG. 1



M-MLV His_6 H- RT (2154bp)

FIG. 2A

FIG. 2B

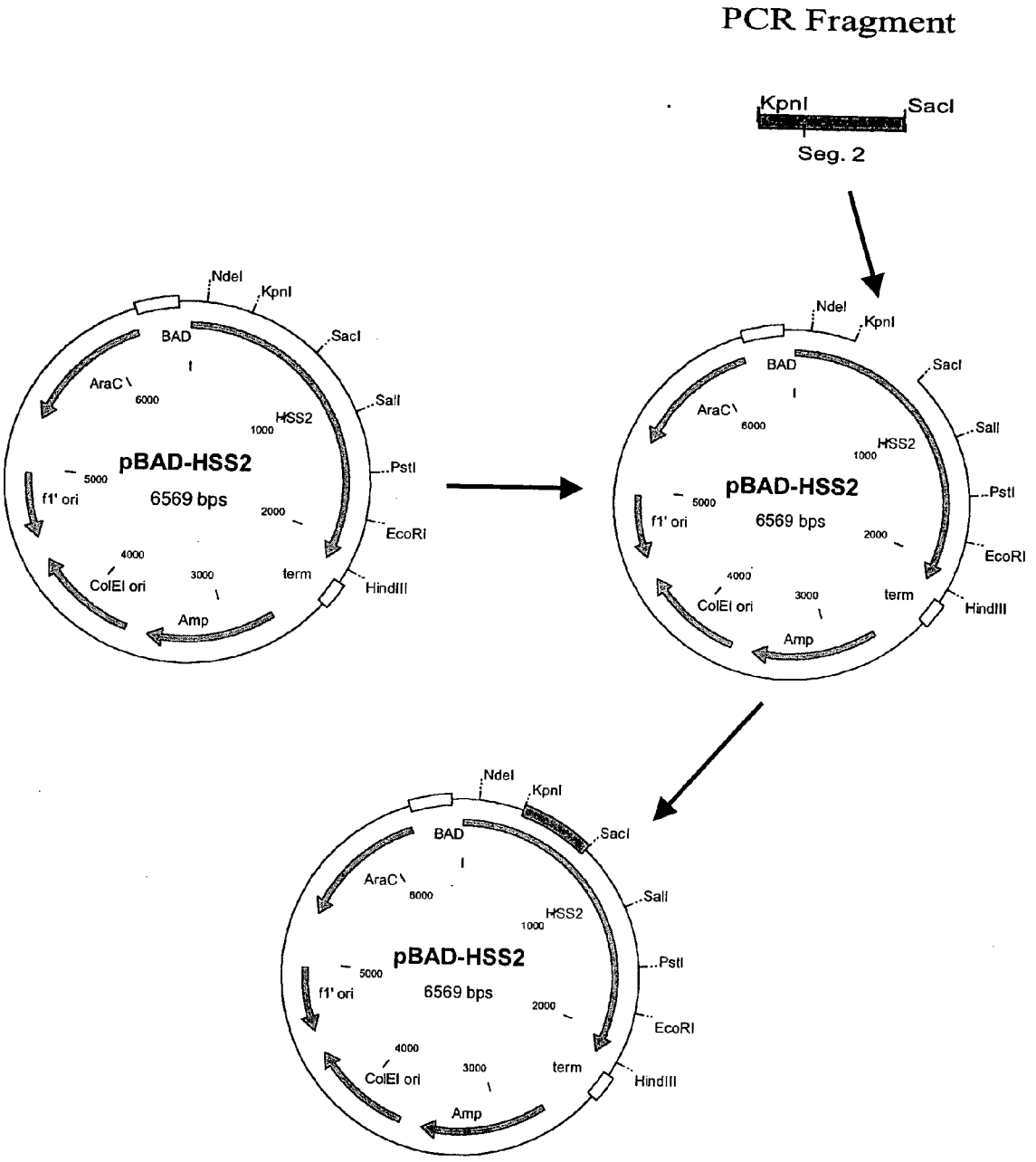

PCR Fragment
KpnI
SacI
Seg. 2
NdeI
KpnI
BAD
SacI
AraC
6000
SalI
HSS2
1000
pBAD-HSS2
5000
PstI
f1' ori
6569 bps
2000
EcoRI
4000
ColEI ori
3000
term
HindIII
Amp

FIG.3

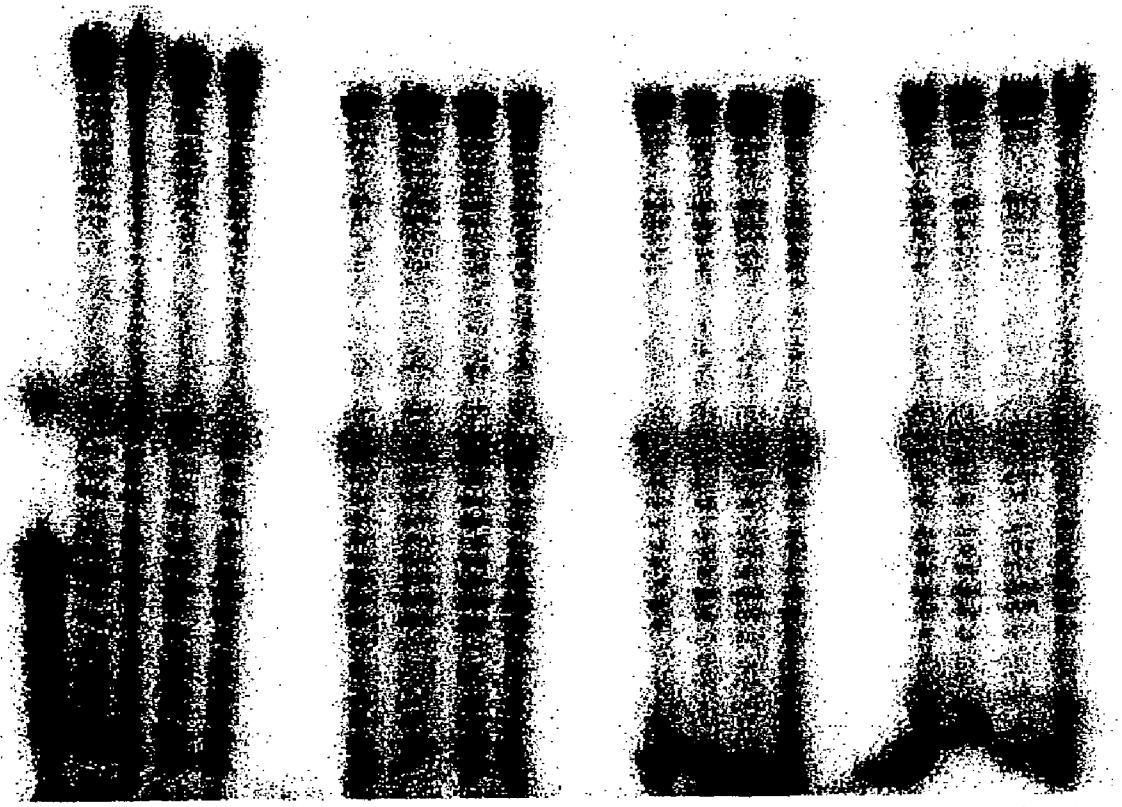


FIG.4

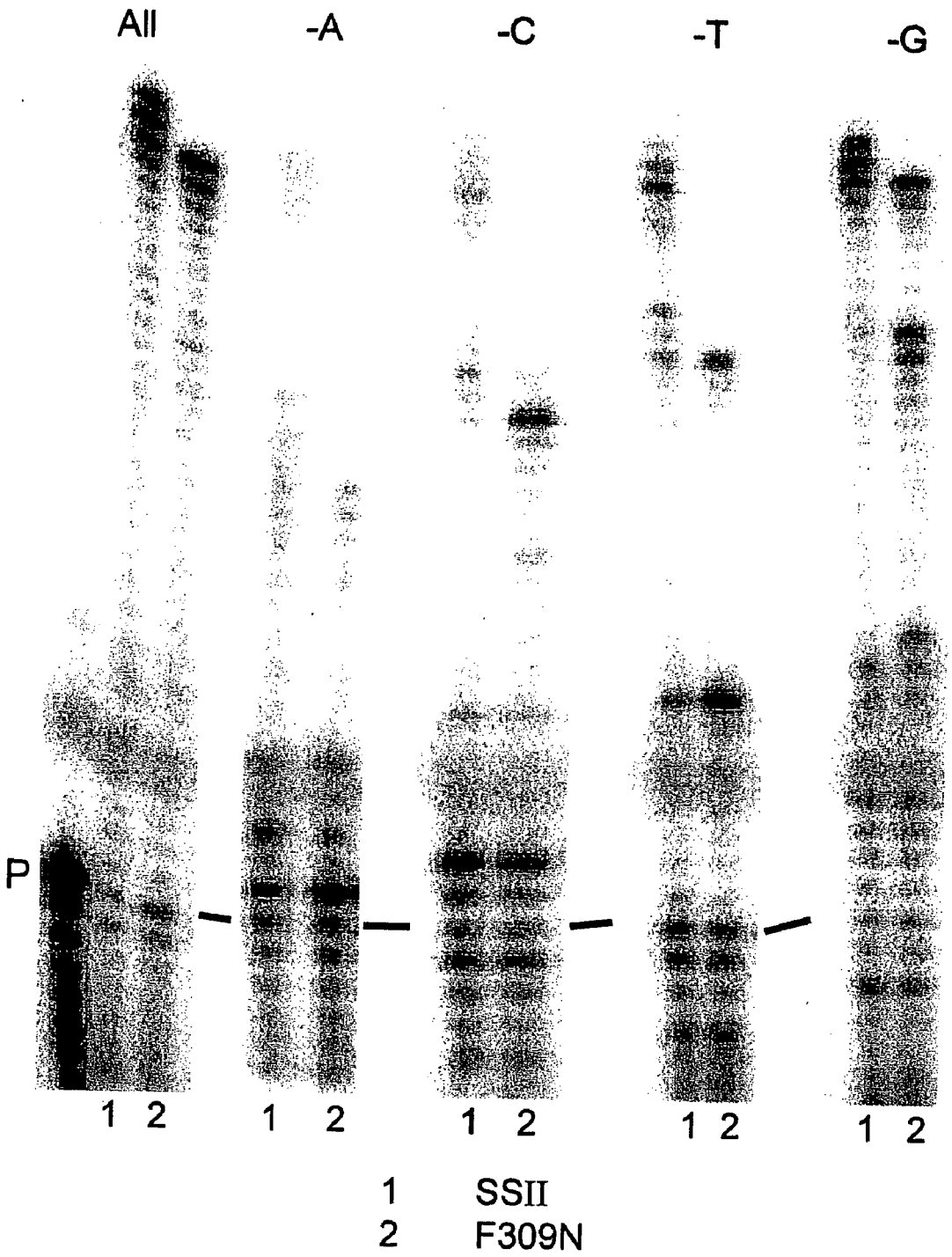


FIG.5

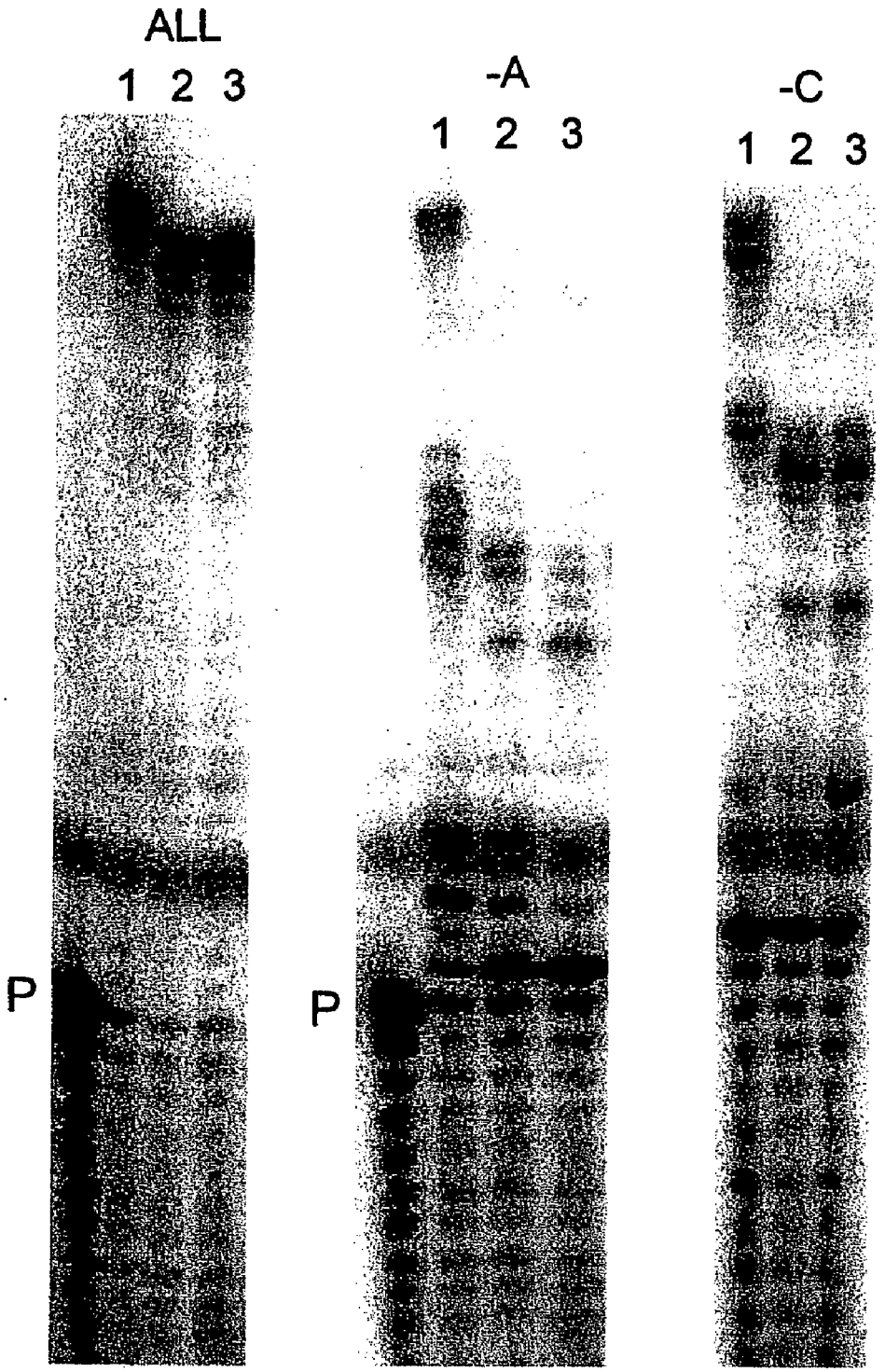


FIG.6

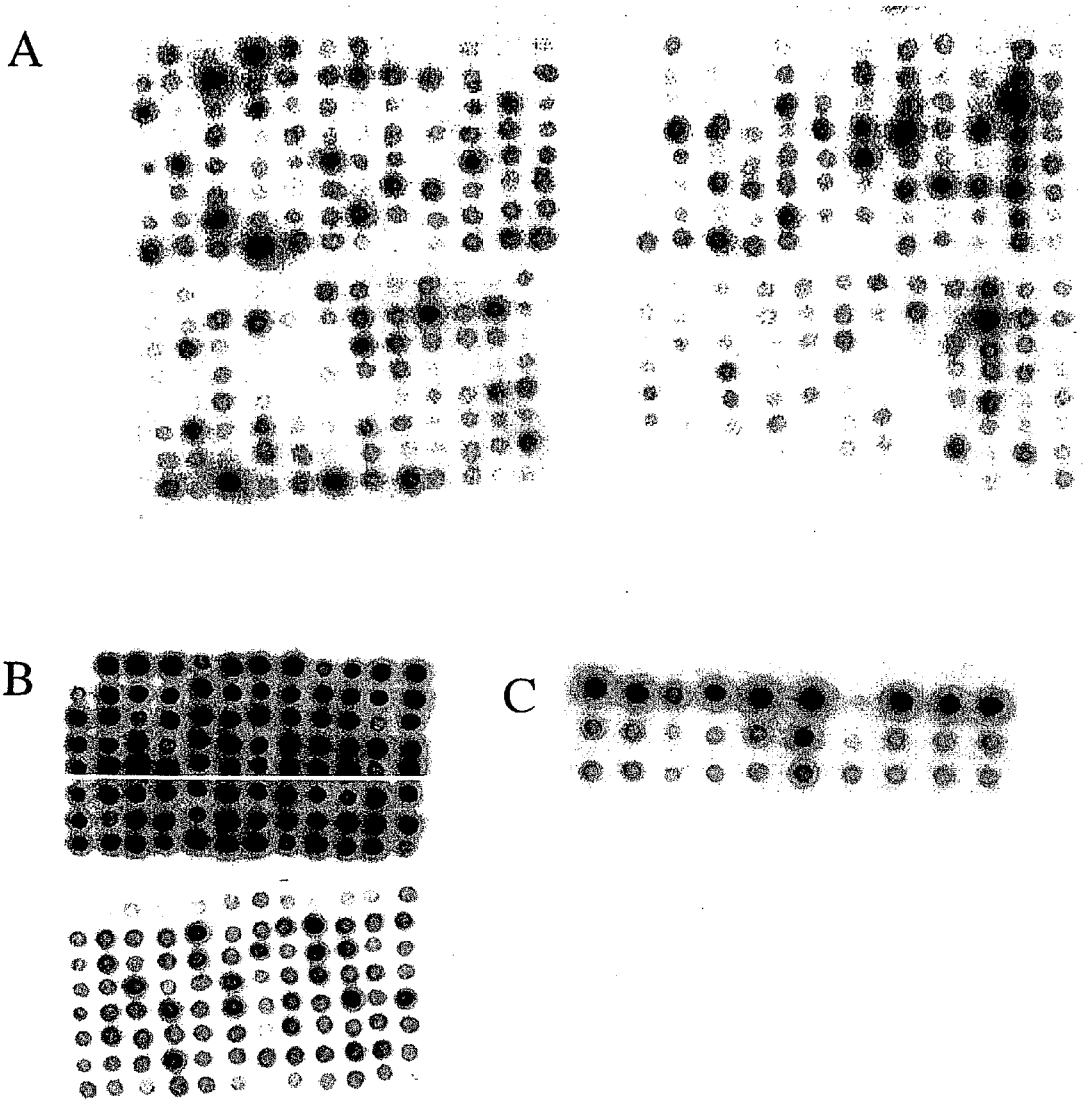


FIG. 7

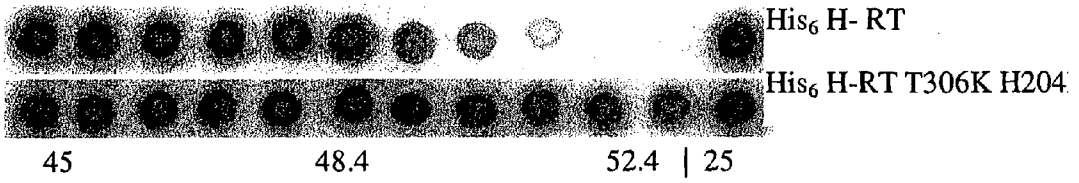


FIG. 8

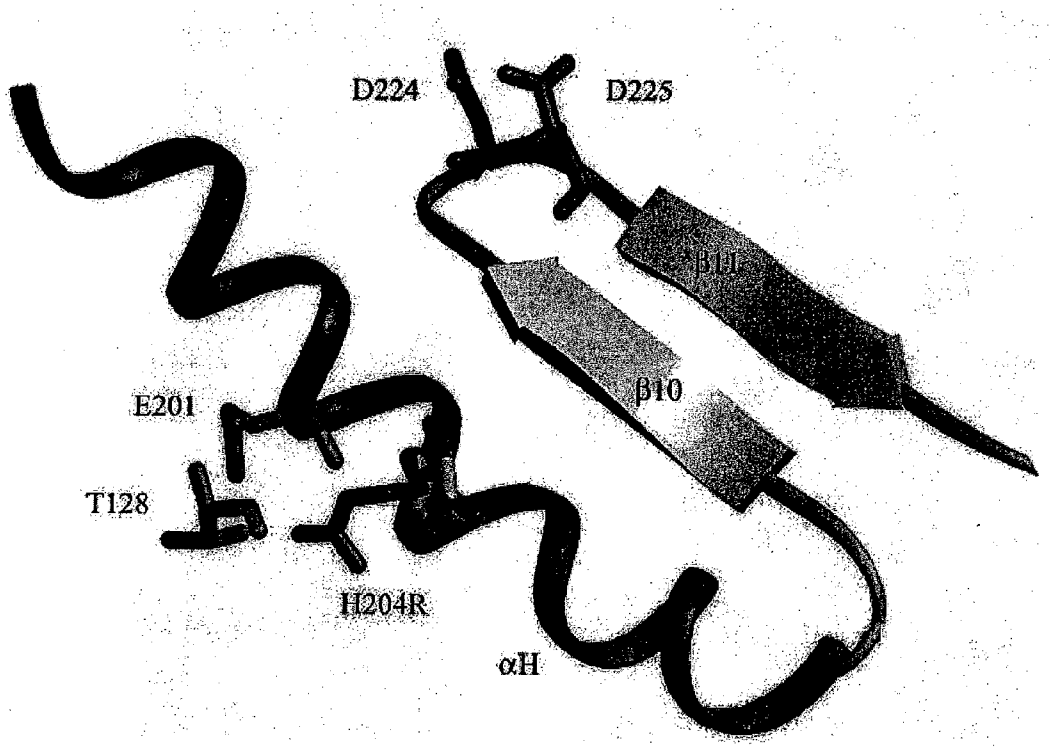


FIG. 9

FIG. 10A

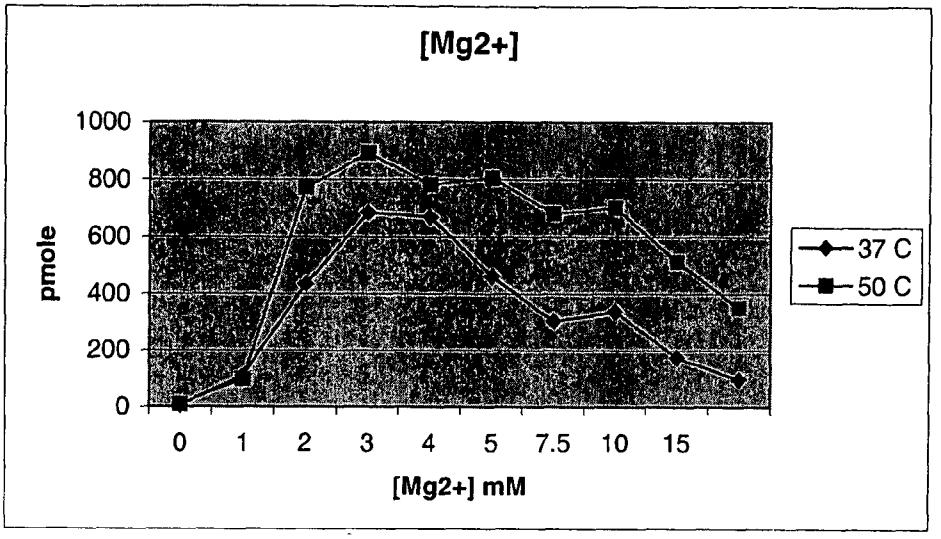


FIG. 10B

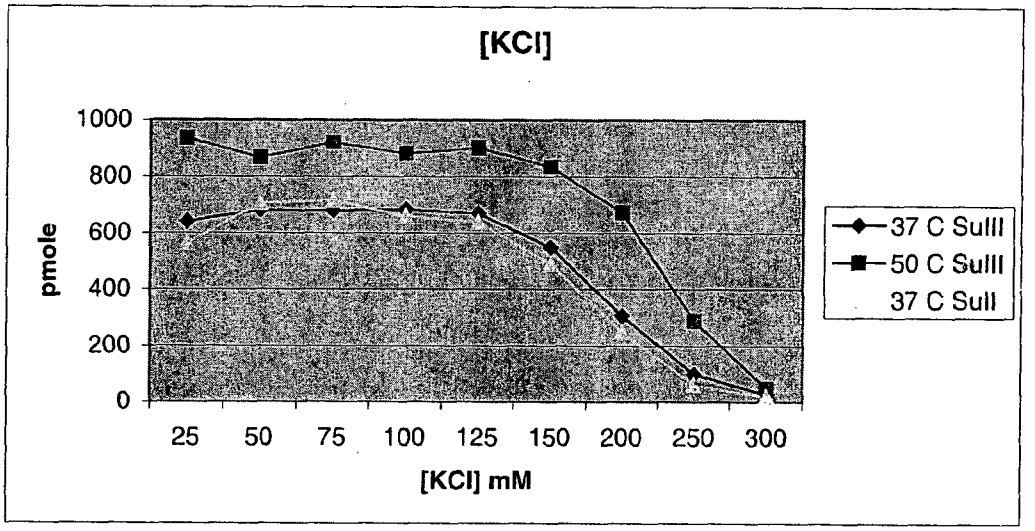
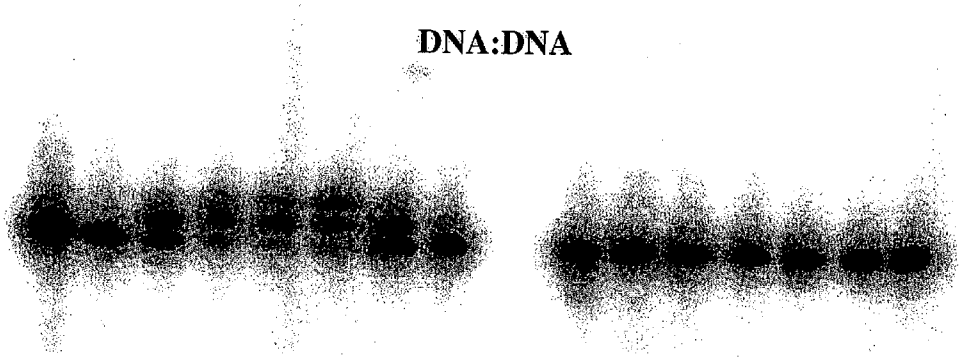


FIG. 11A

SuperScript II SuperScript III

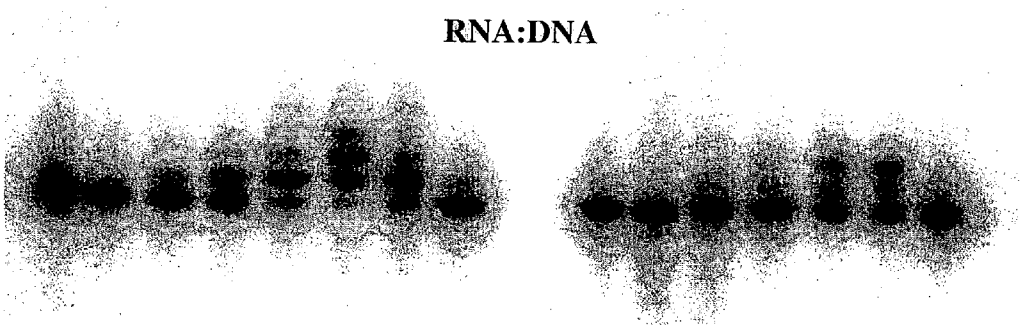
DNA:DNA



Temp	T	-	25	30	37	45	50	55	-	25	30	37	45	50	55
% extension			53	69	75	78	53	3		1	2	5	4	4	6

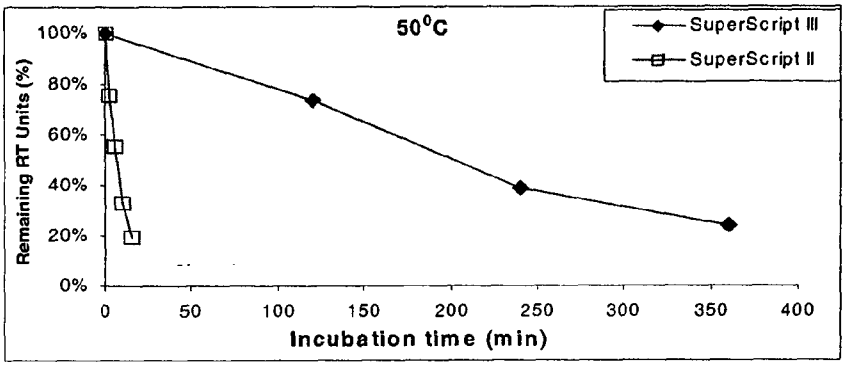
FIG. 11B

RNA:DNA

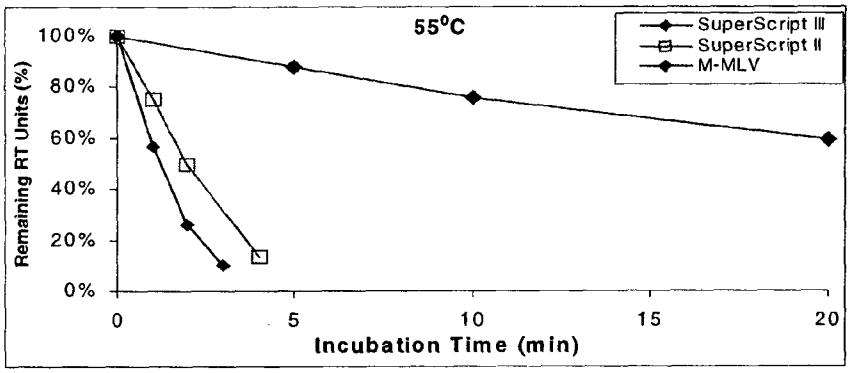


Temp	T	-	25	30	37	45	50	55	-	25	30	37	45	50	55
% extension			27	48	72	87	71	25		2	8	27	53	54	18

A


50°C
SuperScript III
SuperScript II
Remaining RT Units (%)
100%
80%
60%
40%
20%
0%
0
50
100
150
200
250
300
350
400
Incubation time (min)

B


55°C
SuperScript III
SuperScript II
M-MLV
Remaining RT Units (%)
100%
80%
60%
40%
20%
0%
0
5
10
15
20
Incubation Time (min)

C

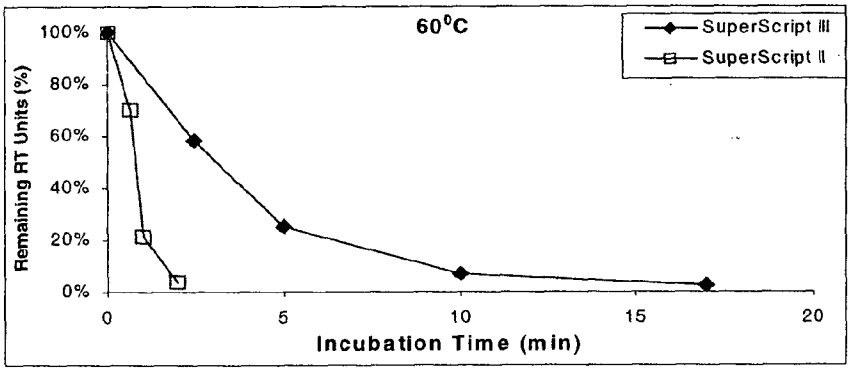

60°C
SuperScript III
SuperScript II
Remaining RT Units (%)
100%
80%
60%
40%
20%
0%
0
5
10
15
20
Incubation Time (min)

FIG. 12

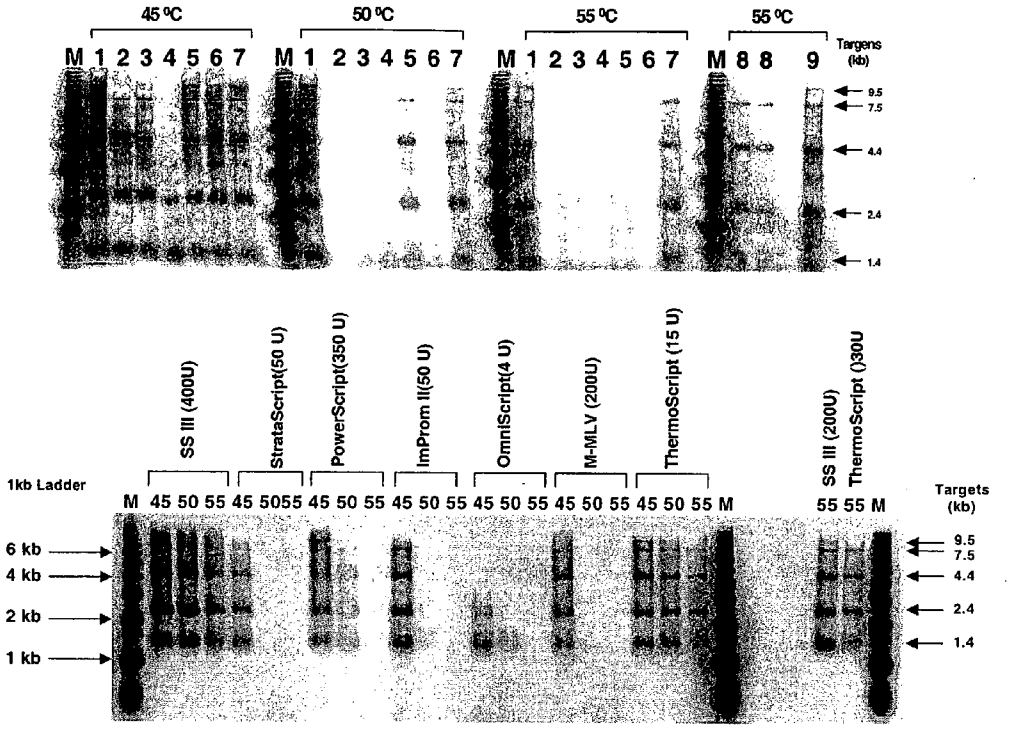

45 °C
50 °C
55 °C
55 °C
M 1 2 3 4 5 6 7 M 1 2 3 4 5 6 7 M 1 2 3 4 5 6 7 M 8 8 9
Targens (kb)
9.5
7.5
4.4
2.4
1.4
SS III (400U)
StrataScript(50 U)
PowerScript(350 U)
ImProm II(50 U)
OmniScript(4 U)
M-MLV (200U)
ThermoScript (15 U)
SS III (200U)
ThermoScript ()30U
1kb Ladder
M 45 50 55 45 5055 45 50 55 45 50 55 45 50 55 45 50 55 45 50 55 M 55 55 M
Targets (kb)
6 kb
4 kb
2 kb
1 kb
9.5
7.5
4.4
2.4
1.4

FIG. 13

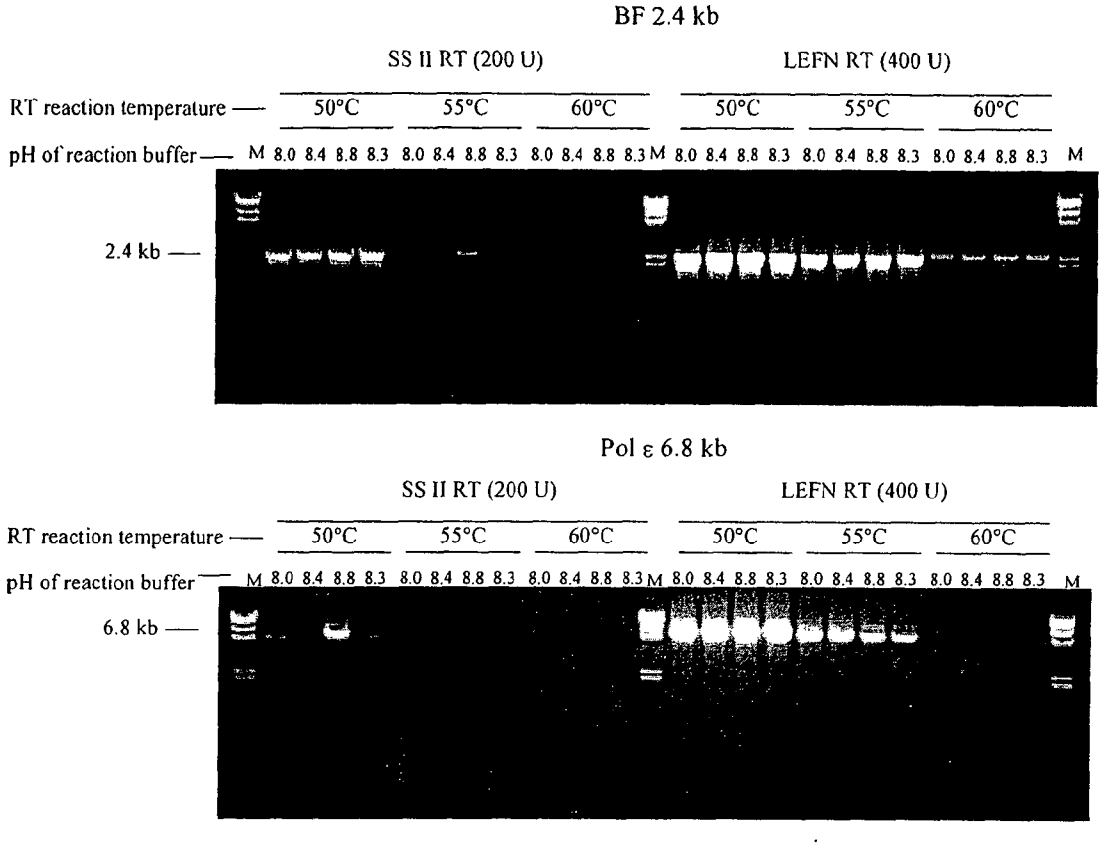

BF 2.4 kb
SS II RT (200 U)
LEFN RT (400 U)
RT reaction temperature
50°C
55°C
60°C
50°C
55°C
60°C
pH of reaction buffer
M 8.0 8.4 8.8 8.3 8.0 8.4 8.8 8.3 8.0 8.4 8.8 8.3 M 8.0 8.4 8.8 8.3 8.0 8.4 8.8 8.3 8.0 8.4 8.8 8.3 M
2.4 kb
Pol ε 6.8 kb
SS II RT (200 U)
LEFN RT (400 U)
RT reaction temperature
50°C
55°C
60°C
50°C
55°C
60°C
pH of reaction buffer
M 8.0 8.4 8.8 8.3 8.0 8.4 8.8 8.3 8.0 8.4 8.8 8.3 M 8.0 8.4 8.8 8.3 8.0 8.4 8.8 8.3 8.0 8.4 8.8 8.3 M
6.8 kb

FIG. 14

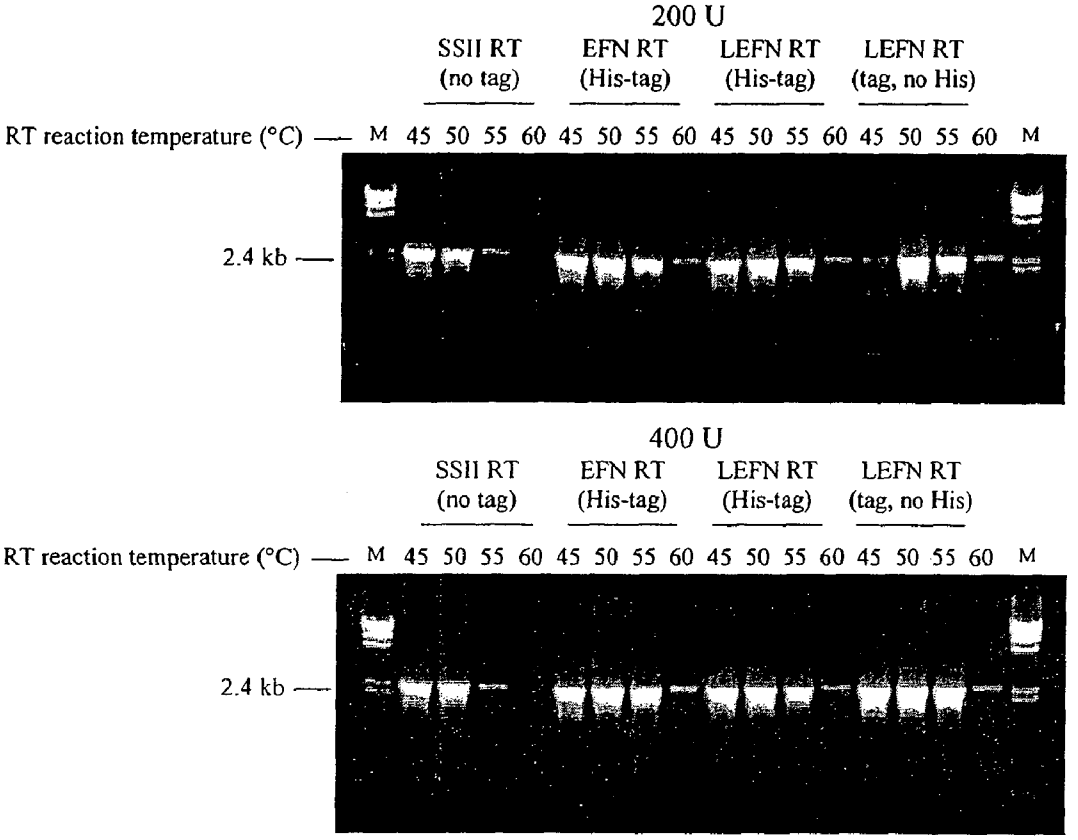

200 U
SSII RT (no tag)
EFN RT (His-tag)
LEFN RT (His-tag)
LEFN RT (tag, no His)
RT reaction temperature (°C)
M 45 50 55 60 45 50 55 60 45 50 55 60 45 50 55 60 M
2.4 kb
400 U
SSII RT (no tag)
EFN RT (His-tag)
LEFN RT (His-tag)
LEFN RT (tag, no His)
RT reaction temperature (°C)
M 45 50 55 60 45 50 55 60 45 50 55 60 45 50 55 60 M
2.4 kb

FIG. 15

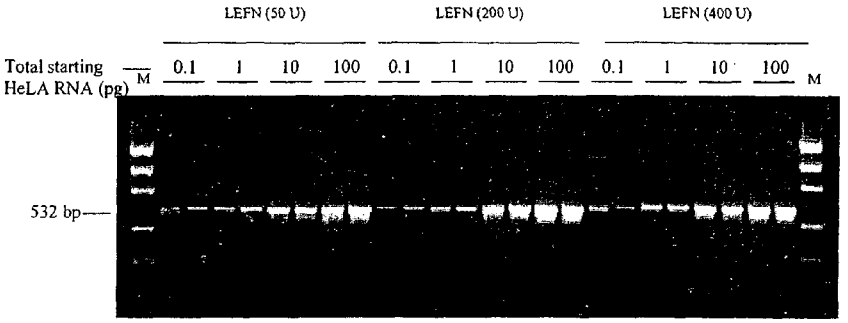

LEFN (50 U)
LEFN (200 U)
LEFN (400 U)
Total starting HeLA RNA (pg)
M
0.1
1
10
100
0.1
1
10
100
0.1
1
10
100
M
532 bp

FIG. 16

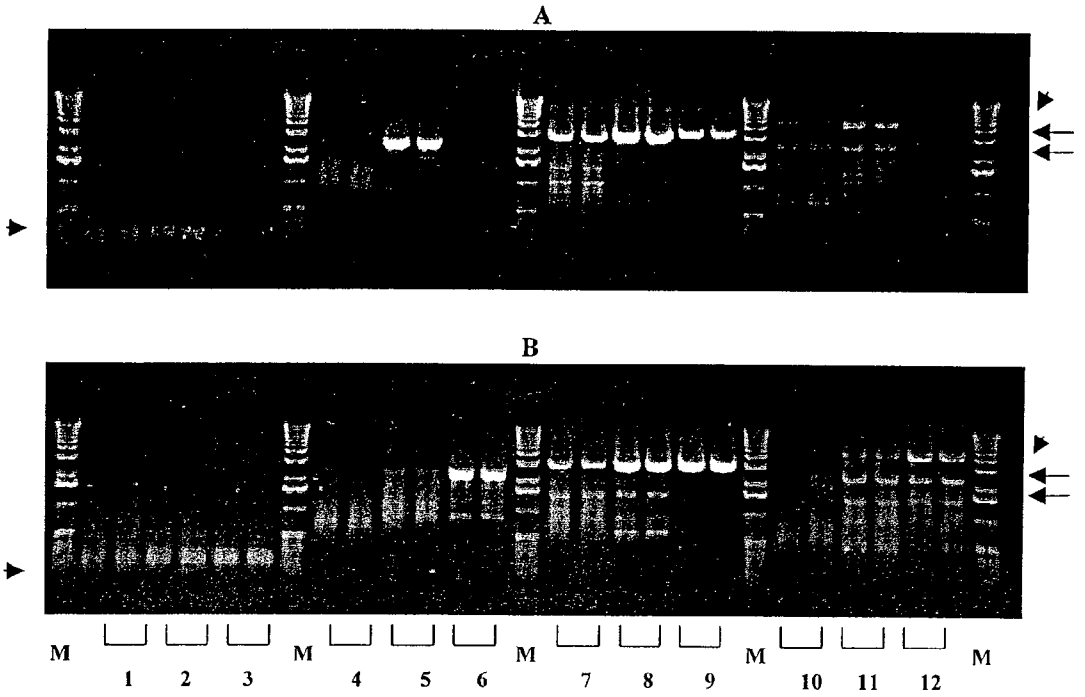


FIG. 17

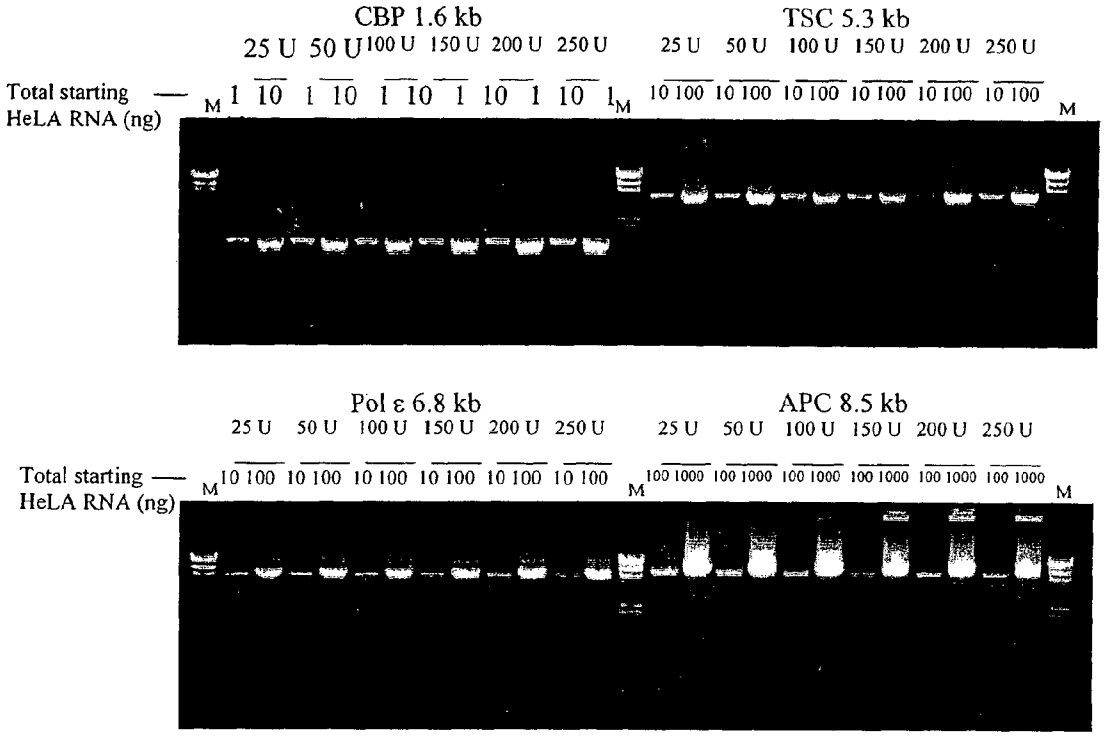

CBP 1.6 kb
25 U 50 U 100 U 150 U 200 U 250 U
TSC 5.3 kb
25 U 50 U 100 U 150 U 200 U 250 U
Total starting HeLA RNA (ng)
M 1 10 1 10 1 10 1 10 1 10 1 M
10 100 10 100 10 100 10 100 10 100 10 100 M
Pol ε 6.8 kb
25 U 50 U 100 U 150 U 200 U 250 U
APC 8.5 kb
25 U 50 U 100 U 150 U 200 U 250 U
Total starting HeLA RNA (ng)
M 10 100 10 100 10 100 10 100 10 100 10 100 M
100 1000 100 1000 100 1000 100 1000 100 1000 100 1000 M

FIG. 18

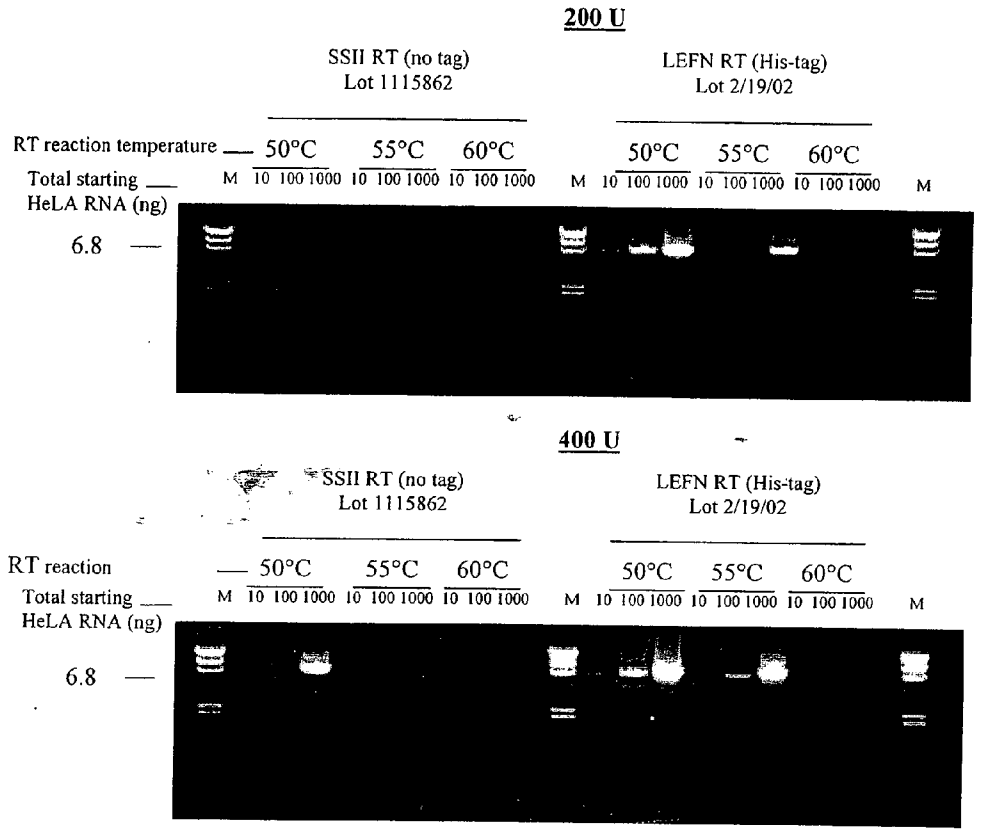

200 U
SSII RT (no tag)
Lot 1115862
LEFN RT (His-tag)
Lot 2/19/02
RT reaction temperature
50°C
55°C
60°C
50°C
55°C
60°C
Total starting
HeLA RNA (ng)
M 10 100 1000 10 100 1000 10 100 1000 M 10 100 1000 10 100 1000 10 100 1000 M
6.8
400 U
SSII RT (no tag)
Lot 1115862
LEFN RT (His-tag)
Lot 2/19/02
RT reaction
50°C
55°C
60°C
50°C
55°C
60°C
Total starting
HeLA RNA (ng)
M 10 100 1000 10 100 1000 10 100 1000 M 10 100 1000 10 100 1000 10 100 1000 M
6.8

FIG. 19

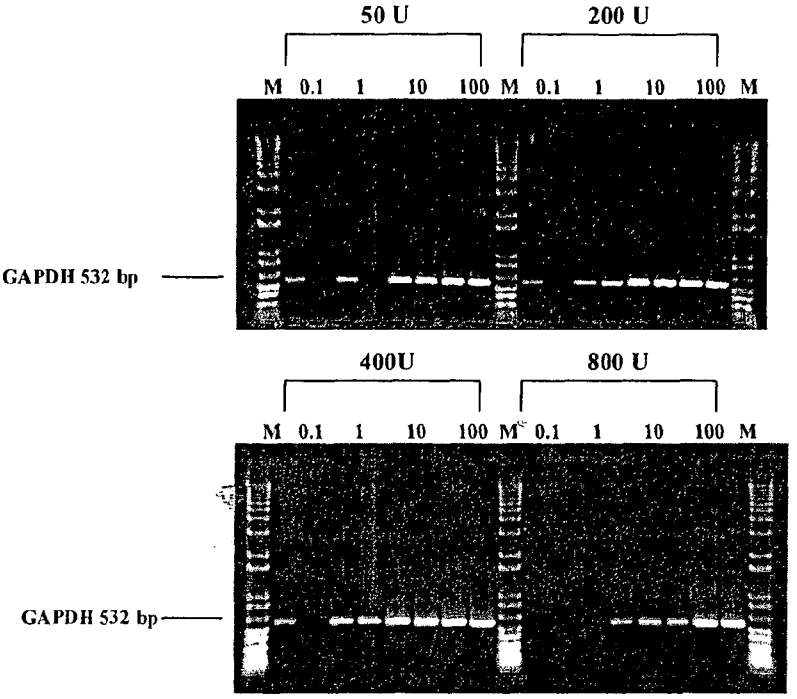

50 U
200 U
M 0.1 1 10 100 M 0.1 1 10 100 M
GAPDH 532 bp
400U
800 U
M 0.1 1 10 100 M 0.1 1 10 100 M
GAPDH 532 bp

FIG. 20

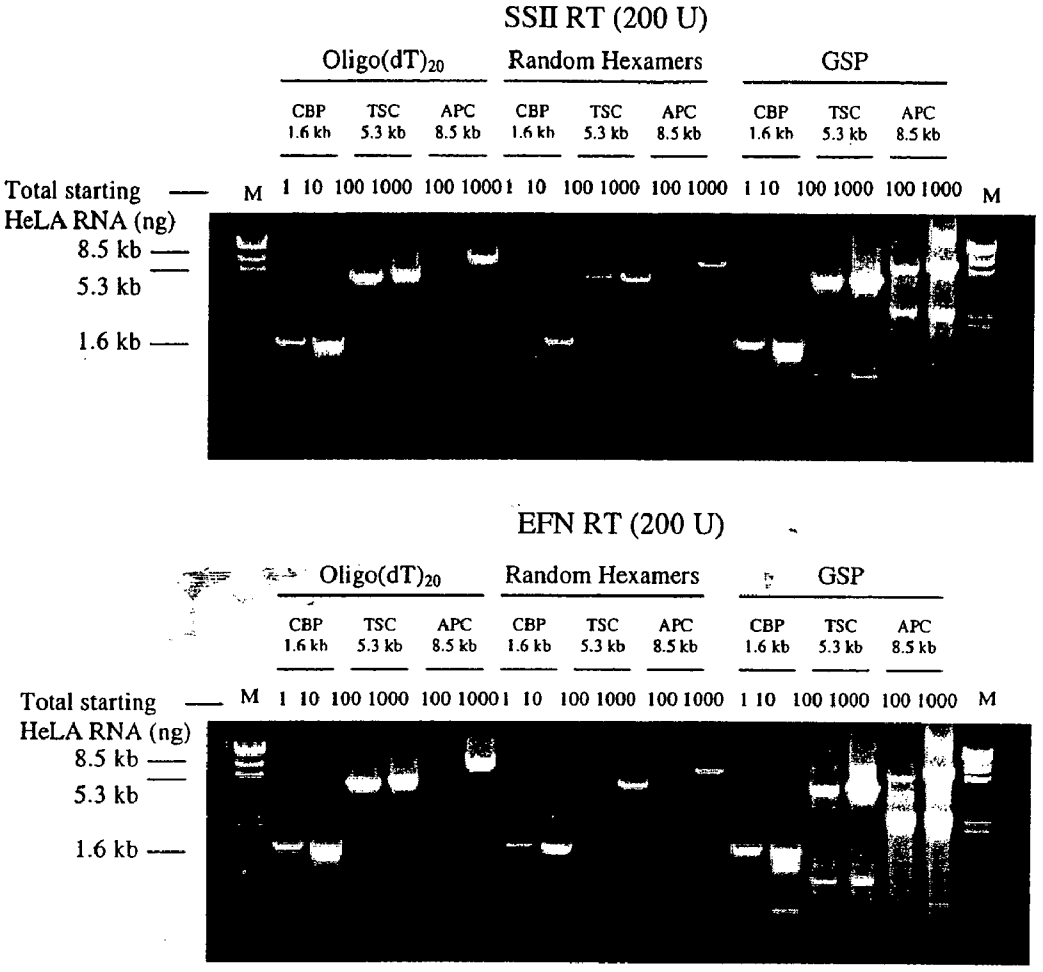

SSII RT (200 U)
Oligo(dT)20
Random Hexamers
GSP
CBP 1.6 kb
TSC 5.3 kb
APC 8.5 kb
Total starting HeLA RNA (ng)
M 1 10 100 1000 100 1000 1 10 100 1000 100 1000 1 10 100 1000 100 1000 M
8.5 kb
5.3 kb
1.6 kb
EFN RT (200 U)
Oligo(dT)20
Random Hexamers
GSP
CBP 1.6 kb
TSC 5.3 kb
APC 8.5 kb
Total starting HeLA RNA (ng)
M 1 10 100 1000 100 1000 1 10 100 1000 100 1000 1 10 100 1000 100 1000 M
8.5 kb
5.3 kb
1.6 kb

FIG. 21

THERMOSTABLE REVERSE TRANSCRIPTASES AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of U.S. Provisional patent application No. 60/410,283, filed Sep. 13, 2002, the entire disclosure of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] The present invention is in the fields of molecular and cellular biology. The invention is generally related to reverse transcriptase enzymes and methods for the reverse transcription of nucleic acid molecules, especially messenger RNA molecules. Specifically, the invention relates to reverse transcriptase enzymes which have been mutated or modified to increase thermostability, decrease terminal deoxynucleotidyl transferase activity, and/or increase fidelity, and to methods of producing, amplifying or sequencing nucleic acid molecules (particularly cDNA molecules) using these reverse transcriptase enzymes or compositions. The invention also relates to nucleic acid molecules produced by these methods and to the use of such nucleic acid molecules to produce desired polypeptides. The invention also relates to nucleic acid molecules encoding the reverse transcriptases of the invention, to vectors containing such nucleic acid molecules, and to host cells containing such nucleic acid molecules. The invention also concerns kits or compositions comprising such enzymes.

[0004] 2. Related Art

[0005] cDNA and cDNA Libraries

[0006] In examining the structure and physiology of an organism, tissue or cell, it is often desirable to determine its genetic content. The genetic framework of an organism is encoded in the double-stranded sequence of nucleotide bases in the deoxyribonucleic acid (DNA) which is contained in the somatic and germ cells of the organism. The genetic content of a particular segment of DNA, or gene, is typically manifested upon production of the protein which the gene encodes. In order to produce a protein, a complementary copy of one strand of the DNA double helix is produced by RNA polymerase enzymes, resulting in a specific sequence of ribonucleic acid (RNA). This particular type of RNA, since it contains the genetic message from the DNA for production of a protein, is called messenger RNA (mRNA).

[0007] Within a given cell, tissue or organism, there exist myriad mRNA species, each encoding a separate and specific protein. This fact provides a powerful tool to investigators interested in studying genetic expression in a tissue or cell. mRNA molecules may be isolated and further manipulated by various molecular biological techniques, thereby allowing the elucidation of the full functional genetic content of a cell, tissue or organism.

[0008] One common approach to the study of gene expression is the production of complementary DNA (cDNA) clones. In this technique, the mRNA molecules from an organism are isolated from an extract of the cells or tissues of the organism. This isolation often employs solid chromatography matrices, such as cellulose or agarose, to which oligomers of thymidine (T) have been complexed. Since the 3' termini on most eukaryotic mRNA molecules contain a string of adenosine (A) bases, and since A base pairs with T, the mRNA molecules can be rapidly purified from other molecules and substances in the tissue or cell extract. From these purified mRNA molecules, cDNA copies may be made using the enzyme reverse transcriptase (RT), which results in the production of single-stranded cDNA molecules. This reaction is typically referred to as the first strand reaction. The single-stranded cDNAs may then be converted into a complete double-stranded DNA copy (i.e., a double-stranded cDNA) of the original mRNA (and thus of the original double-stranded DNA sequence, encoding this mRNA, contained in the genome of the organism) by the action of a DNA polymerase. The protein-specific double-stranded cDNAs can then be inserted into a plasmid or viral vector, which is then introduced into a host bacterial, yeast, animal or plant cell. The host cells are then grown in culture media, resulting in a population of host cells containing (or in many cases, expressing) the gene of interest.

[0009] This entire process, from isolation of mRNA from a source organism or tissue to insertion of the cDNA into a plasmid or vector to growth of host cell populations containing the isolated gene, is termed "cDNA cloning." The set of cDNAs prepared from a given source of mRNAs is called a "cDNA library." The cDNA clones in a cDNA library correspond to the genes transcribed in the source tissue. Analysis of a cDNA library can yield much information on the pattern of gene expression in the organism or tissue from which it was derived.

[0010] Retroviral Reverse Transcriptase Enzymes

[0011] Three prototypical forms of retroviral reverse transcriptase have been studied thoroughly. Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase contains a single subunit of 78 kDa with RNA-dependent DNA polymerase and RNase H activity. This enzyme has been cloned and expressed in a fully active form in *E. coli* (reviewed in Prasad, V. R., *Reverse Transcriptase*, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press, p.135 (1993)). Human Immunodeficiency Virus (HIV) reverse transcriptase is a heterodimer of p66 and p51 subunits in which the smaller subunit is derived from the larger by proteolytic cleavage. The p66 subunit has both a RNA-dependent DNA polymerase and an RNase H domain, while the p51 subunit has only a DNA polymerase domain. Active HIV p66/p51 reverse transcriptase has been cloned and expressed successfully in a number of expression hosts, including *E. coli* (reviewed in Le Grice, S. F. J., *Reverse Transcriptase*, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory press, p. 163 (1993)). Within the HIV p66/p51 heterodimer, the 51-kD subunit is catalytically inactive, and the 66-kD subunit has both DNA polymerase and RNase H activity (Le Grice, S. F. J., el al., *EMBO Journal* 10:3905 (1991); Hostomsky, Z., et al., *J. Virol.* 66:3179 (1992)). Avian Sarcoma-Leukosis Virus (ASLV) reverse transcriptase, which includes but is not limited to Rous Sarcoma Virus (RSV) reverse transcriptase, Avian Myeloblastosis Virus (AMV) reverse transcriptase, Avian Erythroblastosis Virus (AEV) Helper Virus MCAV reverse transcriptase, Avian Myelocytomatosis Virus MC29 Helper Virus MCAV reverse transcriptase, Avian Reticuloendotheliosis Virus (REV-T) Helper Virus REV-A reverse transcriptase, Avian Sarcoma

Virus UR2 Helper Virus UR2AV reverse transcriptase, Avian Sarcoma Virus Y73 Helper Virus YAV reverse transcriptase, Rous Associated Virus (RAV) reverse transcriptase, and Myeloblastosis Associated Virus (MAV) reverse transcriptase, is also a heterodimer of two subunits, α (approximately 62 kDa) and β (approximately 94 kDa), in which a is derived from 13 by proteolytic cleavage (reviewed in Prasad, V. R., *Reverse Transcriptase, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press* (1993), p. 135). ASLV reverse transcriptase can exist in two additional catalytically active structural forms, Ad and a (Hizi, A. and Joklik, W. K., *J. Biol. Chem.* 252: 2281 (1977)). Sedimentation analysis suggests αβ and ββ are dimers and that the a form exists in an equilibrium between monomeric and dimeric forms (Grandgenett, D. P., et al., *Proc. Nat. Acad. Sci. USA* 70:230 (1973); Hizi, A. and Joklik, W. K., *J. Biol. Chem.* 252:2281 (1977); and Soltis, D. A. and Skalka, A. M., *Proc. Nat. Acad. Sci. USA* 85:3372 (1988)). The ASLV αβ and ββ reverse transcriptases are the only known examples of retroviral reverse transcriptase that include three different activities in the same protein complex: DNA polymerase, RNase H, and DNA endonuclease (integrase) activities (reviewed in Skalka, A. M., *Reverse Transcriptase*, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press (1993), p. 193). The a form lacks the integrase domain and activity.

[0012] Various forms of the individual subunits of ASLV reverse transcriptase have been cloned and expressed. These include a 98-kDa precursor polypeptide that is normally processed proteolytically to β and a 4 kDa polypeptide removed from the β carboxy end (Alexander, F., et al., *J. Virol.* 61:534 (1987) and Anderson, D. et al., *Focus* 17:53 (1995)), and the mature β subunit (Weis, J. H. and Salstrom, J. S., U.S. Pat. No. 4,663,290 (1987); and Soltis, D. A. and Skalka, A. M., *Proc. Nat. Acad. Sci. USA* 85:3372 (1988)). (See also Werner S. and Wohrl B. M., *Eur. J. Biochem.* 267:4740-4744 (2000); Werner S. and Wohrl B. M., *J. Virol.* 74:3245-3252 (2000); Werner S. and Wohrl B. M., *J. Biol. Chem.* 274:26329-26336 (1999).) Heterodimeric RSV αβ reverse transcriptase has also been purified from *E. coli* cells expressing a cloned RSV β gene (Chernov, A. P., et al., *Biomed. Sci.* 2:49 (1991)).

[0013] Reverse Transcription Efficiency

[0014] As noted above, the conversion of mRNA into cDNA by reverse transcriptase-mediated reverse transcription is an essential step in the study of proteins expressed from cloned genes. However, the use of unmodified reverse transcriptase to catalyze reverse transcription is inefficient for a number of reasons. First, reverse transcriptase sometimes degrades an RNA template before the first strand reaction is initiated or completed, primarily due to the intrinsic RNase H activity present in reverse transcriptase. In addition, mis-priming of the mRNA template molecule can lead to the introduction of errors in the cDNA first strand while secondary structure of the mRNA molecule itself may make some mRNAs refractory to first strand synthesis.

[0015] Removal of the RNase H activity of reverse transcriptase can eliminate the first problem and improve the efficiency of reverse transcription (Gerard, G. F., et al., *FOCUS* 11(4):60 (1989); Gerard, G. F., et al., *FOCUS* 14(3):91 (1992)). However such reverse transcriptases ("RNase H−" forms) do not address the additional problems of mis-priming and mRNA secondary structure.

[0016] Another factor which influences the efficiency of reverse transcription is the ability of RNA to form secondary structures. Such secondary structures can form, for example, when regions of RNA molecules have sufficient complementarity to hybridize and form double stranded RNA. Generally, the formation of RNA secondary structures can be reduced by raising the temperature of solutions which contain the RNA molecules. Thus, in many instances, it is desirable to reverse transcribe RNA at temperatures above 37° C. However, art known reverse transcriptases generally lose activity when incubated at temperatures much above 37° C. (e.g., 50° C.).

SUMMARY OF THE INVENTION

[0017] The present invention provides, in part, reverse transcriptase enzymes, compositions comprising such enzymes and methods useful in overcoming limitations of reverse transcription discussed above. In general, the invention provides compositions for use in reverse transcription of a nucleic acid molecule, these compositions comprising one or more (e.g., one, two, three, four, five, ten, fifteen, etc.) polypeptides having at least one reverse transcriptase activity. Such compositions may further comprise one or more (e.g., one, two, three, four, five, etc.) nucleotides (e.g. one or more fluorescent-labeled nucleotides, one or more radiolabeled nucleotides, etc.), a suitable buffer, and/or one or more (e.g., one, two, three, four, five, ten, fifteen, etc.) DNA polymerases. Compositions of the invention may also comprise one or more (e.g., one, two, three, four, five, ten, fifteen, etc.) oligonucleotide primers, and/or one or more templates, and/or one or more nucleic acid molecules (which may be complementary to all or a portion of such templates).

[0018] Reverse transcriptases of the invention are preferably modified or mutated such that the thermostability of the enzyme is increased or enhanced and/or the fidelity of the enzyme is increased or enhanced. In specific embodiments, reverse transcriptases of the invention may be single chained (single subunit) or multi-chained (multi-subunit) and may be reduced or substantially reduced in RNase H activity or may have no detectable RNase H activity or may be lacking in RNase H activity. Preferably enzymes of the invention are enzymes selected from the group consisting of Moloney Murine Leukemia Virus (M-MLV) RNase H− reverse transcriptase, Rous Sarcoma Virus (RSV) RNase H− reverse transcriptase, Avian Myeloblastosis Virus (AMV) RNase H− reverse transcriptase, Rous Associated Virus (RAV) RNase H− reverse transcriptase, Myeloblastosis Associated Virus (MAV) RNase H− reverse transcriptase or other ASLV RNase H− reverse transcriptases and Human Immunodeficiency Virus (HIV) RNase H− reverse transcriptase and mutants thereof. In preferred compositions, the reverse transcriptases are present at working concentrations.

[0019] In certain aspects, the invention includes reverse transcriptases which have been modified or mutated to increase or enhance thermostability. Examples of such reverse transcriptases include enzymes comprising one or more modifications or mutations at positions corresponding to. amino acids selected from the group consisting of:

[0020] (a) leucine 52 of M-MLV reverse transcriptase;

[0021] (b) tyrosine 64 of M-MLV reverse transcriptase;

[0022] (c) lysine 152 of M-MLV reverse transcriptase;

[0023] (d) histidine 204 of M-MLV reverse transcriptase;

[0024] (e) methionine 289 of M-MLV reverse transcriptase;

[0025] (f) threonine 306 of M-MLV reverse transcriptase; and

[0026] (g) phenylalanine 309 of M-MLV reverse transcriptase.

[0027] In some embodiments, a modification or mutation may be the addition of an N- and/or C-terminal tag sequence.

[0028] In specific embodiments, the invention is directed to M-MLV reverse transcriptases wherein leucine 52 is replaced with proline, tyrosine 64 is replaced with arginine, lysine 152 is replaced with methionine, histidine 204 is replaced with arginine, methionine 289 is replaced with leucine, threonine 306 is replaced with either lysine or arginine, and/or phenylalanine 309 is replaced with asparagine or serine. Further included within the scope of the invention are reverse transcriptases, other than M-MLV reverse transcriptase, which contain alterations corresponding to those set out above.

[0029] In additional aspects, the invention also include thermostable reverse transcriptases which retain at least about 50%, at least about 60%, at least about 70%, at least about 85%, at least about 95%, at least about 97%, at least about 98%, at least about 99%, at least about 100%, at least about 150%, at least about 200%, at least about 250%, or at least about 300% of reverse transcriptase activity after heating to 50° C. for 5 minutes.

[0030] As noted above, enzymes of the invention include reverse transcriptases which exhibit reverse transcriptase activity either upon the formation of multimers (e.g., dimers) or as individual protein molecules (i.e., in monomeric form). Examples of reverse transcriptases which exhibit reverse transcriptase activity upon the formation of multimers include AMV, RSV and HIV reverse transcriptases. One example of a reverse transcriptase which exhibits reverse transcriptase activity as separate, individual proteins (i.e., in monomeric form) is M-MLV reverse transcriptase.

[0031] Multimeric reverse transcriptases of the invention may form homo-multimers or hetero-multimers. In other words, the subunits of the multimeric protein complex may be identical or different. One example of a hetero-dimeric reverse transcriptase is AMV reverse transcriptase, which is composed of two subunits that differ in primary amino acid sequence. More specifically, as already discussed, AMV reverse transcriptase may be composed of two subunits wherein one of these subunits is generated by proteolytic processing of the other. Thus, dimeric AMV reverse transcriptase may be composed of subunits of differing size which share regions of amino acid sequence identity.

[0032] The present invention relates in particular to mutant or modified reverse transcriptases wherein one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, twenty, etc.) amino acid changes have been made which renders the enzyme more thermostable in nucleic acid synthesis, as compared to the unmutated or unmodified reverse transcriptases. Sites for mutation or modification to produce the thermostable reverse transcriptase enzymes of the present invention and/or reverse transcriptases which exhibit other characteristics (e.g., increased fidelity, decreased TdT activity, etc.) are listed for some reverse transcriptases in Table 1. As will be appreciated by those skilled in the art, one or more of the amino acids identified may be deleted and/or replaced with one or a number of amino acid residues. In a preferred aspect, any one or more of the amino acids identified in Table 1 may be substituted with any one or more amino acid residues such as Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and/or Val. The modifications described in Table 1 preferably produce thermostable reverse transcriptases of the invention. Similar or equivalent sites or corresponding sites in other reverse transcriptases can be mutated or modified to produce additional thermostable reverse transcriptases, as well as reverse transcriptases which exhibit other characteristics (e.g., increased fidelity, decreased TdT activity, etc.). Thus, a reverse transcriptase of the present invention may have one or more of the following properties: (a) increased thermostability or increased half-life at elevated temperatures; (b) reduced, substantially reduced, or no detectable RNase H activity, (c) reduced or substantially reduced terminal deoxynucleotidyl transferase activity, and/or (d) increased fidelity. In some embodiments, a reverse transcriptase of the invention may have a plurality of the properties listed above (e.g., a reverse transcriptase may have enhanced thermostability, reduced RNase H activity, and enhanced fidelity).

TABLE 1

RT	Amino Acids
M-MLV	L52, Y64, L135, H143, K152, Q165, G181, H204, I218, N249, M289, T306, F309, A517, D524, T544, V546, W548, E562, H577, D583, L604, S606, G608, F625, L626, H629, H631, H638, G641
AMV	V2, L4, W12, P14, H16, T17, W20, I21, Q23, W24, L26, P27, G29, V32, Q36, L42, Q43, L44, G45, H46, I47, P49, S50, L51, S52, C53, W54, F59, I61, A64, S65, G66, S67, Y68, L70, L71, A76, A79, P83, A86, V87, Q88, Q89, G90, A91, W101, P102, L108, Q120, S131, V132, N133, N134, Q135, P137, A138, Q142, Q148, T151, Y180, M181, S190, H191, G193, A196, I201, S202, P214, V217, Q218, P221, G222, Q224, L226, G227, Y228, G231, T233, Y234, A236, P237,

TABLE 1-continued

RT	Amino Acids
	G239, L240, P244, I246, T248, W250, Q252, G257, Q260, W261, P264, L266, G267, L272, Y277, Q279, L280, G282, S283, P285, N286, A288, N292, L293, M297, I302, V303, L305, S306, T308, L311, L320, I332, G333, V334, G336, Q337, G338, P345, W348, L349, F350, S351, P354, A357, F358, A360, W361, L362, V364, L365, T366, T370, A374, V377, G381, C392, P400, G402, L405, G412, I414, F423, I425, A426, P428, L433, H440, P441, V443, G444, P445, A451, S453, S454, T455, H456, G458, V459, V460, W462, W468, I470, I473, A474, L476, G477, A478, S479, V480, Q481, Q482, L483, A491, W495, P496, T497, T498, P499, T500, A507, F508, M512, L513, G520, V521, P522, S523, T524, A525, A527, F528, L534, S535, Q536, S538, V543, S548, H549, S550, V552, P553, F556, T557, N560, A562
RSV	V2, L4, W12, P14, H16, T17, W20, I21, Q23, W24, L26, P27, G29, V32, Q36, L42, Q43, L44, G45, H46, I47, P49, S50, L51, S52, C53, W54, F59, I61, A64, S65, G66, S67, Y68, L70, L71, A76, A79, P83, A86, V87, Q88, Q89, G90, A91, W101, P102, L108, Q120, S131, V132, N133, N134, Q135, P137, A138, Q142, Q148, T151, Y180, M181, S190, H191, G193, A196, I201, S202, P214, V217, Q218, P221, G222, Q224, L226, G227, Y228, G231, T233, Y234, A236, P237, G239, L240, P244, I246, T248, W250, Q252, G257, Q260, W261, P264, L266, G267, L272, Y277, Q279, L280, G282, S283, P285, N286, A288, N292, L293, M297, I302, V303, L305, S306, T308, L311, L320, I332, G333, V334, G336, Q337, G338, P345, W348, L349, F350, S351, P354, A357, F358, A360, W361, L362, V364, L365, T366, T370, A374, V377, G381, C392, P400, G402, L405, G412, I414, F423, I425, A426, P428, L433, H440, P441, V443, G444, P445, A451, S453, S454, T455, H456, G458, V459, V460, W462, W468, I470, I473, A474, L476, G477, A478, S479, V480, Q481, Q482, L483, A491, W495, P496, T497, T498, P499, T500, A507, F508, M512, L513, G520, V521, P522, S523, T524, A525, A527, F528, L534, S535, Q536, S538, V543, S548, H549, S550, V552, P553, F556, T557, N560, A562
HIV	I1, P3, L11, P13, G14, M15, Q22, W23, L25, T26, T38, G44, I46, S47, G50, P51, N53, P54, Y55, F60, I62, S67, T68, W70, L73, V89, Q90L91, G92, I93, S104, V110, G111, S133, I134, N135, N136, P139, G140, I141, Q144, N146, Q150, Y182, M183, I194, G195, Q196, T199, Q206, L209, P216, Q221, P224, P225, L227, M229, G230, Y231, H234, Q241, P242, V244, L245, S250, T252, N254, Q257, G261, N264, W265, Q268, P271, G272, Q277, C279, L281, L282, G284, T285, A287, L288, T289, V291, P293, L294, T295, L300, A303, I308, L309, P312, H314, Y317, L324, I328, Q329, G332, Q333, G334, Y341, P344, F345, Y353, M356, G358, A359, H360, T361, Q372, T376, V380, Q392, W405, Q406, A407, F415, V416, N417, T418, P419, P420, L424, W425, P432, V434, G435, A436, A444, A445, N446, T449, L451, N459, G461, Q463, V465, V466, P467, L468, T469, N470, T471, T472, N473, Q474, Y482, Q486, S488, G489, L490, Q499, Y500, G503, I504, S512, S514, L516, N518, Q519, Q523, I525, W534, P536, A537, H538, G540, I541, G542, Q546, L550, S552, A553, V554, I555

[0033] Those skilled in the art will appreciate that a different isolate of virus may encode a reverse transcriptase enzyme having a different amino acid at the positions identified above. Such isolates may be modified to produce the reverse transcriptases (e.g., thermostable reverse transcriptases) of the present invention.

[0034] Reverse transcriptases of the invention may have one or more of the following properties: (a) increased thermostability or increased half-life at elevated temperatures; (b) reduced, substantially reduced, or no detectable RNase H activity, (c) reduced or substantially reduced terminal deoxynucleotidyl transferase activity, and/or (d) increased fidelity.

[0035] Enzymes of the invention which have reduced or substantially reduced terminal deoxynucleotidyl transferase activity may comprise one or more modifications or mutations at positions corresponding to amino acids selected from the group consisting of:

[0036] (a) tyrosine 133 of M-MLV reverse transcriptase;

[0037] (b) threonine 197 of M-MLV reverse transcriptase; and

[0038] (c) phenylalanine 309 of M-MLV reverse transcriptase.

[0039] In specific embodiments, the invention is directed to M-MLV reverse transcriptases wherein tyrosine 133 is replaced with alanine, threonine 197 is replaced with glutamic acid, and/or phenylalanine 309 is replaced with asparagine. As will be appreciated, one or more of the amino acids identified may be deleted and/or replaced with one or a number of amino acid residues. Further included within the scope of the invention are reverse transcriptases, other than M-MLV reverse transcriptase, which contain alterations corresponding to those set out above.

[0040] Additionally, enzymes which exhibit increased fidelity may comprise one or more modifications or mutations at positions corresponding to amino acids selected from the group consisting of:

[0041] (a) tyrosine 64 of M-MLV reverse transcriptase;

[0042] (b) arginine 116 of M-MLV reverse transcriptase;

[0043] (c) glutamine 190 of M-MLV reverse transcriptase; and

[0044] (d) valine 223 of M-MLV reverse transcriptase.

[0045] As will be appreciated, one or more of the amino acids identified may be deleted and/or replaced with any one or a number of amino acid residues. Further, included in the invention are reverse transcriptases, other than M-MLV reverse transcriptase, that contain alterations corresponding to those set out above.

[0046] In some embodiments, the present invention provides a modified or mutated reverse transcriptase (e.g., preferably a modified or mutated retroviral reverse transcriptase) having a reverse transcriptase activity that has a half-life of greater than that of the corresponding unmodified or un-mutated reverse transcriptase at an elevated temperature, i.e., greater than 37° C. In some embodiments, the half-life of a reverse transcriptase of the present invention may be 5 minutes or greater and preferably 10 minutes or greater at 50° C. In some embodiments, the reverse transcriptases of the invention may have a half-life (e.g., at 50° C.) equal to or greater than about 25 minutes, preferably equal to or greater than about 50 minutes, more preferably equal to or greater than about 100 minutes, and most preferably, equal to or greater than about 200 minutes.

[0047] In some embodiments, the reverse transcriptases of the invention may have a half-life at 50° C. that is from about 10 minutes to about 200 minutes, from about 10 minutes to about 150 minutes, from about 10 minutes to about 100 minutes, from about 10 minutes to about 75 minutes, from about 10 minutes to about 50 minutes, from about 10 minutes to about 40 minutes, from about 10 minutes to about 30 minutes, or from about 10 minutes to about 20 minutes.

[0048] A modified or mutated reverse transcriptase of the invention (e.g., one having a half-life at 50° C. as described above) may be a modified or mutated retroviral reverse transcriptase. A reverse transcriptase according to the invention may be selected from a group consisting of M-MLV reverse transcriptase, ASV reverse transcriptase, HIV reverse transcriptase, Avian Sarcoma-Leukosis Virus (ASLV) reverse transcriptase, Rous Sarcoma Virus (RSV) reverse transcriptase, Avian Myeloblastosis Virus (AMV) reverse transcriptase, Avian Erythroblastosis Virus (AEV) Helper Virus MCAV reverse transcriptase, Avian Myelocytomatosis Virus MC29 Helper Virus MCAV reverse transcriptase, Avian Reticuloendotheliosis Virus (REV-T) Helper Virus REV-A reverse transcriptase, Avian Sarcoma Virus UR2 Helper Virus UR2AV reverse transcriptase, Avian Sarcoma Virus Y73 Helper Virus YAV reverse transcriptase, Rous Associated Virus (RAV) reverse transcriptase, and Myeloblastosis Associated Virus (MAV) reverse transcriptase, and fragments of any of the above having reverse transcriptase activity.

[0049] Mutated or modified reverse transcriptases of the present invention may have a reverse transcriptase activity (e.g., RNA-dependent DNA polymerase activity) that has a longer half-life at 55° C. than the reverse transcriptase activity of a corresponding un-mutated or unmodified reverse transcriptases. For example, introduction of the H204R, M289K, T306K, and F309N mutation into His_6-H$^-$ RT increases the half-life at 55° C. from 1.6 minutes to 8.1 minutes (see Table 9). At 55° C., the half-life of reverse transcriptase activity of a mutated or modified reverse transcriptase of the invention may be greater than about 2 minutes, greater than about 3 minutes, greater than about 4 minutes, greater than about 5 minutes, greater than about 6 minutes, greater than about 7 minutes, greater than about 8 minutes, greater than about 10 minutes, greater than about 15 minutes, greater than about 20 minutes, or greater than about 30 minutes. At 55° C., the half-life of reverse transcriptase activity of a reverse transcriptase of the invention may be from about 2 minutes to about 60 minutes, from about 2 minutes to about 45 minutes, from about 2 minutes to about 30 minutes, from about 2 minutes to about 20 minutes, from about 2 minutes to about 15 minutes, from about 2 minutes to about 10 minutes, from about 2 minutes to about 8 minutes, from about 2 minutes to about 7 minutes, from about 2 minutes to about 6 minutes, from about 2 minutes to about 5 minutes, from about 2 minutes to about 4 minutes, or from about 2 minutes to about 3 minutes. Such a reverse transcriptase may be a modified or mutant retroviral reverse transcriptase.

[0050] A modified or mutated reverse transcriptase of the invention (e.g., one having a half-life at 55° C. as described above) may be a modified or mutated retroviral reverse transcriptase. A mutated reverse transcriptase according to the present invention may be selected from a group consisting of M-MLV reverse transcriptase, ASV reverse transcriptase, HIV reverse transcriptase, Avian Sarcoma-Leukosis Virus (ASLV) reverse transcriptase, Rous Sarcoma Virus (RSV) reverse transcriptase, Avian Myeloblastosis Virus (AMV) reverse transcriptase, Avian Erythroblastosis Virus (AEV) Helper Virus MCAV reverse transcriptase, Avian Myelocytomatosis Virus MC29 Helper Virus MCAV reverse transcriptase, Avian Reticuloendotheliosis Virus (REV-T) Helper Virus REV-A reverse transcriptase, Avian Sarcoma Virus UR2 Helper Virus UR2AV reverse transcriptase, Avian Sarcoma Virus Y73 Helper Virus YAV reverse transcriptase, Rous Associated Virus (RAV) reverse transcriptase, and Myeloblastosis Associated Virus (MAV) reverse transcriptase and fragments of any of the above having reverse transcriptase activity.

[0051] Reverse transcriptases of the present invention may produce more product (e.g., full length product) at elevated temperatures than other reverse transcriptases. In one aspect, comparisons of full length product synthesis is made at different temperatures (e.g., one temperature being lower, such as between 37° C. and 50° C., and one temperature being higher, such as between 50° C. and 78° C.) while keeping all other reaction conditions similar or the same. The amount of full length product produced may be determined using techniques well known in the art, for example, by conducting a reverse transcription reaction at a first

temperature (e.g., 37° C., 38° C., 39° C., 40° C., etc.) and determining the amount of full length transcript produced, conducting a second reverse transcription reaction at a temperature higher than the first temperature (e.g., 45° C., 50° C., 52.5° C., 55° C., etc.) and determining the amount of full length product produced, and comparing the amounts produced at the two temperatures. A convenient form of comparison is to determine the percentage of the amount of full length product at the first temperature that is produced at the second (i.e., elevated) temperature. The reaction conditions used for the two reactions (e.g., salt concentration, buffer concentration, pH, divalent metal ion concentration, nucleoside triphosphate concentration, template concentration, reverse transcriptase concentration, primer concentration, length of time the reaction is conducted, etc.) are preferably the same for both reactions. Suitable reaction conditions include, but are not limited to, a template concentration of from about 1 nM to about 1 μM, from about 100 nM to 1 μM, from about 300 nM to about 750 nM, or from about 400 nM to about 600 nM, and a reverse transcriptase concentration of from about 1 nM to about 1 μM, from about 10 nM to 500 nM, from about 50 nM to about 250 nM, or from about 75 nM to about 125 nM. The ratio of the template concentration to the reverse transcriptase concentration may be from about 100:1 to about 1:1, from about 50:1 to about 1:1, from about 25:1 to about 1:1, from about 10:1 to about 1:1, from about 5:1 to about 1:1, or from about 2.5:1 to 1:1. A reaction may be conducted from about 5 minutes to about 5 hours, from about 10 minutes to about 2.5 hours, from about 30 minutes to about 2 hours, from about 45 minutes to about 1.5 hours, or from about 45 minutes to about 1 hour. A suitable reaction time is about one hour. Other suitable reaction conditions may be determined by those skilled in the art using routine techniques and examples of such conditions are provided below.

[0052] When the amount of full length product produced by a reverse transcriptase of the invention at an elevated temperature is compared to the amount of full length product produced by the same reverse transcriptase at a lower temperature, at an elevated temperature, the reverse transcriptases of the invention may produce not less than about 25%, 35%, 45%, 55%, 65%, 75%, 85%, 95%, 100% of the amount of full length product produced at the lower temperature. In some cases, the reverse transcriptases of the invention may produce an amount of full length product at a higher temperature that is greater than the amount of full length product produced by the reverse transcriptase at a lower temperature (e.g., 1% to about 100% greater). In one aspect, reverse transcriptases of the invention produce approximately the same amount (e.g., no more than a 25% difference) of full length product at the lower temperature compared to the amount of full length product made at the higher temperature.

[0053] A reverse transcriptase of the present invention may be one that synthesizes an amount of full length product, wherein the amount of full length product synthesized at 50° C. is no less than 10% (e.g., from about 10% to about 95%, from about 10% to about 80%, from about 10% to about 70%, from about 10% to about 60%, from about 10% to about 50%, from about 10% to about 40%, from about 10% to about 30%, or from about 10% to about 20%) of the amount of full length product it synthesizes at 40° C. In some embodiments, a reverse transcriptase of the invention is one wherein the amount of full length product synthesized at 50° C. is no less than 50% (e.g., from about 50% to about 95%, from about 50% to about 80%, from about 50% to about 70%, or from about 50% to about 60%) of the amount of full length product it synthesizes at 40° C. In some embodiments, a reverse transcriptase of the invention is one wherein the amount of full length product synthesized at 50° C. is no less than 75% (e.g., from about 75% to about 95%, from about 75%, to about 90%, from about 75% to about 85%, or from about 75% to about 80%) of the amount of full length product it synthesizes at 40° C. In other embodiments, a reverse transcriptase of the invention is one wherein the amount of full length product synthesized at 50° C. is no less than 85% (e.g., from about 85% to about 95%, or from about 85% to about 90%) of the amount of full length product it synthesizes at 40° C.

[0054] A reverse transcriptase of the invention may be one that synthesizes an amount of full length product, wherein the amount of full length product synthesized at 52.5° C. is no less than 10% (e.g., from about to about 30%, from about 10% to about to about 25%, from about 10% to about 20%, from about 10% to about 15%, from about 20% to about 60%, from about 20% to about 40%, from about 20% to about 30%, from about 30% to about 80%, from about 30% to about 60%, from about 30% to about 45%, from about 40% to about 90%, from about 40% to about 80%, from about 40% to about 60%, from about 40% to about 50% from about 50% to about 90%, or from about 50% to about 70%), of the amount of full length product it synthesizes at 40° C. In some embodiments, the amount of full length product synthesized at 52.5° C. is no less than 30% (e.g., from about 30% to about 70%, from about 30% to about 60%, from about 30% to about 50%, or from about 30% to about 40%) of the amount of full length product it synthesizes at 40° C. In some embodiments, the amount of full length product synthesized at 52.5° C. is no less than 50% (e.g., from about 50% to about 70%, from about 50% to about 65%, from about 50% to about 60%, or from about 50% to about 55%), of the amount of full length product it synthesizes at 40° C.

[0055] A reverse transcriptase of the invention may be one that synthesizes an amount of full length product, wherein the amount of full length product synthesized at 55° C. is no less than 1% (e.g., from about 1% to about 30%, from about 1% to about 25%, from about 1% to about 20%, from about 1% to about 15%, from about 1% to about 10%, or from about 1% to about 5%) of the amount of full length product it synthesizes at 40° C. In some embodiments, the amount of full length product synthesized at 55° C. is no less than 5% (e.g., from about 5% to about 30%, from about 5% to about to about 25%, from about 5% to about 20%, from about 5% to about 15%, or from about 5% to about 10%) of the amount of full length product it synthesizes at 40° C. In some embodiments, the amount of full length product synthesized at 55° C. is no less than 10% (e.g., from about 10% to about 30%, from about 10% to about to about 25%, from about 10% to about 20%, from about 10% to about 15%, from about 20% to about 60%, from about 20% to about 40%, from about 20% to about 30%, from about 30% to about 80%, from about 30% to about 60%, from about 30% to about 45%, from about 40% to about 90%, from about 40% to about 80%, from about 40% to about 60%, from about 40% to about 50% from about 50% to about 90%, or from about 50% to about 70%) of the amount of full length product it synthesizes at 40° C.

[0056] In another aspect, the reverse transcriptases of the invention are capable of producing more nucleic acid product (e.g., cDNA) and, preferably, more full length product, at one or a number of elevated temperatures (typically between 40° C. an 78° C.) compared to the corresponding un-mutated or unmodified reverse transcriptase (e.g., the control reverse transcriptase). Such comparisons are typically made under similar or the same reaction conditions and the amount of product synthesized by the control reverse transcriptase is compared to the amount of product synthesized by the reverse transcriptase of the invention. Preferably, the reverse transcriptases of the invention produce at least about 5%, at least 10%, at least 15%, at least 25%, at least 50%, at least 75%, at least 100%, or at least 200% more product or full length product compared to the corresponding control reverse transcriptase under the same reaction conditions and temperature. The reverse transcriptases of the invention preferably produce from about 10% to about 200%, from about 25% to about 200%, from about 50% to about 200%, from about 75% to about 200%, or from about 100% to about 200% more product or full length product compared to a control reverse transcriptase under the same reaction conditions and incubation temperature. The reverse transcriptases of the invention preferably produce at least 2 times, at least 3 times, at least 4 times, at least 5 times, at least 6 times, at least 7 times, at least 8 times, at least 9 times, at least 10 times, at least 25 times, at least 50 times, at least 75 times, at least 100 times, at least 150 times, at least 200 times, at least 300 times, at least 400 times, at least 500 times, at least 1000 times, at least 5,000 times, or at least 10,000 times more product or full length product compared to a control reverse transcriptase (e.g., the corresponding un-mutated or unmodified reverse transcriptase) under the same reaction conditions and temperature. The reverse transcriptases of the invention preferably produce from 2 to 10,000, 5 to 10,000, 10 to 5,000, 50 to 5,000, 50 to 500, 2 to 500, 5 to 500, 5 to 200, 5 to 100, or 5 to 75 times more product or full length product than a control reverse transcriptase under the same reaction conditions and temperature.

[0057] In one aspect, the reverse transcriptases of the invention produce, at 50° C., at least 25% more, preferably at least 50% more and more preferably at least 100% more nucleic acid product or full length product than a control reverse transcriptase (which is preferably the corresponding wild-type reverse transcriptase). In another aspect, at 52.5° C., the reverse transcriptases of the invention produce at least 1.5 times, at least 2 times, at least 2.5 times, at least 3 times, at least 4 times, at least 5 times, at least 6 times, at least 7 times, at least 8 times, at least 9 times, at least 10 times the amount of nucleic acid product or full length product compared to a control reverse transcriptase. In another aspect, at 55° C., the reverse transcriptases of the invention produce at least 2 times, at least 5 times, at least 10 times, at least 15 times, at least 20 times, at least 25 times, at least 50 times, at least 75 times, at least 100 times the amount of nucleic acid product or full length product compared to a control reverse transcriptase. Such comparisons are preferably made under the same reaction conditions and temperature.

[0058] Modified or mutated reverse transcriptases of the present invention may have an increased thermostability at elevated temperatures as compared to corresponding unmodified or un-mutated reverse transcriptases. They may show increased thermostability in the presence or absence an RNA template. In some instances, reverse transcriptases of the invention may show an increased thermostability in both the presence and absence of an RNA template. Those skilled in the art will appreciate-that reverse transcriptase enzymes are typically more thermostable in the presence of an RNA template. The increase in thermostability may be measured by comparing suitable parameters of the modified or mutated reverse transcriptase of the invention to those of a corresponding unmodified or un-mutated reverse transcriptase. Suitable parameters to compare include, but are not limited to, the amount of product and/or full length product synthesized by the modified or mutated reverse transcriptase at an elevated temperature compared to the amount or product and/or full length product synthesized by the corresponding un-modified or un-mutated reverse transcriptase at the same temperature, and/or the half-life of reverse transcriptase activity at an elevated temperature of a modified or mutated reverse transcriptase at an elevated temperature compared to that of a corresponding unmodified or un-mutated reverse transcriptase.

[0059] A modified or mutated reverse transcriptase of the invention may have an increase in thermostability at 50° C. of at least about 1.5 fold (e.g., from about 1.5 fold to about 100 fold, from about 1.5 fold to about 50 fold, from about 1.5 fold to about 25 fold, from about 1.5 fold to about 10 fold) compared, for example, to the corresponding un-mutated or unmodified reverse transcriptase. A reverse transcriptase of the invention may have an increase in thermostability at 50° C. of at least about 10 fold (e.g., from about 10 fold to about 100 fold, from about 10 fold to about 50 fold, from about 10 fold to about 25 fold, or from about 10 fold to about 15 fold) compared, for example, to the corresponding un-mutated or unmodified reverse transcriptase. A reverse transcriptase of the invention may have an increase in thermostability at 50° C. of at least about 25 fold (e.g., from about 25 fold to about 100 fold, from about 25 fold to about 75 fold, from about 25 fold to about 50 fold, or from about 25 fold to about 35 fold) compared to a corresponding un-mutated or unmodified reverse transcriptase.

[0060] The present invention also contemplates a modified or mutated thermostable reverse transcriptase, wherein the reverse transcriptase has an increase in thermostability of greater than about 1.5 fold at 52.5° C. (e.g., from about 1.5 fold to about 100 fold, from about 1.5 fold to about 50 fold, from about 1.5 fold to about 25 fold, or from about 1.5 fold to about 10 fold) compared, for example, to the corresponding un-mutated or unmodified reverse transcriptase. A reverse transcriptase of the invention may have an increase in thermostability at 52.5° C. of at least about 10 fold (e.g., from about 10 fold to about 100 fold, from about 10 fold to about 50 fold, from about 10 fold to about 25 fold, or from about 10 fold to about 15 fold) compared, for example, to the corresponding un-mutated or unmodified reverse transcriptase. A reverse transcriptase of the invention may have an increase in thermostability at 52.5° C. of at least about 25 fold (e.g., from about 25 fold to about 100 fold, from about 25 fold to about 75 fold, from about 25 fold to about 50 fold, or from about 25 fold to about 35 fold) compared, for example, to the corresponding un-mutated or unmodified reverse transcriptase.

[0061] In other embodiments, the present invention provides a reverse transcriptase, wherein the reverse tran-

scriptase has an increase in thermostability of greater than about 1.5 fold at 55° C. (e.g., from about 1.5 fold to about 100 fold, from about 1.5 fold to about 50 fold, from about 1.5 fold to about. 25 fold, or from about 1.5 fold to about 10 fold) compared to a corresponding un-mutated or unmodified reverse transcriptase. In some embodiments, a reverse transcriptase of the invention may have an increase in thermostability at 55° C. of at least about 10 fold (e.g., from about 10 fold to about 100 fold, from about 10 fold to about 50 fold, from about 10 fold to about 25 fold, or from about 10 fold to about 15 fold) compared to a corresponding un-mutated or unmodified reverse transcriptase. In some embodiments, a reverse transcriptase of the invention may have an increase in thermostability at 55° C. of at least about 25 fold (e.g., from about 25 fold to about 100 fold, from about 25 fold to about 75 fold, from about 25 fold to about 50 fold, or from about 25 fold to about 35 fold) compared to a corresponding un-mutated or unmodified reverse transcriptase.

[**0062**] The present invention provides reverse transcriptase enzymes, compositions and kits comprising such enzymes, and methods useful in preparing labeled nucleic acid molecules by reverse transcription. In general, the invention relates to the use of polypeptides of the invention (e.g., reverse transcriptase enzymes having one or more of the mutations identified above) to synthesized labeled nucleic acid molecules. In some embodiments, polypeptides of the invention may be heterodimers and more specifically two subunit enzymes (e.g., dimers) such as HIV RT and ASLV RTs. In some embodiments, polypeptides of the invention may be single sub-unit enzymes (e.g., M-MLV reverse transcriptase). Preferably, such labeling involves the use of modified nucleotides (e.g., labeled nucleotides, particularly fluorescently labeled nucleotides, nucleotide analogs and the like) and one or more nucleic acid templates (preferably RNA and most preferably mRNA). In accordance with the invention, one or more labeled nucleic acid molecules are synthesized which are complementary to all or a portion of the one or more templates. The labeled nucleic acid molecules preferably have one or more labeled nucleotides incorporated into the synthesized molecule and in a preferred aspect, the labels are one or more fluorescent labels (which may be the same or different). In another aspect, nucleotides are used during nucleic acid synthesis using the reverse transcriptases of the invention to produce one or more nucleic acid molecules complementary to all or a portion of one or more templates. In such aspects, such nucleotides, which are incorporated in the synthesized nucleic acid molecules, may be modified (before or after incorporation) to contain one or more labels , which may then be detected.

[**0063**] The invention also relates to compositions for use in the invention and such compositions may comprise one or more polypeptides of the invention (e.g., single sub-unit such as M-MLV RT and/or multi-subunit RTs such as HIV and ASLV RTs). Such compositions may further comprise one or more nucleotides, a suitable buffer, and/or one or more DNA polymerases. The compositions of the invention may also comprise one or more primers. The reverse transcriptases in these compositions preferably have RNase H activity or are reduced or substantially reduced in RNase H activity, and most preferably are enzymes selected from the group consisting of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, Rous Sarcoma Virus (RSV) reverse transcriptase, Avian Myeloblastosis Virus (AMV) reverse transcriptase, Rous Associated Virus (RAV) reverse transcriptase, Myeloblastosis Associated Virus (MAV) reverse transcriptase and Human Immunodeficiency Virus (HIV) reverse transcriptase or other ASLV reverse transcriptases. The reverse transcriptases of the invention may be composed of one or more subunits (which may be the same or different). When two subunit RTs are use in the practice of the invention, such enzymes may contain various forms and combinations of such subunits such as αβ, αα, ββ, etc. and mutants, variants or derivatives thereof. In preferred compositions, the reverse transcriptases are present at working concentrations.

[**0064**] The invention is also directed to methods for making one or more nucleic acid molecules and/or labeled nucleic acid molecules, comprising mixing one or more nucleic acid templates (preferably one or more RNA templates and most preferably one or more messenger RNA templates) with one or more polypeptides of the invention having reverse transcriptase activity and incubating the mixture under conditions sufficient to synthesize one or more first nucleic acid molecules complementary to all or a portion of the one or more nucleic acid templates, wherein said at least one of said synthesized molecules are optionally labeled and/or comprise one or more labeled nucleotides and/or wherein said synthesized molecules may optionally be modified to contain one or more labels. In a preferred embodiment, the one or more first nucleic acid molecules are single-stranded cDNA molecules. Nucleic acid templates suitable for reverse transcription according to this aspect of the invention include any nucleic acid molecule or population of nucleic acid molecules (preferably RNA and most preferably mRNA), particularly those derived from a cell or tissue. In a preferred aspect, a population of mRNA molecules (a number of different mRNA molecules, typically obtained from cells or tissue) are used to make a labeled cDNA library, in accordance with the invention. Preferred sources of nucleic acid templates include viruses, virally infected cells, bacterial cells, fungal cells, plant cells and animal cells.

[**0065**] The invention also concerns methods for making one or more double-stranded nucleic acid molecules (which may optionally be labeled). Such methods comprise (a) mixing one or more nucleic acid templates (preferably RNA or mRNA, and more preferably a population of mRNA templates) with one or more polypeptides of the invention having reverse transcriptase activity; (b) incubating the mixture under conditions sufficient to make one or more first nucleic acid molecules complementary to all or a portion of the one or more templates; and (c) incubating the one or more first nucleic acid molecules under conditions sufficient to make one or more second nucleic acid molecules complementary to all or a portion of the one or more first nucleic acid molecules, thereby forming one or more double-stranded nucleic acid molecules comprising the first and second nucleic acid molecules. In accordance with the invention, the first and/or second nucleic acid molecules may be labeled (e.g., may comprise one or more of the same or different labeled nucleotides and/or may be modified to contain one or more of the same or different labels). Thus, labeled nucleotides may be used at one or both synthesis steps. Such methods may include the use of one or more DNA polymerases as part of the process of making the one or more double-stranded nucleic acid molecules. The inven-

tion also concerns compositions useful for making such double-stranded nucleic acid molecules. Such compositions comprise one or more reverse transcriptases of the invention and optionally one or more DNA polymerases, a suitable buffer and/or one or more nucleotides (preferably including labeled nucleotides).

[0066] The invention is also directed to nucleic acid molecules and/or labeled nucleic acid molecules (particularly single- or double-stranded cDNA molecules) produced according to the above-described methods and to kits comprising these nucleic acid molecules. Such molecules or kits may be used to detect nucleic acid molecules (for example by hybridization) or for diagnostic purposes.

[0067] The invention is also directed to kits for use in the methods of the invention. Such kits can be used for making nucleic acid molecules and/or labeled nucleic acid molecules (single- or double-stranded). Kits of the invention may comprise a carrier, such as a box or carton, having in close confinement therein one or more containers, such as vials, tubes, bottles and the like. In kits of the invention, a first container may contain one or more of the reverse transcriptase enzymes of the invention or one or more of the compositions of the invention. Kits of the invention may also comprise, in the same or different containers, at least one component selected from one or more DNA polymerases (preferably thermostable DNA polymerases), a suitable buffer for nucleic acid synthesis and one or more nucleotides. Alternatively, the components of the kit may be divided into separate containers. In one aspect, kits of the invention comprise reverse transcriptases which have RNase H activity or are reduced or substantially reduced in RNase H activity (or lacking or having undetectable RNase H activity). Such RTs preferably are selected from the group consisting of M-MLV reverse transcriptase, RSV reverse transcriptase, AMV reverse transcriptase, RAV reverse transcriptase, MAV reverse transcriptase and HIV reverse transcriptase. In additional preferred kits of the invention, the enzymes (e.g. reverse transcriptases and/or DNA polymerases) in the containers are present at working concentrations.

[0068] In specific embodiments, reverse transcriptases of the invention may not include M-MLV reverse transcriptases, HIV reverse transcriptases, AMV reverse transcriptases, and/or RSV reverse transcriptases. Thus, for example, in certain embodiments, the invention is directed to reverse transcriptases with increased thermostability that are not a HIV reverse transcriptase. In other embodiments, the invention is directed to reverse transcriptases with increased thermostability that are not a M-MLV reverse transcriptase. In yet other embodiments, the invention is directed to reverse transcriptases with increased thermostability that are not an AMV reverse transcriptase. In still other embodiments, the invention is directed to reverse transcriptases with increased thermostability that are not a RSV reverse transcriptase.

[0069] The present invention is also directed to nucleic acid molecules (e.g., vectors) containing a gene or nucleic acid molecules encoding the mutant or modified reverse transcriptases of the present invention (or fragments thereof including fragments having polymerase activity) and to host cells containing such DNA or other nucleic acid molecules. Any number of hosts may be used to express the gene or nucleic acid molecule of interest, including prokaryotic and eukaryotic cells. In specific embodiments, prokaryotic cells are used to express the reverse transcriptases of the invention. One example of a prokaryotic host suitable for use with the present invention is *Escherichia coli*. Examples of eukaryotic hosts suitable for use with the present invention include fungal cells (e.g., *Saccharomyces cerevisiae* cells, *Pichia pastoris* cells, etc.), plant cells, and animal cells (e.g., *Drosophila melanogaster* cells, *Spodoptera frugiperda* Sf9 and Sf21 cells, *Trichoplusa* High-Five cells, *C. elegans* cells, *Xenopus laevis* cells, CHO cells, COS cells, VERO cells, BHK cells, etc.). Preferably, polypeptides of the invention may be purified and/or isolated from a cell or organism expressing them, which may be a wild type cell or organism or a recombinant cell or organism. In some embodiments, such polypeptides may be substantially isolated from the cell or organism in which they are expressed.

[0070] The invention also relates to a method of producing reverse transcriptases of the invention, said method comprising:

[0071] (a) culturing a host cell comprising a gene or other nucleic acid molecule encoding a reverse transcriptase of the invention (preferably such reverse transcriptase gene or other nucleic acid, molecule is contained by a vector within the host cell);

[0072] (b) expressing the gene or nucleic acid molecule; and

[0073] (c) isolating or purifying said reverse transcriptase.

[0074] The invention is also directed to methods for making one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, etc.) nucleic acid molecules, comprising mixing one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, etc.) nucleic acid templates (preferably one or more RNA templates and most preferably one or more messenger RNA templates or a population of messenger RNA templates) with one or more (e.g., one, two, three, four, five, ten, fifteen, etc.) reverse transcriptases of the invention and incubating the mixture under conditions sufficient to make a first nucleic acid molecule or molecules complementary to all or a portion of the one or more nucleic acid templates. In some embodiments, the mixture is incubated at an elevated temperature, i.e., greater than 37° C. In specific embodiments, the elevated temperature may be from about 40° C. or greater, from about 45° C. or greater, from about 50° C. or greater, from about 51° C. or greater, from about 52° C. or greater, from about 53° C. or greater, from about 54° C. or greater, from about 55° C. or greater, from about 56° C. or greater, from about 57° C. or greater, from about 58° C. or greater, from about 59° C. or greater, from about 60° C. or greater, from about 61° C. or greater, from about 62° C. or greater, from about 63° C. or greater, from about 64° C. or greater, from about 65° C. or greater, from about 66° C. or greater, from about 67° C. or greater, from about 68° C. or greater, from about 69° C. or greater, from about 70° C. or greater, from about 71° C. or greater, from about 72° C. or greater, from about 73° C. or greater, from about 74° C. or greater, from about 75° C. or greater, from about 76° C. or greater, from about 77° C. or greater, or from about 78° C. or greater. An elevated temperature may be within a temperature range of from about 40° C. to about 45° C., from about 40° C. to about 48° C., from about 40° C. to

about 50° C., from about 40° C. to about 52° C., from about 40° C. to about 55° C., from about 40° C. to about 58° C., from about 40° C. to about 60° C., from about 40° C. to about 65° C., from about 42° C. to about 45° C., from about 42° C. to about 48° C., from about 42° C. to about 50° C., from about 42° C. to about 52° C., from about 42° C. to about 55° C., from about 42° C. to about 58° C., from about 42° C. to about 60° C., from about 42° C. to about 65° C., from about 45° C. to about 48° C., from about 45° C. to about 50° C., from about 45° C. to about 52° C., from about 45° C. to about 55° C., from about 45° C. to about 58° C., from about 45° C. to about 60° C., from about 45° C. to about 65° C., from about 48° C. to about 50° C., from about 48° C. to about 52° C., from about 48° C. to about 55° C., from about 48° C. to about 58° C., from about 48° C. to about 60° C., from about 48° C. to about 65° C., from about 50° C. to about 52° C., from about 50° C. to about 55° C., from about 50° C. to about 58° C., from about 50° C. to about 60° C., from about 50° C. to about 65° C., from about 52° C. to about 55° C., from about 52° C. to about 58° C., from about 52° C. to about 60° C., from about 52° C. to about 65° C., from about 55° C. to about 58° C., from about 55° C. to about 60° C., from about 55° C. to about 65° C., from about 55° C. to about 70° C., from about 58° C. to about 60° C., from about 58° C. to about 65° C., from about 58° C. to about 70° C. An elevated temperature may be within a temperature range from about 37° C. to about 75° C., from about 40° C. to about 75° C., from about 45° C. to about 75° C., from about 50° C. to about 75° C., from about 51° C. to about 75° C., from about 52° C. to about 75° C., from about 53° C. to about 75° C., from about 54° C. to about 75° C., from about 55° C. to about 75° C. In other embodiments, the elevated temperature may be within the range of about 50° C. to about 70° C., from about 51° C. to about 70° C., from about 52° C. to about 70° C., from about 53° C. to about 70° C., from about 54° C. to about 70° C., from about 55° C. to about 70° C., from about 56° C. to about 65° C., from about 56° C. to about 64° C. or about 56° C. to about 62° C. In other embodiments, the elevated temperature may be within the range of about 46° C. to about 60° C., from about 47° C. to about 60° C., from about 49° C. to about 60° C., from about 51° C. to about 60° C., from about 53° C. to about 60° C., or from about 54° C. to about 60° C. In additional specific embodiments, the first nucleic acid molecule is a single-stranded cDNA. The invention further includes nucleic acid molecules prepared by the above methods and reaction mixtures used in and formed by such methods. Such conditions for incubation may include the use of one or more buffers or buffering salts, one or more primers (such as oligo dT primers) and/or one or more nucleotides (e.g.; one or more nucleoside triphosphates). The invention also concerns compositions for making one or more nucleic acid molecules comprising one or more components selected from the group consisting of one or more reverse transcriptases of the invention, one or more primers, one or more nucleotides and one or more suitable buffers.

[0075] Nucleic acid templates suitable for reverse transcription according to this aspect of the invention include any nucleic acid molecule or population of nucleic acid molecules (preferably RNA and most preferably mRNA), particularly those derived from a cell or tissue. In a specific aspect, a population of mRNA molecules (a number of different mRNA molecules, typically obtained from a particular cell or tissue type) is used to make a cDNA library, in accordance with the invention. Examples of cellular sources of nucleic acid templates include bacterial cells, fungal cells, plant cells and animal cells.

[0076] The invention also concerns methods for making one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, etc.) double-stranded nucleic acid molecules. Such methods comprise (a) mixing one or more nucleic acid templates (preferably RNA or mRNA, and more preferably a population of mRNA templates) with one or more (e.g., one, two, three, four, five, ten, fifteen, etc.) reverse transcriptases of the invention; (b) incubating the mixture under conditions sufficient to make a first nucleic acid molecule or molecules complementary to all or a portion of the one or more templates; and (c) incubating the first nucleic acid molecule or molecules under conditions sufficient to make a second nucleic acid molecule or molecules complementary to all or a portion of the first nucleic acid molecule or molecules, thereby forming one or more double-stranded nucleic acid molecules comprising the first and second nucleic acid molecules. In some embodiments, the incubation of step (b) is performed at an elevated temperature. In some embodiments, conditions may comprise the use of one or more labeled nucleotides and the double stranded nucleic acid molecules may be labeled. In specific embodiments, the elevated temperature may be from about 40° C. or greater, from about 45° C. or greater, from about 50° C. or greater, from about 51° C. or greater, from about 52° C. or greater, from about 53° C. or greater, from about 54° C. or greater, from about 55° C. or greater, from about 56° C. or greater, from about 57° C. or greater, from about 58° C. or greater, from about 59° C. or greater, from about 60° C. or greater, from about 61° C. or greater, from about 62° C. or greater, from about 63° C. or greater, from about 64° C. or greater, from about 65° C. or greater, from about 66° C. or greater, from about 67° C. or greater, from about 68° C. or greater, from about 69° C. or greater, from about 70° C. or greater, from about 71° C. or greater, from about 72° C. or greater, from about 73° C. or greater, from about 74° C. or greater, from about 75° C. or greater, from about 76° C. or greater, from about 77° C. or greater, or from about 78° C. or greater. An elevated temperature may be within a temperature range of from about 40° C. to about 45° C., from about 40° C. to about 48° C., from about 40° C. to about 50° C., from about 40° C. to about 52° C., from about 40° C. to about 55° C., from about 40° C. to about 58° C., from about 40° C. to about 60° C., from about 40° C. to about 65° C., from about 42° C. to about 45° C., from about 42° C. to about 48° C., from about 42° C. to about 50° C., from about 42° C. to about 52° C., from about 42° C. to about 55° C., from about 42° C. to about 58° C., from about 42° C. to about 60° C., from about 42° C. to about 65° C., from about 45° C. to about 48° C., from about 45° C. to about 50° C., from about 45° C. to about 52° C., from about 45° C. to about 55° C., from about 45° C. to about 58° C., from about 45° C. to about 60° C., from about 45° C. to about 65° C., from about 48° C. to about 50° C., from about 48° C. to about 52° C., from about 48° C. to about 55° C., from about 48° C. to about 58° C., from about 48° C. to about 60° C., from about 48° C. to about 65° C., from about 50° C. to about 52° C., from about 50° C. to about 55° C., from about 50° C. to about 58° C., from about 50° C. to about 60° C., from about 50° C. to about 65° C., from about 52° C. to about 55° C., from about 52° C. to about 58° C, from about 52° C to about

60° C., from about 52° C. to about 65° C., from about 55° C. to about 58° C., from about 55° C. to about 60° C., from about 55° C. to about 65° C., from about 55° C. to about 70° C., from about 58° C. to about 60° C., from about 58° C. to about 65° C., from about 58° C. to about 70° C. An elevated temperature may be within a temperature range from about 37° C. to about 75° C., from about 40° C. to about 75° C., from about 45° C. to about 75° C., from about 50° C. to about 75° C., from about 51° C. to about 75° C., from about 52° C. to about 75° C., from about 53° C. to about 75° C., from about 54° C. to about 75° C., from about 55° C. to about 75° C. In other embodiments, the elevated temperature may be within the range of about 50° C. to about 70° C., from about 51° C. to about 70° C., from about 52° C. to about 70° C., from about 53° C. to about 70° C., from about 54° C. to about 70° C., from about 55° C. to about 70° C., from about 56° C. to about 65° C., from about 56° C. to about 64° C. or about 56° C. to about 62° C. In other embodiments, the elevated temperature may be within the range of about 46° C. to about 60° C., from about 47° C. to about 60° C., from about 49° C. to about 60° C., from about 51° C. to about 60° C., from about 53° C. to about 60° C., or from about 54° C. to about 60° C. Such conditions may involve the use of one or more suitable buffers or buffer salts, on or more primers (such as oligo dT primers), and one or more nucleotides. Such methods may include the use of one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, etc.) DNA polymerases as part of the process of making the one or more double-stranded nucleic acid molecules. Such DNA polymerases are preferably thermostable DNA polymerases and most preferably the nucleic acid synthesis accomplished with such DNA polymerases is conducted at elevated temperatures, i.e., greater than 37° C. The invention also concerns compositions useful for making such double-stranded nucleic acid molecules. Such compositions comprise one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, twenty, etc.) reverse transcriptases of the invention and optionally one or more DNA polymerases, a suitable buffer, one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, etc.) primers, and/or one or more (e.g., one, two, three, four, five, etc.) nucleotides. The invention further includes nucleic acid molecules prepared by the above methods and reaction mixtures used in and formed by such methods.

[0077] The invention also relates to methods for amplifying a nucleic acid molecule. Such amplification methods comprise mixing the double-stranded nucleic acid molecule or molecules produced as described above with one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, etc.) DNA polymerases (preferably thermostable DNA polymerases) and incubating the mixture under conditions sufficient to amplify the double-stranded nucleic acid molecule. In a first embodiment, the invention concerns a method for amplifying a nucleic acid molecule, the method comprising (a) mixing one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, twenty, etc.) nucleic acid templates (preferably one or more RNA or mRNA templates and more preferably a population of mRNA templates) with one or more reverse transcriptases of the invention and with one or more DNA polymerases and (b) incubating the mixture under conditions sufficient to amplify nucleic acid molecules complementary to all or a portion of the one or more templates. In some embodiments, the incubation of step (b) is performed at an elevated temperature. In specific embodiments, the elevated temperature may be from about 40° C. or greater, from about 45° C. or greater, from about 50° C. or greater, from about 51° C. or greater, from about 52° C. or greater, from about 53° C. or greater, from about 54° C. or greater, from about 55° C. or greater, from about 56° C. or greater, from about 57° C. or greater, from about 58° C. or greater, from about 59° C. or greater, from about 60° C. or greater, from about 61° C. or greater, from about 62° C. or greater, from about 63° C. or greater, from about 64° C. or greater, from about 65° C. or greater, from about 66° C. or greater, from about 67° C. or greater, from about 68° C. or greater, from about 69° C. or greater, from about 70° C. or greater, from about 71° C. or greater, from about 72° C. or greater, from about 73° C. or greater, from about 74° C. or greater, from about 75° C. or greater, from about 76° C. or greater, from about 77° C. or greater, or from about 78° C. or greater. An elevated temperature may be within a temperature range of from about 40° C. to about 45° C., from about 40° C. to about 48° C., from about 40° C. to about 50° C., from about 40° C. to about 52° C., from about 40° C. to about 55° C., from about 40° C. to about 58° C., from about 40° C. to about 60° C., from about 40° C. to about 65° C., from about 42° C. to about 45° C., from about 42° C. to about 48° C., from about 42° C. to about 50° C., from about 42° C. to about 52° C., from about 42° C. to about 55° C., from about 42° C. to about 58° C., from about 42° C. to about 60° C., from about 42° C. to about 65° C., from about 45° C. to about 48° C., from about 45° C. to about 50° C., from about 45° C. to about 52° C., from about 45° C. to about 55° C., from about 45° C. to about 58° C., from about 45° C. to about 60° C., from about 45° C. to about 65° C., from about 48° C. to about 50° C., from about 48° C. to about 52° C., from about 48° C. to about 55° C., from about 48° C. to about 58° C., from about 48° C. to about 60° C., from about 48° C. to about 65° C., from about 50° C. to about 52° C., from about 50° C. to about 55° C., from about 50° C. to about 58° C., from about 50° C. to about 60° C., from about 50° C. to about 65° C., from about 52° C. to about 55° C., from about 52° C. to about 58° C., from about 52° C. to about 60° C., from about 52° C. to about 65° C., from about 55° C. to about 58° C, from about 55° C to about 60° C., from about 55° C. to about 65° C., from about 55° C. to about 70° C., from about 58° C. to about 60° C., from about 58° C. to about 65° C., from about 58° C. to about 70° C. An elevated temperature may be within a temperature range from about 37° C. to about 75° C., from about 40° C. to about 75° C., from about 45° C. to about 75° C., from about 50° C. to about 75° C., from about 51° C. to about 75° C., from about 52° C. to about 75° C., from about 53° C. to about 75° C., from about 54° C. to about 75° C., from about 55° C. to about 75° C. In other embodiments, the elevated temperature may be within the range of about 50° C. to about 70° C., from about 51° C. to about 70° C., from about 52° C. to about 70° C., from about 53° C. to about 70° C., from about 54° C. to about 70° C., from about 55° C. to about 70° C., from about 56° C. to about 65° C., from about 56° C. to about 64° C. or about 56° C. to about 62° C. In other embodiments, the elevated temperature may be within the range of about 46° C. to about 60° C., from about 47° C. to about 60° C., from about 49° C. to about 60° C., from about 51° C. to about 60° C., from about 53° C. to about 60° C., or from about 54° C. to about 60° C.

[0078] Preferably, reverse transcriptases of the invention, used in methods of the invention, and/or present in compositions of the invention (1) are reduced or substantially reduced in RNase H activity, (2) are reduced or substantially reduced in TdT activity, and/or (3) exhibit increased fidelity. Preferably, DNA polymerases used with the invention may comprise a first DNA polymerase having 3' exonuclease activity and a second DNA polymerase having substantially reduced 3' exonuclease activity. The invention further includes nucleic acid molecules prepared by the above methods and reaction mixtures used in and formed by such methods.

[0079] The invention also concerns compositions comprising one or more reverse transcriptases of the invention and one or more DNA polymerases for use in amplification reactions. Such compositions may further comprise one or more nucleotides and/or a buffer suitable for amplification. Compositions of the invention may also comprise one or more oligonucleotide primers. Compositions of the invention may further include nucleic acid molecules prepared by the above methods and reaction mixtures used in and formed by such methods.

[0080] The invention is also directed to nucleic acid molecules (particularly single- or double-stranded cDNA molecules) or amplified nucleic acid molecules produced according to the above-described methods and to vectors (particularly expression vectors) comprising these nucleic acid molecules or amplified nucleic acid molecules.

[0081] The invention is further directed to recombinant host cells comprising the above-described nucleic acid molecules, amplified nucleic acid molecules or vectors. Examples of such host cells include bacterial cells, yeast cells, plant cells and animal cells (including insect cells and mammalian cells).

[0082] The invention is additionally directed to methods of producing polypeptides encoded by the nucleic acid molecules produced by the methods of the invention. Such methods include those comprising culturing the above-described recombinant host cells and isolating the encoded polypeptides. The invention further includes polypeptides produced by such methods.

[0083] The invention also concerns methods for sequencing one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, etc.) nucleic acid molecules using compositions or enzymes of the invention. Such methods comprise (a) mixing one or more nucleic acid molecules (e.g., one or more RNA or DNA molecules) to be sequenced with one or more reverse transcriptases of the invention, and, optionally, one or more nucleotides, one or more terminating agents, such as one or more dideoxynucleoside triphosphates, and one or more primers; (b) incubating the mixture under conditions sufficient to synthesize a population of nucleic acid molecules complementary to all or a portion of the one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, twenty, thirty, fifty, one hundred, two hundred, etc.) nucleic acid molecules to be sequenced; and (c) separating the population of nucleic acid molecules to determine the nucleotide sequence of all or a portion of the one or more nucleic acid molecules to be sequenced. Such methods may also comprise (a) mixing a nucleic acid molecule (e.g., one or more RNA or DNA molecules) to be sequenced with one or more primers, one or more reverse transcriptases of the invention, one or more nucleotides and one or more terminating agents, such as one or more dideoxynucleoside triphosphates; (b) incubating the mixture under conditions sufficient to synthesize a population of nucleic acid molecules complementary to all or a portion of the nucleic acid molecule to be sequenced; and (c) separating members of the population of nucleic acid molecules to determine the nucleotide sequence of all or a portion of the nucleic acid molecule to be sequenced. In some embodiments, such incubation may be performed at elevated temperatures as described herein. The invention further includes sequence data generated by the above methods, as well as methods for generating such sequence data, and reaction mixtures used in and formed by such methods.

[0084] The invention is also directed to kits for use in methods of the invention. Such kits can be used for making, sequencing or amplifying nucleic acid molecules (single- or double-stranded), preferably at the elevated temperatures described herein. Kits of the invention may comprise a carrier, such as a box or carton, having in close confinement therein one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, etc.) containers, such as vials, tubes, bottles and the like. In kits of the invention, a first container contains one or more of the reverse transcriptase enzymes of the present invention. Kits of the invention may also comprise, in the same or different containers, one or more DNA polymerases (preferably thermostable DNA polymerases), one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, etc.) suitable buffers for nucleic acid synthesis, one or more nucleotides and one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, etc.) oligonucleotide primers. Alternatively, the components of the kit may be divided into separate containers (e.g., one container for each enzyme and/or component). Kits of the invention also may comprise instructions or protocols for carrying out the methods of the invention. In preferred kits of the invention, the reverse transcriptases are reduced or substantially reduced in RNase H activity (or lacking or having undetectable RNase H activity), and are most preferably selected from the group consisting of M-MLV RNase H- reverse transcriptase, RSV RNase H- reverse transcriptase, AMV RNase H- reverse transcriptase, RAV RNase H- reverse transcriptase, MAV RNase H- reverse transcriptase and HIV RNase H- reverse transcriptase. In other preferred kits of the invention, the reverse transcriptases are reduced or substantially reduced in TdT activity, and/or exhibit increased fidelity, as described elsewhere herein.

[0085] In additional preferred kits of the invention, the enzymes (reverse transcriptases and/or DNA polymerases) in the containers are present at working concentrations.

[0086] Thus, the invention is further directed to kits for use in reverse transcription, amplification or sequencing of a nucleic acid molecule, the kit comprising one or more reverse transcriptases of the invention.

[0087] As indicated above, kits of the invention may contain any number of various components for practicing methods of the invention. One example of such a component is instructions for performing methods of the invention.

[0088] Example of such instructions include those which direct individuals using the kits to perform methods for amplifying nucleic acid molecules using one or more reverse transcriptases of the invention.

[0089] As one skilled in the art would recognize, the full text of these instructions need not be included with the kit. One example of a situation in which kits of the invention would not contain such full length instructions is where directions are provided which inform individuals using the kits where to obtain instructions for using the kit. Thus, instructions for performing methods of the invention may be obtain from internet web pages, separately sold or distributed manuals or other product literature, etc. The invention thus includes kits which direct kit users to locations where they can find instructions which are not directly packaged and/or distributed with the kits. These instructions may be in any form including, but not limited to, electronic or printed forms.

[0090] The invention thus also provides, in part, kits for performing methods using the reverse transcriptases of the invention. In specific embodiments, kits of the invention contain instructions for performing methods for amplifying and/or sequencing nucleic acid molecules. These methods will often involve reacting RNA molecules with one or more reverse transcriptases of the invention.

[0091] In specific embodiments, reverse transcriptases of kits of the invention may comprise one or more modifications or mutations at positions corresponding to amino acids selected from the group consisting of:

[0092] (a) leucine 52 of M-MLV reverse transcriptase;

[0093] (b) tyrosine 64 of M-MLV reverse transcriptase;

[0094] (c) lysine 152 of M-MLV reverse transcriptase;

[0095] (d) arginine 204 of M-MLV reverse transcriptase;

[0096] (e) methionine 289 of M-MLV reverse transcriptase;

[0097] (f) threonine 306 of M-MLV reverse transcriptase; and

[0098] (g) phenylalanine 309 of M-MLV reverse transcriptase.

[0099] Reverse transcriptases of the invention include any reverse transcriptase having one or a combination of the properties described herein. Such properties include, but are not limited to, enhanced thermostability, reduced or eliminated RNase H activity, reduced terminal deoxynucleotidyl transferase activity, and/or increased fidelity. Such reverse transcriptases include retroviral reverse transcriptases, bacterial reverse transcriptases, retrotransposon reverse transcriptases (e.g., reverse transcriptases of the Ty1 and/or Ty3 retrotransposons), and DNA polymerases having reverse transcriptase activity. Preferred reverse transcriptases of the invention include a single and multi-subunit reverse transcriptase and preferably retroviral reverse transcriptases. In particular, the invention relates to M-MLV-reverse transcriptases and ASLV-reverse transcriptases (such as AMV-RT and RSV-RT). Such reverse transcriptases of the invention preferably have reduced, substantially reduced, or no detectable RNase H activity.

[0100] Other embodiments of the present invention will be apparent to one of ordinary skill in light of the following drawings and description of the invention, and of the claims.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[0101] **FIG. 1** is a map of plasmid pBAD-6-His-M-MLV H− (F1).

[0102] **FIG. 2A** is a linear representation of the coding sequence of the M-MLV reverse transcriptase showing the locations of the restriction enzyme cleavage sites used to generate the segments of the gene used to generate mutations. **FIG. 2B** is a schematic representation showing the insertion of a mutagenized PCR fragment into the coding sequence of the remaining portion of the reverse transcriptase gene.

[0103] **FIG. 3** represents a scanned phosphoimage of an extension assay using (1) SUPERSCRIPT™ II reverse transcriptase, and (2) F309N. The [^{32}P]-labeled 18-mer primer annealed to a 47-mer DNA template (5 nM) was extended by equal units of reverse transcriptase at 37° C. for 30 minutes as seen in the extension reactions with all 4 nucleotides. The extension reactions were analyzed by denaturing 6% gel electrophoresis. P, non-extended primer.

[0104] **FIG. 4** represents a scanned phosphoimage showing a TdT extension assay of SUPERSCRIPT™ II reverse transcriptase and the mutants F309N, T197E, and Y133A. The [^{32}P]-labeled 18-mer primer annealed to a 47-mer DNA template (5 nM) was extended with decreasing units of reverse transcriptase (lane (1) 646 units, lane (2) 200 units, lane (3) 50 units, and lane (4) 20 units) at 37° C. for 30 minutes with all four nucleotides (see the Methods section below in Example 3). The extension reactions were analyzed by denaturing 6% gel electrophoresis. In this assay, extension past the 47 nucleotide templates is considered non-template directed addition or TdT activity. P, non-extended primer.

[0105] **FIG. 5** represents a scanned phosphoimage showing misinsertion assays of SUPERSCRIPT™ II reverse transcriptase (1) and mutant protein F309N reverse transcriptase (2) with DNA template. The [^{32}P]-labeled 18-mer primer annealed to a 47-mer DNA template (5 nM) was extended by equal units of reverse transcriptase protein at 37° C. for 30 min. as seen in the extension reactions with all four nucleotides. The extension reactions were also performed in the presence of only 3 complementary dNTPs; minus dCTP, minus dATP, minus TTP, and minus dGTP. The extension reactions were analyzed by denaturing 6% gel electrophoresis. In this assay, the higher efficiency of elongation of terminated primer with only three nucleotides will reflect the lower fidelity of the SUPERSCRIPT™ II reverse transcriptase assayed. P, non-extended primer.

[0106] **FIG. 6** represents a scanned phosphoimage showing a misinsertion assay of SUPERSCRIPT™ II reverse transcriptase (1) and mutant protein T197A/F309N reverse transcriptase (2) and V223H/F309N (3) with DNA template. The [^{32}P]-labeled 18-mer primer annealed to a 47-mer DNA template (5 nM) was extended by equal units of reverse transcriptase protein at 37° C. for 30 min. as seen in the extension reactions with all four nucleotides. The extension reactions were also performed in the presence of only 3 complementary dNTPs; minus dATP, and minus dCTP. The extension reactions were analyzed by denaturing 6% gel electrophoresis. In this assay, the higher efficiency of elongation of terminated primer with only three nucleotides will

reflect the lower fidelity of the SUPERSCRIPT™ II reverse transcriptase assayed. P, non-extended primer.

[0107] **FIGS. 7A-7C** show representative results obtained from the screen for thermal stable RT mutants. Lysates of mutants were assayed for RT activity in a 96-well plate format. ^{32}P-Labeled DNA product was trapped on a membrane and the amount of radioactivity present was quantified with a phosphorimager. **FIG. 7A** shows the results of an initial screen of RT mutants in 4, 96-well plates. Heat pretreatment of lysates was at 58° C. for 10 min. RT mutants that retained the most activity after heat treatment at 58° C. were selected and lysates were screened again and the results are shown in 7B. A duplicate screen was performed with no heat pretreatment (**FIG. 7B** upper panel) and heat pretreatment at 58° C. (**FIG. 7B** lower panel). RT mutants with the highest resistance to heat inactivation in crude extracts were purified by nickel-affinity chromatography and screened again for RT activity and the results are shown in **FIG. 7C**. The results after heat treatment at 37° C. are shown in **FIG. 7C** in the upper row, after heat treatment at 53° C. in **FIG. 7C** middle row, and after heat treatment at 58° C. in 7C bottom row.

[0108] **FIG. 8** shows a comparison of the thermal inactivation profiles of His_6 H− RT and His_6 H− H204R T306K RT in crude extracts. Crude extracts were subjected to a heat treatment in a 96-well plate for 5 min. The temperature of the heat treatment increased from left to right, except that the wells on the far right were not heat treated.

[0109] **FIG. 9** is a ribbon diagram of the crystal structure of amino acids 193 to 232 of M-MLV RT showing the sites of some of the amino acids identified by the methods of the present invention. Potential interactions of arginine substituted for histidine at M-MLV RT position 204 in αhelix H with E201 or T128. The catalytic site amino acids D224 and D225 in the turn between β10 and β11 are also shown. The three-dimensional structure is taken from Georgiadis, et al., (1995) *Structure* 3, 879-892. Thus, the invention also includes reverse transcriptases having one or more mutations or modifications in various regions including the α-helix H region.

[0110] **FIGS. 10A and 10B** are graphs of reverse transcriptase activity as a function of Mg^{2+} concentration (**FIG. 10A**) and KCl concentration (**FIG. 10B**). The DNA polymerase assay for SUPERSCRIPT™ III (SuIII) RT was conducted at 37° C. or 50° C. for 10 minutes under various concentrations of A) Mg^{2+} or B) KCl. SUPERSCRIPT™ II (SuII) at 37° C. included for comparison).

[0111] **FIGS. 11A and 11B** show autoradiograms of TdT activity measure by extension for 60 minutes at various temperatures of a labeled DNA primer on DNA (**FIG. 11A**) or RNA (**FIG. 11B**) template forming a blunt end. T is template only, Lanes marked (−) is T-P plus enzyme without dNTPs. Since SUPERSCRIPT™ III is more thermostable, its TdT activity appears greater at 50 degrees than SUPERSCRIPT™ II.

[0112] **FIGS. 12A, 12B** and 12C are graphs of RT activity as a function of incubation time. **FIG. 12A** shows the data obtained at 50° C., **FIG. 12B** shows the data obtained at 55° C., and **FIG. 12C** shows the data obtained at 60° C.

[0113] **FIG. 13** is an autoradiogram comparing reverse transcriptase activity of a variety of commercially available reverse transcriptase enzymes at 45° C., 50° C., and 55° C. SUPERSCRIPT™ III is designated SS III and SUPERSCRIPT™ II is designated SS II.

[0114] **FIG. 14** is a photograph of ethidium bromide stained gels showing the results of the evaluation of the pH of the first strand buffer. RT reactions with first-strand buffers at pHs from 8.0 to 8.8 were performed with 500 ng of total Hela RNA and 200 units of SUPERSCRIPT™ II (SS II) or 400 units of SUPERSCRIPT™ III (LEFN RT, which contains an N-terminal tag sequence=MASGTGGQQMGRDLYDDDDKH (SEQ ID NO:3) and the following point mutations H204R, T306K, M289L, and F309N). 2 μl of the resulting cDNA were then added to 50 μl PCR reactions containing the BF 2.4 kb or Polε6.8 kb primer set. Resulting PCR products were then run on a 0.8% agarose gel containing 0.4 mg/ml ethidium bromide.

[0115] **FIG. 15** is a photograph of ethidium bromide stained gels showing the results of the evaluation of the effect of temperature on the reverse transcription reaction with various reverse transcriptases. SUPERSCRIPT™ II (SS II) was compared to the His-tagged EFN reverse transcriptase (His tag sequence=MGGSHHHHHHGMASMTGGQQMGRDLYDDDDKH, amino acids 1-32 of SEQ ID NO:2 and Table 3, EFN mutations are H204R, T306K, and F309N), His-tagged LEFN reverse transcriptase (same His tag sequence, LEFN mutations are H204R, T306K, M289L, and F309N), and to SUPERSCRIPT™ III, which is the tagged, no His LEFN reverse transcriptase (tag sequence= MASGTGGQQMGRDLYDDDDKH (SEQ ID NO:3), LEFN mutations are H204R, T306K, M289L, and F309N).

[0116] **FIG. 16** is a photograph of ethidium bromide stained gels showing the results of the evaluation of the effect of reverse transcriptase concentration on the reverse transcription reaction with SUPERSCRIPT™ III designated LEFN which contains the tag sequence=MASGTGGQQMGRDLYDDDDKH (SEQ ID NO: 1), and LEFN mutations, which are H204R, T306K, M289L, and F309N.

[0117] **FIG. 17** is a photograph of ethidium bromide stained gels showing the results of the comparison of hot start RT-PCR amplification by SUPERSCRIPT™ II (Panel A) or SUPERSCRIPT™ III (Panel B). Lanes (in duplicate) 1, 4, 7, and 10 are products reverse transcribed at 42° C. Lanes 2, 5, 8, and 11 are products reverse transcribed at 50° C. Lanes 3, 6, 9, and 12 are products transcribed at 55° C. Lanes 1-3 are the result of RNAs reverse transcribed by gene-specific priming from FGF, lanes 4-6 CBS 2.4, lanes 7-9 from TOP 3.2, lanes 10-12 VIN 4.6. Arrows indicate expected product sizes of 240 bp, 2390 bp, 3162 bp, and 4641 bp. SUPERSCRIPT™ III contains the tag sequence= MASGTGGQQMGRDLYDDDDKH (SEQ ID NO:3), and the LEFN mutations, which are H204R, T306K, M289L, and F309N.

[0118] **FIG. 18** shows the results of RT-PCR performed with varying amounts SUPERSCRIPT™ III from 25 units to 250 units per reaction with a variety of primer sets.

[0119] **FIG. 19** shows a comparison of SUPERSCRIPT™ II (SS II) and His tagged LEFN RT in RT-PCR using 200 or 400 units in the first strand reaction. His tagged LEFN has the His tag sequence=MGGSHHHHHHGMASMTGGQQMGRDLYDDDDKH, amino acids 1-32 of SEQ ID NO:2 and Table 3, and the LEFN mutations, which are H204R, T306K, M289L, and F309N).

[0120] **FIG. 20** shows the use of SUPERSCRIPT™ III (LEFN RT) in RT-PCR with varying amounts of RT in the first strand reaction. SUPERSCRIPT™ III contains the tag sequence=MASGTGGQQMGRDLYDDDDKH (SEQ ID NO:3), and the LEFN mutations, which are H204R, T306K, M289L, and F309N.

[0121] **FIG. 21** shows the results of a comparison of various primers in RT-PCR reactions using the polypeptides of the invention. EFN contains the tag sequence=MASGTGGQQMGRDLYDDDDKH (SEQ ID NO:3), and the EFN mutations, which are H204R, T306K, and F309N.

DETAILED DESCRIPTION OF THE INVENTION

[0122] In the description that follows, a number of terms used in recombinant DNA, virology and immunology are utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

[0123] Cloning Vector.

[0124] As used herein "cloning vector" means a nucleic acid molecule such as plasmid, cosmid, phage, phagemid or other nucleic acid molecule which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of recognition sequences, (e.g., restriction endonuclease recognition sites, recombination sites, topoisomerase recognition sites, etc.) , at which such nucleic acid sequences may be manipulated in a determinable fashion, and into which a nucleic acid segment of interest may be inserted in order to bring about its replication and cloning.

[0125] The cloning vector may further contain a marker suitable for use in the identification of cells transformed with the cloning vector. Markers, for example, are genes that confer a recognizable phenotype on host cells in which such markers are expressed. Commonly used markers include, but are not limited to, antibiotic resistance genes such as tetracycline resistance or ampicillin resistance.

[0126] Expression Vector.

[0127] As used herein "expression vector" means a nucleic acid molecule similar to a cloning vector but which may additionally comprise nucleic acid sequences capable of enhancing and/or controlling the expression of a gene or other nucleic acid molecule which has been cloned into it, after transformation into a host. The additional nucleic acid sequences may comprise promoter sequences, repressor binding sequences and the like. The cloned gene or nucleic acid molecule is usually operably linked to one or more (e.g., one, two, three, four, etc.) of such control sequences such as promoter sequences.

[0128] Recombinant Host.

[0129] As used herein "recombinant" means any prokaryotic or eukaryotic or microorganism which contains the desired cloned genes or nucleic acid molecules, for example, in an expression vector, cloning vector or any nucleic acid molecule. The term "recombinant host" is also meant to include those host cells which have been genetically engineered to contain the desired gene or other nucleic acid molecule on the host chromosome or genome.

[0130] Host.

[0131] As used herein "host" means any prokaryotic or eukaryotic cell or organism that is the recipient of a replicable expression vector, cloning vector or any nucleic acid molecule. The nucleic acid molecule may contain, but is not limited to, a structural gene, a promoter and/or an origin of replication.

[0132] Promoter.

[0133] As used herein "promoter" means a nucleic acid sequence generally described as the 5' region of a gene, located proximal to the start codon which is capable of directing the transcription of a gene or other nucleic acid molecule. At the promoter region, transcription of an adjacent gene(s) or nucleic acid(s) is initiated.

[0134] Gene.

[0135] As used herein "gene" means a nucleic acid sequence that contains information necessary for expression of a polypeptide or protein. It includes the promoter and the structural gene as well as other sequences involved in expression of the protein.

[0136] Structural Gene.

[0137] As used herein "structural gene" means a DNA or other nucleic acid sequence that is transcribed into messenger RNA that is then translated into a sequence of amino acids characteristic of a specific polypeptide.

[0138] Operably Linked.

[0139] As used herein "operably linked" means that a nucleic acid element is positioned so as to influence the initiation of expression of the polypeptide encoded by the structural gene or other nucleic acid molecule.

[0140] Expression.

[0141] As used herein "expression" refers to the process by which a gene or other nucleic acid molecule produces a polypeptide. It includes transcription of the gene or nucleic acid molecule into messenger RNA (mRNA) and the translation of such mRNA into polypeptide(s).

[0142] Substantially Pure.

[0143] As used herein "substantially pure" means that the desired material is essentially free from contaminating cellular components which are associated with the desired material in nature. In a preferred aspect, a reverse transcriptase of the invention has 25% or less, preferably 15% or less, more preferably 10% or less, more preferably 5% or less, and still more preferably 1% or less contaminating cellular components. In another aspect, the reverse transcriptases of the invention have no detectable protein contaminants when 200 units of reverse transcriptase are run on a protein gel (e.g., SDS-PAGE) and stained with Comassie blue. Contaminating cellular components may include, but are not limited to, enzymatic activities such as phosphatases, exonucleases, endonucleases or undesirable DNA polymerase enzymes. Preferably, reverse transcriptases of the invention are substantially pure.

[0144] Substantially Isolated.

[0145] As used herein "substantially isolated" means that the polypeptide of the invention is essentially free from contaminating proteins, which may be associated with the

polypeptide of the invention in nature and/or in a recombinant host. In one aspect, a substantially isolated reverse transcriptase of the invention has 25% or less, preferably 15% or less, more preferably 10% or less, more preferably 5% or less, and still more preferably 1% or less contaminating proteins. In another aspect, in a sample of a substantially isolated polypeptide of the invention, 75% or greater (preferably 80%, 85%, 90%, 95%, 98%, or 99% or greater) of the protein in the sample is the desired reverse transcriptase of the invention. The percentage of contaminating protein and/or protein of interest in a sample may be determined using techniques known in the art, for example, by using a protein gel (e.g., SDS-PAGE) and staining the gel with a protein dye (e.g., Coomassie blue, silver stain, amido black, etc.). In another aspect, the reverse transcriptases of the invention have no detectable protein contaminants when 200 units of reverse transcriptase are run on a protein gel (e.g., SDS-PAGE) and stained with Comassie blue.

[0146] Primer.

[0147] As used herein "primer" refers to a single-stranded oligonucleotide that is extended by covalent bonding of nucleotide monomers during amplification or polymerization of a DNA molecule.

[0148] Template.

[0149] The term "template" as used herein refers to a double-stranded or single-stranded nucleic acid molecule which is to be amplified, copied or sequenced. In the case of a double-stranded DNA molecule, denaturation of its strands to form single-stranded first and second strands may be performed before these molecules are amplified, copied or sequenced. A primer complementary to a portion of a nucleic acid template is hybridized under appropriate conditions and a nucleic acid polymerase, such as the reverse transcriptase enzymes of the invention, may then add nucleotide monomers to the primer thereby synthesizing a nucleic acid molecule complementary to said template or a portion thereof. The newly synthesized nucleic acid molecule, according to the invention, may be equal or shorter in length than the original template. Mismatch incorporation during the synthesis or extension of the newly synthesized nucleic acid molecule may result in one or a number of mismatched base pairs. Thus, the synthesized nucleic acid molecule need not be exactly complementary to the template.

[0150] Incorporating.

[0151] The term "incorporating" as used herein means becoming a part of a nucleic acid molecule or primer.

[0152] Oligonucleotide.

[0153] "Oligonucleotide" refers to a synthetic or natural molecule comprising a covalently linked sequence of nucleotides which are joined by a phosphodiester bond between the 3' position of the pentose of one nucleotide and the 5' position of the pentose of the adjacent nucleotide.

[0154] Nucleotide.

[0155] As used herein "nucleotide" refers to a base-sugar-phosphate combination. Nucleotides are monomeric units of a nucleic acid sequence (DNA and RNA). The term nucleotide includes ribonucleoside triphosphates ATP, UTP, CTG, GTP and deoxyribonucleoside triphosphates such as dATP, dCTP, dITP, dUTP, dGTP, dTTP, or derivatives thereof. Such derivatives include, for example, [αS]dATP, 7-deaza-dGTP and 7-deaza-dATP, and nucleotide derivatives that confer nuclease resistance on the nucleic acid molecule containing them. The term nucleotide as used herein also refers to dideoxyribonucleoside triphosphates (ddNTPs) and their derivatives. Illustrated examples of dideoxyribonucleoside triphosphates include, but are not limited to, ddATP, ddCTP, ddGTP, ddITP, and ddTTP. According to the present invention, a "nucleotide" may be unlabeled or detectably labeled by well known techniques. Detectable labels include, for example, radioactive isotopes, fluorescent labels, chemiluminescent labels, bioluminescent labels and enzyme labels. Fluorescent labels of nucleotides may include but are not limited fluorescein, 5-carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5-dichloro-6-carboxyfluorescein (JOE), rhodamine, 6-carboxyrhodamine (R6G), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX), 4-(4'dimethylaminophenylazo) benzoic acid (DABCYL), Cascade Blue, Oregon Green, Texas Red, Cyanine and 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS). Specific examples of fluroescently labeled nucleotides include [R6G]dUTP, [TAMRA]dUTP, [R110]dCTP, [R6G]dCTP, [TAMRA]dCTP, [JOE]ddATP, [R6G]ddATP, [FAM]ddCTP, [R110]ddCTP, [TAMRA]ddGTP, [ROX]ddTTP, [dR6G]ddATP, [dR110]ddCTP, [dTAMRA]ddGTP, and [dROX]ddTTP available from Perkin Elmer, Foster City, Calif.. FluoroLink DeoxyNucleotides, FluoroLink Cy3-dCTP, FluoroLink Cy5-dCTP, FluoroLink FluorX-dCTP, FluoroLink Cy3-dUTP, and FluoroLink Cy5-dUTP available from Amersham Arlington Heights, Ill.; Fluorescein-15-dATP, Fluorescein-12-dUTP, Tetramethylrodamine-6-dUTP, IR_{770}-9-dATP, Fluorescein-12-ddUTP, Fluorescein-12-UTP, and Fluorescein-15-2'-dATP available from Boehringer Mannheim Indianapolis, Ind.; and ChromaTide Labeled Nucleotides, BODIPY-FL-14-UTP, BODIPY-FL-4-UTP, BODIPY-TMR-14-UTP, BODIPY-TMR-14-dUTP, BODIPY-TR-14-UTP, BODIPY-TR-14-dUTP, Cascade Blue-7-UTP, Cascade Blue-7-dUTP, fluorescein-12-UTP, fluorescein-12-dUTP, Oregon Green 488-5-dUTP, Rhodamine Green-5-UTP, Rhodamine Green-5-dUTP, tetramethylrhodamine-6-UTP, tetramethylrhodamine-6-dUTP, Texas Red-5-UTP, Texas Red-5-dUTP, and Texas Red-12-dUTP available from Molecular Probes, Eugene, Oreg..

[0156] Probes.

[0157] The term probes refer to single or double stranded nucleic acid molecules or oligonucleotides which are detectably labeled by one or more detectable markers or labels. Such labels or markers may be the same or different and may include radioactive labels, fluorescent labels, chemiluminescent labels, bioluminescent labels and enzyme labels, although one or more fluorescent labels (which are the same or different) are preferred in accordance with the invention. Probes have specific utility in the detection of nucleic acid molecules by hybridization and thus may be used in diagnostic assays.

[0158] Hybridization.

[0159] As used herein, hybridization (hybridizing) refers to the pairing of two complementary single-stranded nucleic acid molecules (RNA and/or DNA) to give a double-stranded molecule. As one skilled in the art will recognize, two nucleic acid molecules may be hybridized, although the

base pairing is not completely complementary. Accordingly, mismatched bases do not prevent hybridization of two nucleic acid molecules provided that appropriate conditions, well known in the art, are used.

[0160] Thermostable Reverse Transcriptase.

[0161] For the purposes of this disclosure, a thermostable reverse transcriptase includes a reverse transcriptase which retains a greater percentage or amount of its activity after a heat treatment than is retained by a reverse transcriptase that has wild-type thermostability after an identical treatment. Thus, a reverse transcriptase having increased/enhanced thermostability may be defined as a reverse transcriptase having any increase in thermostability, preferably from about 1.2 to about 10,000 fold, from about 1.5 to about 10,000 fold, from about 2 to about 5,000 fold, or from about 2 to about 2000 fold (preferably greater than about 5 fold, more preferably greater than about 10 fold, still more preferably greater than about 50 fold, still more preferably greater than about 100 fold, still more preferably greater than about 500 fold, and most preferably greater than about 1000 fold) retention of activity after a heat treatment sufficient to cause a reduction in the activity of a reverse transcriptase that is wild-type for thermostability. Preferably, the mutant or modified reverse transcriptase of the invention is compared to the corresponding unmodified or wild-type reverse transcriptase to determine the relative enhancement or increase in thermostability. For example, after a heat treatment at 52° C. for 5 minutes, a thermostable reverse transcriptase may retain approximately 90% of the activity present before the heat treatment, whereas a reverse transcriptase that is wild-type for thermostability may retain 10% of its original activity. Likewise, after a heat treatment at 53° C. for five minutes, a thermostable reverse transcriptase may retain approximately 80% of its original activity, whereas a reverse transcriptase that is wild-type for thermostability may have no measurable activity. Similarly, after a heat treatment at 50° C. for five minutes, a thermostable reverse transcriptase may retain approximately 50%, approximately 55%, approximately 60%, approximately 65%, approximately 70%, approximately 75%, approximately 80%, approximately 85%, approximately 90%, or approximately 95% of its original activity, whereas a reverse transcriptase that is wild-type for thermostability may have no measurable activity or may retain 10%, 15% or 20% of its original activity. In the first instance (i.e., after heat treatment at 52° C. for 5 minutes), the thermostable reverse transcriptase would be said to be 9-fold more thermostable than the wild-type reverse transcriptase. Examples of conditions which may be used to measure thermostability of reverse transcriptases are set out below, for example, in the Examples.

[0162] The thermostability of a reverse transcriptase can be determined by comparing the residual activity of a sample of the reverse transcriptase that has been subjected to a heat treatment, i.e., incubated at 52° C. for a given period of time, for example, five minutes, to a control sample of the same reverse transcriptase that has been incubated at room temperature for the same length of time as the heat treatment. Typically the residual activity may be measured by following the incorporation of a radiolabled deoxyribonucleotide into an oligodeoxyribonucleotide primer using a complementary oligoribonucleotide template. For example, the ability of the reverse transcriptase to incorporate [α-^{32}P]-dGTP into an oligo-dG primer using a poly(riboC) template may be assayed to determine the residual activity of the reverse transcriptase.

[0163] In another aspect, thermostable reverse transcriptases of the invention may include any reverse transcriptase which is inactivated at a higher temperature compared to the corresponding wild-type, unmutated, or unmodified reverse transcriptase. Preferably, the inactivation temperature for the thermostable reverse transcriptases of the invention is from about 2° C. to about 50° C. (e.g., about 2° C., about 4° C., about 5° C., about 8° C., about 10° C., about 12° C., about 14° C., about 16° C., about 18° C, about 20° C, about 24° C, about 26° C., about 28° C., about 30° C., about 33° C, about 35° C, about 38° C., about 40° C., about 42° C., about 44° C., about 46° C, about 48° C, or about 50° C.) higher than the inactivation temperature for the corresponding wild-type, unmutated, or unmodified reverse transcriptase. More preferably, the inactivation temperature for the reverse transcriptases of the invention is from about 5° C. to about 50° C., from about 5° C. to about 40° C., from about 5° C. to about 30° C., or from about 5° C. to about 25° C. greater than the inactivation temperature for the corresponding wild-type, unmutated or unmodified reverse transcriptase, when compared under the same conditions.

[0164] The difference in inactivation temperature for the reverse transcriptase of the invention compared to its corresponding wild-type, unmutated or unmodified reverse transcriptase can be determined by treating samples of such reverse transcriptases at different temperatures for a defined time period and then measuring residual reverse transcriptase activity, if any, after the samples have been heat treated. Determination of the difference or delta in the inactivation temperature between the test reverse transcriptase compared to the wild-type, unmutated or unmodified control is determined by comparing the difference in temperature at which each reverse transcriptase is inactivated (i.e., no residual reverse transcriptase activity is measurable in the particular assay used). As will be recognized, any number of reverse transcriptase assays may be used to determine the different or delta of inactivation temperatures for any reverse transcriptases tested.

[0165] In another aspect, thermostability of a reverse transcriptase of the invention may be determined by measuring the half-life of the reverse transcriptase activity of a reverse transcriptase of interest. Such half-life may be compared to a control or wild-type reverse transcriptase to determine the difference (or delta) in half-life. Half-lifes of the reverse transcriptases of the invention are preferably determined at elevated temperatures (e.g., greater than 37° C.) and preferably at temperatures ranging from 40° C. to 80° C., more preferably at temperatures ranging from 45° C. to 75° C., 50° C. to 70° C., 50° C. to 65° C., and 50° C. to 60° C. Preferred half-lifes of the reverse transcriptases of the invention may range from 4 minutes to 10 hours, 4 minutes to 7.5 hours, 4 minutes to 5 hours, 4 minutes to 2.5 hours, or 4 minutes to 2 hours, depending upon the temperature used. For example, the reverse transcriptase activity of the reverse transcriptases of the invention may have a half-life of at least 4 minutes, at least 5 minutes, at least 6 minutes, at least 7 minutes, at least 8 minutes, at least 9 minutes, at least 10 minutes, at least 1 minutes, at least 12 minutes, at least 13 minutes, at least 14 minutes, at least 15 minutes, at

least 20 minute, at least 25minutes, at least 30 minutes, at least 40 minutes, at least 50 minutes, at least 60 minutes, at least 70 minutes, at least 80 minutes, at least 90 minutes, at least 100 minutes, at least 115 minutes, at least 125 minutes, at least 150 minutes, at least 175 minutes, at least 200 minutes, at least 225 minutes, at least 250 minutes, at least 275 minutes, at least 300 minutes, at least 400 minutes, at least 500 minutes at temperatures of 48° C., 50° C., 52° C., 52.5° C., 55° C., 57° C., 60° C., 62° C., 65° C., 68° C., and/or 70° C.

[0166] Terminal Eextension Activity.

[0167] As used herein, terminal extension activity refers to the ability of a reverse transcriptase (RT) to add additional bases on to the 3' end of a newly synthesized cDNA strand beyond the 5' end of the DNA or mRNA template. Terminal extension activity may add bases specifically (with a nucleotide bias) or randomly.

[0168] Terminal extension activity is also known as terminal deoxynucleotidyl transferase (TdT) activity. A reverse transcriptase having reduced TdT activity is defined as any reverse transcriptase having lower TdT specific activity than the TdT specific activity of the corresponding wild-type, unmutated, or unmodified enzyme, for example, less than about 90% of the TdT specific activity of the corresponding wild-type, unmutated, or unmodified enzyme, less than about 85% of the TdT specific activity of the corresponding wild-type, unmutated, or unmodified enzyme, less than about 80% of the TdT specific activity of the corresponding wild-type, unmutated, or unmodified enzyme, less than about 75% of the TdT specific activity of the corresponding wild-type, unmutated, or unmodified enzyme, less than about 50% of the TdT specific activity of the corresponding wild-type, unmutated, or unmodified enzyme, less than about 25% of the TdT specific activity of the corresponding wild-type, unmutated, or unmodified enzyme, less than about 15% of the TdT specific activity of the corresponding wild-type, unmutated, or unmodified enzyme, less than 10% of the TdT specific activity of the corresponding wild-type, unmutated, or unmodified enzyme, less than about 5% of the TdT specific activity of the corresponding wild-type, unmutated, or unmodified enzyme, or less than about 1% of the TdT specific activity of the corresponding wild-type, unmutated, or unmodified enzyme. A reverse transcriptase of the invention having substantially reduced TdT activity refers to a reverse transcriptase having a TdT specific activity level of 30% or less than the TdT specific activity of the corresponding wild-type or TdT^+ reverse transcriptase. Eliminated TdT activity is defined as a level of activity that is undetectable by the assay methods set out herein in Example 3.

[0169] As noted below in Example 3, reverse transcriptases are known in the art which extend nucleic acid molecules 2-3 nucleotides past the end of templates (e.g., RNA or DNA templates). Further, in any one reaction mixture in which reverse transcription occurs, mixtures of molecules may be present which contain different numbers of nucleotides that extend beyond the end of the template. TdT activity may be determined herein in reference to the number or percentage of molecules which contain one or more nucleotides which extend beyond the end of the template. For example, if a wild-type reverse transcriptase adds 1 or more nucleotides past the end of a template to 90% of the molecules generated during reverse transcription and a modified reverse transcriptase adds 1 or more nucleotides past the end of a template to 45% of the molecules under the same or similar conditions, then the modified reverse transcriptase would be said to exhibit a 50% decrease in TdT activity as compared to the wild-type enzyme. Further, an F309N, T306K, H204R mutant of M-MLV SUPERSCRIPT™ II has been generated which exhibits about 0% of the TdT activity exhibited by SUPERSCRIPT™ II when DNA is used as a template and about 10-20% of the TdT activity exhibited by SUPERSCRIPT™ II when RNA is used as a template.

[0170] Fidelity.

[0171] Fidelity refers to the accuracy of polymerization, or the ability of the reverse transcriptase to discriminate correct from incorrect substrates, (e.g., nucleotides) when synthesizing nucleic acid molecules which are complementary to a template. The higher the fidelity of a reverse transcriptase, the less the reverse transcriptase misincorporates nucleotides in the growing strand during nucleic acid synthesis; that is, an increase or enhancement in fidelity results in a more faithful reverse transcriptase having decreased error rate or decreased misincorporation rate.

[0172] A reverse transcriptase having increased/enhanced/higher fidelity is defined as a polymerase having any increase in fidelity, preferably about 1.2 to about 10,000 fold, about 1.5 to about 10,000 fold, about 2 to about 5,000 fold, or about 2 to about 2000 fold (preferably greater than about 5 fold, more preferably greater than about 10 fold, still more preferably greater than about 50 fold, still more preferably greater than about 100 fold, still more preferably greater than about 500 fold and most preferably greater than about 100 fold) reduction in the number of misincorporated nucleotides during synthesis of any given nucleic acid molecule of a given length compared to the control reverse transcriptase. Preferably, the mutant or modified reverse transcriptase of the invention is compared to the corresponding unmodified or wild-type reverse transcriptase to determine the relative enhancement or increase in fidelity. For example, a mutated reverse transcriptase may misincorporate one nucleotide in the synthesis of a nucleic acid molecule segment of 1000 bases compared to an unmutated reverse transcriptase misincorporating 10 nucleotides in the same size segment. Such a mutant reverse transcriptase would be said to have an increase of fidelity of 10 fold.

[0173] Fidelity can also be measured by the decrease in the incidence of frame shifting, as described below in Example 5. A reverse transcriptase having increased fidelity may be defined as a polymerase or reverse transcriptase having any increase in fidelity with respect to frame shifting, as compared to a control reverse transcriptase (e.g., a corresponding wild-type and/or a corresponding un-mutated or unmodified reverse transcriptase), for example, a reverse transcriptase having greater than about 1.2 fold increased fidelity with respect to frame shifting, having greater than about 1.5 fold increased fidelity with respect to frame shifting, having greater than about 5 fold increased fidelity with respect to frame shifting, having greater than about 10 fold increased fidelity with respect to frame shifting, having greater than about 20 fold increased fidelity with respect to frame shifting, having greater than about 30 fold increased fidelity with respect to frame shifting, or having greater than about 40 fold increased fidelity with respect to frame shifting.

[0174] A reverse transcriptase having increased/enhanced/higher fidelity, with respect to frame shifting, can also be defined as a reverse transcriptase or polymerase having any increase in fidelity, such as from about 1.5 to about 10,000 fold, from about 2 to about 5,000 fold, from about 2 to about 2000 fold, from about 1.5 to about 40 fold, from about 5 to about 40 fold, from about 10 to about 40 fold, from about 20 to about 40 fold, from about 30 to about 40 fold, from about 5 to about 30 fold, from about 10 to about 30 fold, from about 15 to about 30 fold, from about 20 to about 30 fold, from about 5 to about 20 fold, from about 10 to about 20 fold, from about 15 to about 20 fold, from about 10 to about 100 fold, from about 15 to about 100 fold, from about 20 to about 100 fold, from about 30 to about 100 fold, or from about 50 to about 100 fold increased fidelity with respect to frame shifting.

[0175] A reverse transcriptase having reduced misincorporation is defined herein as either a mutated or modified reverse transcriptase that has about or less than 90%, has about or less than 85%, has about or less than 75%, has about or less than 70%, has about or less than 60%, or preferably has about or less than 50%, preferably has about or less than 25%, more preferably has about or less than 10%, and most preferably has about or less than 1% of relative misincorporation compared to the corresponding wild-type, unmutated, or unmodified enzyme.

[0176] The fidelity or misincorporation rate of a reverse transcriptase can be determined by sequencing or by other methods known in the art (Eckert & Kunkel, 1990, *Nucl. Acids Res.* 18:3739-3744). In one example, the sequence of a DNA molecule synthesized by the unmutated and mutated reverse transcriptases can be compared to the expected (known) sequence. In this way, the number of errors (misincorporation or frame shifts) can be determined for each enzyme and compared. In another example, the unmutated and mutated reverse transcriptases may be used to sequence a DNA molecule having a known sequence. The number of sequencing errors (misincorporation or frame shifts) can be compared to determine the fidelity or misincorporation rate of the enzymes. Other means of determining the fidelity or misincorporation rate include a forward complementation assay using an RNA template as described below and previously in Boyer J. C. et al. *Methods Enzymol.* 275:523 (1996), and are set out in the examples. Other methods of determining the fidelity or misincorporation rate will be recognized by one of skill in the art.

[0177] Strand Jumping.

[0178] Strand jumping, as used herein, refers to a type of random mutation caused by an reverse transcriptase "skipping" more than one (e.g., two, five, ten, fifty, one-hundred, etc.) nucleotides on the mRNA template, resulting in a deletion of the corresponding nucleotides in the resulting cDNA. Sequencing the synthesized nucleic acid molecule and comparing to the expected sequence may allow determination of the level or amount of strand jumping for the reverse transcriptases of the invention. This level or amount may then be compared to the level or amount of strand jumping caused by the corresponding wild type and/or unmodified or un-mutated reverse transcriptase.

[0179] Hand Domain.

[0180] The hand domain, as used herein, refers to those amino acids which are in the area or areas that control the template, primer, or nucleotide interaction of the reverse transcriptase. This domain is further characterized by a group of three regions of secondary structure in a reverse transcriptase enzyme, the thumb, fingers and palm regions. The thumb region is defined as residing between amino acids 240-315 of HIV reverse transcriptase, or between amino acids 280-355 of M-MLV reverse transcriptase. The fingers region is defined as residing between amino acids 1-85 and 120-154 of HIV reverse transcriptase, or between 1-124 and 161-193 of M-MLV reverse transcriptase. The palm region is defined as residing between amino acids 86-199 and 155-239 of HIV reverse transcriptase, or between amino acids 126-160 and 193-279 of M-MLV reverse transcriptase. These areas are generally defined, and the amino acids defining the N-termini and C-termini are approximate. Corresponding regions may also be defined for other reverse transcriptases. Preferred reverse transcriptases of the invention have one or more modifications or mutations within the hand domain. More particularly, reverse transcriptases of the invention comprise one or more mutations or modifications within one or more regions, including the thumb, finger, and palm regions.

[0181] Full Length.

[0182] As used herein, full length when used to describe a product molecule, e.g., a cDNA molecule, indicates that the product molecule is the same length or substantially the same length as the template molecule, e.g., an mRNA molecule, from which it is produced by the activity of polypeptides of the invention. A cDNA molecule may be substantially the same length as the template from which it is copied when it is about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or greater of the length of the portion of the template located 3' to the 3' most nucleotide of the primer used to reverse transcribe the template. Thus, if a primer anneals in the center of a template, a full length product would be one that contains a cDNA copy of half of the template. Template molecules may be from about 100 bases to about 50 kb in length, from about 200 bases to about 50 kb in length, from about 300 bases to about 50 kb in length, from about 400 bases to about 50 kb in length, from about 500 bases to about 50 kb in length, from about 600 bases to about 50 kb in length, from about 700 bases to about 50 kb in length, from about 800 bases to about 50 kb in length, from about 900 bases to about 50 kb in length, and from about 1 kb to about 50 kb in length. In some embodiments, template molecules may be from about 500 bases to about 10 kb in length, from about 600 bases to about 10 kb in length, from about 700 bases to about 10 kb in length, from about 800 bases to about 10 kb in length, from about 900 bases to about 10 kb in length, from about 1000 bases to about 10 kb in length, from about 1100 bases to about 10 kb in length, and/or from about 1200 bases to about 10 kb in length. In some embodiments, template molecules may be from about 250 bases to about 5 kb in length, from about 300 bases to about 5 kb in length, and from about 350 bases to about 5 kb in length, from about 400 bases to about 5 kb in length, from about 450 bases to about 5 kb in length, from about 500 bases to about 5 kb in length, from about 550 bases to about 5 kb in length, from about 600 bases to about 5 kb in length, from about 650 bases to about 5 kb in length, from about 700 bases to about 5 kb in length, from about 750 bases to about 5 kb in length, from about 800 bases to about 5 kb in length, and from about 850 bases to about 5 kb in length.

[0183] In some embodiments, the ability of a reverse transcriptase to synthesize a full length product may be determined using a defined template and primer, for example, a polyadenylated template corresponding to the chloramphenicol acetyl transferase gene and an oligo(dT) primer, under defined reaction conditions, e.g., pH, salt concentration, divalent metal concentration, template concentration, temperature, etc. In some embodiments, a template molecule is greater than about 500 base pairs in length, and the amount of full length product synthesized may determined by separating full length product from truncated product, for example, by gel electrophoresis, and quantifying the full length product, for example, by incorporating a radiolabel in to the product and using a scintillation counter.

[0184] About.

[0185] The term "about" as used herein, means the recited number plus or minus 10%. Thus, "about 100" includes 90-110.

[0186] Overview

[0187] In general, the invention provides, in part, compositions for use in reverse transcription of a nucleic acid molecule comprising a reverse transcriptase with one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, twenty, thirty, etc.) mutations or modification which render the reverse transcriptase more thermostable. The invention also provides compositions for use in reverse transcription of a nucleic acid molecule, the compositions comprising a reverse transcriptase with one or more mutations or modification which render the reverse transcriptase more efficient, that is having higher fidelity, and/or has lower TdT activity than a corresponding un-mutated or un-modified reverse transcriptase. The invention further provides compositions comprising a reverse transcriptase with one or more mutations or modification which render the reverse transcriptase more thermostable and/or more efficient than a corresponding un-mutated or un-modified reverse transcriptase.

[0188] The enzymes in these compositions are preferably present in working concentrations and are also preferably reduced, substantially reduced, or eliminated in RNase H activity. Alternatively, reverse transcriptases used in the compositions of the invention may have RNase H activity. Preferred reverse transcriptases include retroviral reverse transcriptases such as M-MLV reverse transcriptase, HIV reverse transcriptase, RSV reverse transcriptase, AMV reverse transcriptase, RAV reverse transcriptase, and MAV reverse transcriptase or other ASLV reverse transcriptases or their corresponding RNase H− derivatives. Additional reverse transcriptases which may be used to prepare compositions of the invention include bacterial reverse transcriptases (e.g., *Escherichia coli* reverse transcriptase) (see, e.g., Mao et al., *Biochem. Biophys. Res. Commun.* 227:489-93 (1996)) and reverse transcriptases of *Saccharomyces cerevisiae* (e.g., reverse transcriptases of the Ty1 or Ty3 retrotransposons) (see, e.g., Cristofari et al., *Jour. Biol. Chem.* 274:36643-36648 (1999); Mules et al., *Jour. Virol.* 72:6490-6503 (1998)).

[0189] In accordance with the invention, any number of mutations can be made to the reverse transcriptases and, in a preferred aspect, multiple mutations can be made to result in an increased thermostability and/or to confer other desired properties on reverse transcriptases of the invention. Such mutations include point mutations, frame shift mutations, deletions and insertions, with one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, twenty, thirty, etc.) point mutations preferred. Mutations may be introduced into reverse transcriptases of the present invention using any methodology known to those of skill in the art. Mutations may be introduced randomly by, for example, conducting a PCR reaction in the presence of manganese as a divalent metal ion cofactor. Alternatively, oligonucleotide directed mutagenesis may be used to create the mutant polymerases which allows for all possible classes of base pair changes at any determined site along the encoding DNA molecule. In general, this technique involves annealing an oligonucleotide complementary (except for one or more mismatches) to a single stranded nucleotide sequence coding for the reverse transcriptase of interest. The mismatched oligonucleotide is then extended by DNA polymerase, generating a double-stranded DNA molecule which contains the desired change in sequence in one strand. The changes in sequence can, for example, result in the deletion, substitution, or insertion of an amino acid. The double-stranded polynucleotide can then be inserted into an appropriate expression vector, and a mutant or modified polypeptide can thus be produced. The above-described oligonucleotide directed mutagenesis can, for example, be carried out via PCR.

[0190] The invention is also directed to methods for reverse transcription of one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, twenty, etc.) nucleic acid molecules comprising mixing one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, twenty, etc.) nucleic acid templates, which are preferably RNA or messenger RNA (mRNA) and more preferably a population of mRNA molecules, with one or more reverse transcriptase of the present invention and incubating the mixture under conditions sufficient to make a nucleic acid molecule or molecules complementary to all or a portion of the one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, twenty, thirty, etc.) templates. To make the nucleic acid molecule or molecules complementary to the one or more templates, a primer (e.g., an oligo(dT) primer) and one or more nucleotides are preferably used for nucleic acid synthesis in the 5' to 3' direction. Nucleic acid molecules suitable for reverse transcription according to this aspect of the invention include any nucleic acid molecule, particularly those derived from a prokaryotic or eukaryotic cell. Such cells may include normal cells, diseased cells, transformed cells, established cells, progenitor cells, precursor cells, fetal cells, embryonic cells, bacterial. cells, yeast cells, animal cells (including human cells), avian cells, plant cells and the like, or tissue isolated from a plant or an animal (e.g., human, cow, pig, mouse, sheep, horse, monkey, canine, feline, rat, rabbit, bird, fish, insect, etc.). Nucleic acid molecules suitable for reverse transcription may also be isolated and/or obtained from viruses and/or virally infected cells.

[0191] The invention further provides methods for amplifying or sequencing a nucleic acid molecule comprising contacting the nucleic acid molecule with a reverse transcriptase of the present invention. Preferred such methods comprise one or more polymerase chain reactions (PCRs).

[0192] Sources of Reverse Transcriptases

[0193] Enzymes for use in compositions, methods and kits of the invention include any enzyme having reverse tran-

scriptase activity. Such enzymes include, but are not limited to, retroviral reverse transcriptase, retrotransposon reverse transcriptase, hepatitis B reverse transcriptase, cauliflower mosaic virus reverse transcriptase, bacterial reverse transcriptase, Tth DNA polymerase, Taq DNA polymerase (Saiki, R. K., et al., *Science* 239:487-491 (1988); U.S. Pat. Nos. 4,889,818 and 4,965,188), Tne DNA polymerase (PCT Publication No. WO 96/10640), Tma DNA polymerase (U.S. Pat. No. 5,374,553) and mutants, fragments, variants or derivatives thereof (see, e.g., commonly owned U.S. Pat. Nos. 5,948,614 and 6,015,668, which are incorporated by reference herein in their entireties). Preferably, reverse transcriptases for use in the invention include retroviral reverse transcriptases such as M-MLV reverse transcriptase, AMV reverse transcriptase, RSV reverse transcriptase, RAV reverse transcriptase, MAV reverse transcriptase, and generally ASLV reverse transcriptases. As will be understood by one of ordinary skill in the art, modified reverse transcriptases may be obtained by recombinant or genetic engineering techniques that are routine and well-known in the art. Mutant reverse transcriptases can, for example, be obtained by mutating the gene or genes encoding the reverse transcriptase of interest by site-directed or random mutagenesis. Such mutations may include point mutations, deletion mutations and insertional mutations. For example, one or more point mutations (e.g., substitution of one or more amino acids with one or more different amino acids) may be used to construct mutant reverse transcriptases of the invention.

[**0194**] The invention further includes reverse transcriptases which are 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical at the amino acid level to a wild-type reverse transcriptase (e.g., M-MLV reverse transcriptase, AMV reverse transcriptase, RSV reverse transcriptase, HIV reverse transcriptase, etc.) and exhibit increased thermostability and/or other desired properties of the invention. Also included within the invention are reverse transcriptases which are 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical at the amino acid level to a reverse transcriptase comprising the amino acid sequence set out below in Table 3 (SEQ ID NO:2) and exhibit increased thermostability and/or more efficient (e.g., having higher fidelity and/or having lower TdT activity). The invention also includes nucleic acid molecules which encode the above described reverse transcriptases.

[**0195**] The invention also includes fragments of reverse transcriptases which comprise at least 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, or 700 amino acid residues and retain one or more activities associated with reverse transcriptases. Such fragments may be obtained by deletion mutation, by recombinant techniques that are routine and well-known in the art, or by enzymatic digestion of the reverse transcriptase(s) of interest using any of a number of well-known proteolytic enzymes. The invention further includes nucleic acid molecules which encode the above described mutant reverse transcriptases and reverse transcriptase fragments.

[**0196**] Reverse transcriptase fragments of the invention also comprise amino acids 1-10, 11-20, 21-30, 31-40, 41-50, 51-60, 61-70, 71-80, 81-90, 91-100, 101-110, 111-120, 121-130, 131-140, 141-150, 151-160, 161-170, 171-180, 181-190, 191-200, 201-210, 211-220, 221-230, 231-240, 241-250, 251-260, 261-270, 271-280, 281-290, 291-300, 301-310, 311-320, 321-330, 331-340, 341-350, 351-360, 361-370, 371-380, 381-390, 391-400, 401-410, 411-420, 421-430, 431-440, 441-450, 451-460, 461-470, 471-480, 481-490, 491-500, 501-510, 511-520, 521-530, 531-540, and/or 541-550 and/or amino acids 1-355, 1-498, 1-500, and/or 1-550 of M-MLV reverse transcriptase (and more preferably the sequence shown in Table 3, which may further contain one or more of the modifications or mutations discussed herein), as well as corresponding fragments of other reverse transcriptases. Reverse transcriptase fragments of the invention further comprise polypeptides which are 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to one or more of the fragments set out above. The invention also concerns various combinations of any number of these fragments.

[**0197**] By a protein or protein fragment having an amino acid sequence at least, for example, 70% "identical" to a reference amino acid sequence it is intended that the amino acid sequence of the protein is identical to the reference sequence except that the protein sequence may include up to 30 amino acid alterations per each 100 amino acids of the amino acid sequence of the reference protein. In other words, to obtain a protein having an amino acid sequence at least 70% identical to a reference amino acid sequence, up to 30% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 30% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino (N-) and/or carboxy (C-) terminal positions of the reference amino acid sequence and/or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence and/or in one or more contiguous groups within the reference sequence. As a practical matter, whether a given amino acid sequence is, for example, at least 70% identical to the amino acid sequence of a reference protein can be determined conventionally using known computer programs such as those described above for nucleic acid sequence identity determinations, or using the CLUSTAL W program (Thompson, J. D., et al., *Nucleic Acids Res.* 22:4673-4680 (1994)).

[**0198**] Sequence identity may be determined by comparing a reference sequence or a subsequence of the reference sequence to a test sequence. The reference sequence and the test sequence are optimally aligned over an arbitrary number of residues termed a comparison window. In order to obtain optimal alignment, additions or deletions, such as gaps, may be introduced into the test sequence. The percent sequence identity is determined by determining the number of positions at which the same residue is present in both sequences and dividing the number of matching positions by the total length of the sequences in the comparison window and multiplying by 100 to give the percentage. In addition to the number of matching positions, the number and size of gaps is also considered in calculating the percentage sequence identity.

[**0199**] Sequence identity is typically determined using computer programs. A representative program is the BLAST (Basic Local Alignment Search Tool) program publicly accessible at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). This program compares segments in a test sequence to sequences in

a database to determine the statistical significance of the matches, then identifies and reports only those matches that that are more significant than a threshold level. A suitable version of the BLAST program is one that allows gaps, for example, version 2× (Altschul, et al., *Nucleic Acids Res.* 25(17):3389-402, 1997). Standard BLAST programs for searching nucleotide sequences (blastn) or protein (blastp) may be used. Translated query searches in which the query sequence is translated, i.e., from nucleotide sequence to protein (blastx) or from protein to nucleic acid sequence (tbblastn) may also be used as well as queries in which a nucleotide query sequence is translated into protein sequences in all 6 reading frames and then compared to an NCBI nucleotide database which has been translated in all six reading frames (tbblastx).

[**0200**] Additional suitable programs for identifying proteins with sequence identity to the proteins of the invention include, but are not limited to, PHI-BLAST (Pattern Hit Initiated BLAST, Zhang, et al., *Nucleic Acids Res.* 26(17):3986-90, 1998) and PSI-BLAST (Position-Specific Iterated BLAST, Altschul, et al., *Nucleic Acids Res.* 25(17):3389-402, 1997).

[**0201**] Programs may be used with default searching parameters. Alternatively, one or more search parameter may be adjusted. Selecting suitable search parameter values is within the abilities of one of ordinary skill in the art.

[**0202**] Preferred enzymes for use in the invention include those that are reduced, substantially reduced, or lacking in RNase H activity. Such enzymes that are reduced or substantially reduced in RNase H activity may be obtained by mutating, for example, the RNase H domain within the reverse transcriptase of interest, for example, by introducing one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, twenty, thirty, etc.) point mutations, one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, twenty, thirty, etc.) deletion mutations, and/or one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, twenty, thirty, etc.) insertion mutations as described above. In some embodiments, the reverse transcriptase of the invention does not contain a modification or mutation in the RNase H domain and preferably does not contain a modification which reduces RNase H activity. In one aspect, the reverse transcriptase of the invention has 90%, 95%, or 100% of the RNase H activity compared to the corresponding wildtype reverse transcriptase.

[**0203**] By an enzyme "substantially reduced in RNase H activity" is meant that the enzyme has less than about 30%, less than about 25%, less than about 20%, more preferably less than about 15%, less than about 10%, less than about 7.5%, or less than about 5%, and most preferably less than about 5% or less than about 2%, of the RNase H activity of the corresponding wild-type or RNase H^+ enzyme, such as wild-type Moloney Murine Leukemia Virus (M-MLV), Avian Myeloblastosis Virus (AMV) or Rous Sarcoma Virus (RSV) reverse transcriptases. A reduction in RNase H activity means any reduction in the activity compared, for example, to the corresponding wild type or un-mutatated or unmodified reverse transcriptase.

[**0204**] Reverse transcriptases having reduced, substantially reduced, undetectable or lacking RNase H activity have been previously described (see U.S. Pat. No. 5,668,005, U.S. Pat. No. 6,063,608, and PCT Publication No. WO 98/47912). The RNase H activity of any enzyme may be determined by a variety of assays, such as those described, for example, in U.S. Pat. No. 5,244,797, in Kotewicz, M. L., et al., *Nucl. Acids Res.* 16:265 (1988), in Gerard, G. F., et al., *FOCUS* 14(5):91 (1992), in PCT publication number WO 98/47912, and in U.S. Pat. No. 5,668,005, the disclosures of all of which are fully incorporated herein by reference. Reverse transcriptases having no detectable RNase H activity or lacking RNase H activity by one or more of the described assays are particularly preferred.

[**0205**] Particularly preferred enzymes for use in the invention include, but are not limited to, M-MLV RNase H– reverse transcriptase, RSV RNase H– reverse transcriptase, AMV RNase H– reverse transcriptase, RAV RNase H– reverse transcriptase, MAV RNase H– reverse transcriptase and HIV RNase H– reverse transcriptase. It will be understood by one of ordinary skill, however, that any enzyme capable of producing a DNA molecule from a ribonucleic acid molecule (i.e., an enzyme having reverse transcriptase activity) that is reduced, substantially reduced, or lacking in RNase H activity may be equivalently used in the compositions, methods and kits of the invention.

[**0206**] Enzymes for use in the invention also include those in which terminal deoxynucleotidyl transferase (TdT) activity has been reduced, substantially reduced, or eliminated. Such enzymes that are reduced or substantially reduced in terminal deoxynucleotidyl transferase activity, or in which TdT activity has been eliminated, may be obtained by mutating, for example, amino acid residues within the reverse transcriptase of interest which are in close proximity or in contact with the template-primer, for example, by introducing one or more (e.g., one, two, three, four, five, ten, twelve, fifteen, twenty, thirty, etc.) point mutations, one or more deletion mutations, and/or one or more insertion mutations. Reverse transcriptases which exhibit decreased TdT activity are described in U.S. application Ser. No. 09/808,124, filed Mar. 15, 2001 (the entire disclosure of which is incorporated herein by reference), and include reverse transcriptases with one or more alterations at amino acid positions equivalent or corresponding to Y64, M289, F309, T197 and/or Y133 of M-MLV reverse transcriptase.

[**0207**] In one aspect, amino acid substitutions are made at one or more of the above identified positions (i.e., amino acid positions equivalent or corresponding to Y64, M289, F309, T197 or Y133 of M-MLV reverse transcriptase). Thus, the amino acids at these positions may be substituted with any other amino acid including Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. Specific example of reverse transcriptases which exhibit reduced, substantially reduced, or eliminated TdT activity include M-MLV reverse transcriptases (e.g., SUPERSCRIPT™ II) in which (1) the phenylalanine residue at position 309 has been replaced with asparagine, (2) the threonine residue at position 197 has been replaced with either alanine or glutamic acid, and/or (3) the tyrosine residue at position 133 has been replaced with alanine.

[**0208**] Enzymes for use in the invention also include those that exhibit increased fidelity. Reverse transcriptases which exhibit increased fidelity are described in U.S. application Ser. No. 60/189,454, filed Mar. 15, 2000, and U.S. application Ser. No. 09/808,124, filed Mar. 15, 2001 (the entire disclosures of each of which are incorporated herein by

reference), and include reverse transcriptases with one or more alterations at positions equivalent or corresponding to those set out below in Table 2.

TABLE 2

RT	Amino Acid
M-MLV	Y64 (e.g., Y64W, Y64R), R116 (e.g., R116M), K152 (e.g., K152R), Q190 (e.g., Q190F), T197 (e.g., T197A, T197E), V223 (e.g., V223H, V223I, V223F), D124, H126, Y133 (e.g., Y133A, Y133H), F309 (e.g., F309N, F309R)
AMV	W25, R76, K110, Q149, T156, M182
RSV	W25, R76, K110, Q149, T156, M182
HIV	W24, R78, G112, Q151, A158, M184

[0209] In some embodiments of the invention, amino acid substitutions are made at one or more of the above identified positions. Thus, the amino acids at these positions may be substituted with any other amino acid including Ala, Arg, Asn, Arg, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. Specific example of reverse transcriptases which exhibit increased fidelity include M-MLV reverse transcriptase in which (1) the valine residue at position 223 has been replaced with histidine, phenylalanine or isoleucine, (2) the arginine residue at position 116 has been replaced with methionine, (3) the lysine residue at position 152 has been replaced with arginine, (4) the glutamic acid residue at position 190 has been replaced with phenylalanine, (5) the threonine residue at position 197 has been replaced with alanine or glutamic acid, (6) the phenylalanine residue at position 309 has been replaced with asparagine or arginine, (7) the tyrosine residue at position 133 has been replaced with histidine or alanine, and/or (8) the tyrosine residue at position 64 has been replaced with tryptophan or arginine.

[0210] Thus, in specific embodiments, the invention includes reverse transcriptases which exhibit increased thermostability and, optionally, also exhibit one or more of the following characteristics: (1) reduced or substantially reduced RNase H activity, (2) reduced or substantially reduced TdT activity, and/or (3) increased fidelity.

[0211] The invention also relates to reverse transcriptase mutants, where the mutations or substitutions have been made in a recognized region of the reverse transcriptase enzyme. Such regions include, but are not limited to, the fingers, palm, thumb, α-helix H, β-sheet 10, and/or β-sheet 11 regions. Thus, the invention includes reverse transcriptases which exhibit increased thermostability (as well as other properties), as described elsewhere herein, and have one or more (e.g., one, two, three, four, five, ten, fifteen, etc.) mutations or modification in the hand domain and, more specifically, in one or more regions including the fingers, palm and/or thumb regions.

[0212] Polypeptides having reverse transcriptase activity for use in the invention may be isolated from their natural viral or bacterial sources according to standard procedures for isolating and purifying natural proteins that are well-known to one of ordinary skill in the art (see, e.g., Houts, G. E., et al., *J. Virol.* 29:517 (1979); U.S. Pat. No. 5,668,005; and PCT publication number WO 98/47912). In addition, polypeptides having reverse transcriptase activity may be prepared by recombinant DNA techniques that are familiar to one of ordinary skill in the art (see, e.g., Kotewicz, M. L., et al., *Nucl. Acids Res.* 16:265 (1988); Soltis, D. A., and Skalka, A. M., *Proc. Natl. Acad. Sci. USA* 85:3372-3376 (1988)); U.S. Pat. No. 5,668,005; and PCT publication no. WO 98/47912.

[0213] In one aspect of the invention, mutant or modified reverse transcriptases are made by recombinant techniques. A number of cloned reverse transcriptase genes are available or may be obtained using standard recombinant techniques (see U.S. Pat. No. 5,668,005 and PCT Publication No. WO 98/47912).

[0214] To clone a gene or other nucleic acid molecule encoding a reverse transcriptase which will be modified in accordance with the invention, isolated DNA which contains the reverse transcriptase gene or open reading frame may be used to construct a recombinant DNA library. Any vector, well known in the art, can be used to clone the reverse transcriptase of interest. However, the vector used must be compatible with the host in which the recombinant vector will be transformed.

[0215] Prokaryotic vectors for constructing the plasmid library include plasmids such as those capable of replication in *E. coli* such as, for example, pBR322, Co1E1, pSC101, pUC-vectors (pUC18, pUC19, etc.: In: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1982); and Sambrook et al., In: Molecular Cloning A Laboratory Manual (2d ed.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)). *Bacillus* plasmids include pC194, pUB110, pE194, pC221, pC217, etc. Such plasmids are disclosed by Glyczan, T. In: *The Molecular Biology Bacilli*, Academic Press, York (1982), 307-329. Suitable *Streptomyces* plasmids include pIJ101 (Kendall et al., *J. Bacteriol.* 169:4177-4183 (1987)). *Pseudomonas* plasmids are reviewed by John et al., (*Rad. Insec. Dis.* 8:693-704 (1986)), and Igaki, (*Jpn. J. Bacteriol.* 33:729-742 (1978)). Broad-host range plasmids or cosmids, such as pCP13 (Darzins and Chakrabarty, *J. Bacteriol.* 159:9-18 (1984)) can also be used for the present invention. Preferred vectors for cloning the genes and nucleic acid molecules of the present invention are prokaryotic vectors. Preferably, pBAD, pCP13 and pUC vectors are used to clone the genes of the present invention. Other suitable vectors are known to those skilled in the art and are commercially available, for example, from Invitrogen Corporation, Carlsbad, Calif.

[0216] Suitable host for cloning the reverse transcriptase genes and nucleic acid molecules of interest are prokaryotic hosts. One example of a prokaryotic host is *E. coli*. However, the desired reverse transcriptase genes and nucleic acid molecules of the present invention may be cloned in other prokaryotic hosts including, but not limited to, hosts in the genera *Escherichia, Bacillus, Streptomyces, Pseudomonas, Salmonella, Serratia, and Proteus*. Bacterial hosts of particular interest include *E. coli* DH10B, which may be obtained from Invitrogen Corporation (Carlsbad, Calif.).

[0217] Eukaryotic hosts for cloning and expression of the reverse transcriptase of interest include yeast, fungal, and mammalian cells. Expression of the desired reverse transcriptase in such eukaryotic cells may require the use of eukaryotic regulatory regions which include eukaryotic promoters. Cloning and expressing the reverse transcriptase

gene or nucleic acid molecule in eukaryotic cells may be accomplished by well known techniques using well known eukaryotic vector systems.

[0218] Once a DNA library has been constructed in a particular vector, an appropriate host is transformed by well known techniques. Transformed cells are plated at a density to produce approximately 200-300 transformed colonies per petri dish. For selection of reverse transcriptase, colonies are then screened for the expression of a reverse transcriptase or a thermostable reverse transcriptase as described in the Examples below. Briefly, overnight cultures of individual transformant colonies are assayed directly for reverse transcriptase or thermostable reverse transcriptase activity and/or other desirable activities using a labeled deoxynucleotide and analyzed for the presence of labeled product. If thermostable reverse transcriptase activity and/or other desirable activity is detected, the mutant is sequenced to determine which amino acids maintained reverse transcriptase activity. The gene or nucleic acid molecule encoding a reverse transcriptase of the present invention can be cloned using techniques known to those in the art.

[0219] Modifications or Mutations of Polymerases

[0220] In accordance with the invention, one or more mutations may be made in any reverse transcriptase in order to increase the thermostability of the enzyme, or confer other properties described herein upon the enzyme, in accordance with the invention. Such mutations include point mutations, frame shift mutations, deletions and insertions. Preferably, one or more point mutations, resulting in one or more amino acid substitutions, are used to produce reverse transcriptases having enhanced or increased thermostability. In a preferred aspect of the invention, one or more mutations at positions equivalent or corresponding to position H204 (e.g., H204R) and/or T306 (e.g., T306K or T306R) of M-MLV reverse transcriptase may be made to produced the desired result in other reverse transcriptases of interest.

[0221] In specific embodiments, one or more mutations at positions equivalent or corresponding to position L52, Y64, R116, Y133, K152 Q190, T197, H204, V223, M289, T306 and/or F309 of M-MLV reverse transcriptase may be made to produced a desired result (e.g., increased thermostability, increased fidelity, decreased TdT activity, etc.). Thus, in specific embodiments, using amino acid positions of M-MLV reverse transcriptase as a frame of reference, proteins of the invention include reverse transcriptases (e.g., M-MLV reverse transcriptase, AMV reverse transcriptase, HIV reverse transcriptase, RSV reverse transcriptase, etc.) having one or more of the following alterations: L52P, Y64S, Y64W, Y64R, R116M, Y133A, Y133H, K152R, K152M, Q190F, T197R, T197E, T197A, T197K, H204R, V223H, V223F, V223I, M289L, T306K, T306R, F309R, and/or F309N, as well as compositions containing these proteins, nucleic acid molecules which encode these proteins, and host cells which contain these nucleic acid molecules.

[0222] Mutations in reverse transcriptases which alter thermostability properties of these proteins may be present in conjunction with alterations which either have little or no effect on activities normally associated with reverse transcriptases (e.g., RNase H activity, reverse transcriptase or polymerase activity, terminal deoxynucleotidyl transferase (TdTase) activity, etc.) or substantially alter one or more activities normally associated with reverse transcriptases. One example of a reverse transcriptase which has such a combination of mutations is a M-MLV reverse transcriptase which has the following alterations: K152M, V223H.

[0223] One mutation which has been shown to enhanced the fidelity of SUPERSCRIPT™ II (Invitrogen Corporation (Carlsbad, Calif.) Catalog No. 18064-022) is V223H (see U.S. application Ser. No. 60/189,454, filed Mar. 15, 2000, U.S. application Ser. No. 09/808,124, filed Mar. 15, 2001, and PCT publication number WO 01/68895, the entire disclosures of each of which are incorporated herein by reference). However, the V223H alteration decreases the thermostability of this enzyme. One mutant was identified, K152M, which suppress the destabilizing effect of enzymes having the V223H mutation. Thus, the invention includes M-MLV reverse transcriptase which contain alterations at positions K152 and V223 and exhibit both increased fidelity and increased thermostability. Specific examples of such reverse transcriptases are those in which K152 is replaced with methionine and V223 is replaced with histidine. Other reverse transcriptases (e.g., AMV reverse transcriptase, HIV reverse transcriptase, RSV reverse transcriptase, etc.) with corresponding alterations are also included within the scope of the invention.

[0224] SUPERSCRIPT™ II is an RNase H– reverse transcriptase from M-MLV which has the following substitutions: D524G, E562Q, and D583N (see U.S. Pat. Nos. 5,017,492, 5,244,797, 5,405,776, 5,668,005, and 6,063,608, the entire disclosures of which are incorporated herein by reference). The invention includes reverse transcriptases that contain alterations, such as those reference positions (i.e., 524, 562, and/or 583)or equivalent positions.

[0225] One or more amino acid substitutions are made at one or more selected positions for any reverse transcriptase of interest. Thus, the amino acids at the selected positions may be substituted with any other amino acid including Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. In some preferred embodiments, the selected amino acid will be a non-charged surface residue and will be replaced by a charged residue. In some preferred embodiments, the non-charged surface residue may be replaced by a positively charged amino acid (e.g., lysine or arginine). In one aspect, a charged residue will be replaced with an un-charged residue. In one aspect, a non-charged residue will be replaced with a negatively charged residue. In another aspect, a negatively charged residue will be replaced with a positively charged residue and/or a positively charged residue will be replaced with a negatively charged residue.

[0226] The corresponding positions of M-MLV reverse transcriptase identified above may be readily identified for other reverse transcriptases by one with skill in the art. Thus, given the defined region and the assays described in the present application, one with skill in the art can make one or a number of modifications which would result in increased thermostability and/or other desired features of any reverse transcriptase of interest. Residues to be modified in accordance with the present invention may include those listed in Table 1 above.

[0227] The nucleotide sequences for M-MLV reverse transcriptase (Shinnick et al., 1981, *Nature* 293:543-548; Georgiadis et al., 1995, *Structure* 3:879-892), AMV reverse

transcriptase (Joliot et al., 1993, *Virology* 195:812-819), RSV reverse transcriptase (Schwartz et al., 1983, *Cell* 32:853-859), and HIV reverse transcriptase (Wong-Staal et al., 1985, *Nature* 313:277-284) are known and are incorporated herein by reference in their entirety.

[0228] Preferably, oligonucleotide directed mutagenesis is used to create the mutant reverse transcriptases which allows for all possible classes of base pair changes at any determined site along the encoding DNA molecule.

[0229] Enhancing Expression of Reverse Transcriptases

[0230] To optimize expression of reverse transcriptases of the present invention, inducible or constitutive promoters are well known and may be used to express high levels of a reverse transcriptase structural gene in a recombinant host. Similarly, high copy number vectors, well known in the art, may be used to achieve high levels of expression. Vectors having an inducible high copy number may also be useful to enhance expression of the reverse transcriptases of the invention in a recombinant host.

[0231] To express the desired structural gene in a prokaryotic cell (such as *E. coli, B. subtilis, Pseudomonas*, etc.), it is preferable to operably link the desired structural gene to a functional prokaryotic promoter. However, the natural promoter of the reverse transcriptase gene may function in prokaryotic hosts allowing expression of the reverse transcriptase gene. Thus, the natural promoter or other promoters may be used to express the reverse transcriptase gene. Such other promoters that may be used to enhance expression include constitutive or regulatable (i.e., inducible or derepressible) promoters. Examples of constitutive promoters include the int promoter of bacteriophage λ, and the bla promoter of the β-lactamase gene of pBR322. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage λ (P_R and P_L), trp, recA, lacZ, lacI, tet, gal, trc, ara BAD (Guzman, et al., 1995, *J. Bacteriol.* 177(14):4121-4130) and tac promoters of *E. coli*. The *B. subtilis* promoters include α-amylase (Ulmanen et al., *J. Bacteriol* 162:176-182 (1985)) and *Bacillus* bacteriophage promoters (Gryczan, T., In: *The Molecular Biology Of Bacilli*, Academic Press, New York (1982)). *Streptomyces* promoters are described by Ward et al., *Mol. Gen. Genet.* 203:468478 (1986)). Prokaryotic promoters are also reviewed by Glick, *J. Ind. Microbiol.* 1:277-282 (1987); Cenatiempto, Y., *Biochimie* 68:505-516 (1986); and Gottesman, *Ann. Rev. Genet.* 18:415-442 (1984). Expression in a prokaryotic cell also requires the presence of a ribosomal binding site upstream of the gene-encoding sequence. Such ribosomal binding sites are disclosed, for example, by Gold et al., *Ann. Rev. Microbiol.* 35:365404 (1981).

[0232] To enhance the expression of polymerases of the invention in a eukaryotic cell, well known eukaryotic promoters and hosts may be used. Enhanced expression of the polymerases may be accomplished in a prokaryotic host. One example of a prokaryotic host suitable for use with the present invention is *Escherichia coli.*

[0233] Isolation and Purification of Reverse Transcriptases

[0234] The enzyme(s) of the present invention is preferably produced by growth in culture of the recombinant host containing and expressing the desired reverse transcriptase gene. However, the reverse transcriptase of the present invention may be isolated from any strain, organism, or tissue which produces the reverse transcriptase of the present invention. Fragments of the reverse transcriptase are also included in the present invention. Such fragments include proteolytic fragments and fragments having reverse transcriptase activity. Such fragments may also be produced by cloning and expressing portions of the reverse transcriptase gene of interest, creating frame shift mutations and/or by adding one or more stop codons in the gene of interest for expression of a truncated protein or polypeptide. Preferably, polypeptides of the invention may be purified and/or isolated from a cell or organism expressing them, which may be a wild type cell or organism or a recombinant cell or organism. In some embodiments, such polypeptides may be substantially isolated from the cell or organism in which they are expressed.

[0235] Any nutrient that can be assimilated by a host containing the cloned reverse transcriptase gene may be added to the culture medium. Optimal culture conditions should be selected case by case according to the strain used and the composition of the culture medium. Antibiotics may also be added to the growth media to insure maintenance of vector DNA containing the desired gene to be expressed. Media formulations have been described in DSM or ATCC Catalogs and Sambrook et al., In: *Molecular Cloning, a Laboratory Manual* (2nd ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

[0236] Recombinant host cells producing the reverse transcriptases of this invention can be separated from liquid culture, for example, by centrifugation. In general, the collected microbial cells are dispersed in a suitable buffer, and then broken open by ultrasonic treatment or by other well known procedures to allow extraction of the enzymes by the buffer solution. After removal of cell debris by ultracentrifugation. or centrifugation, the reverse transcriptases can be purified by standard protein purification techniques such as extraction, precipitation, chromatography, affinity chromatography, electrophoresis or the like. Assays to detect the presence of the reverse transcriptase during purification are well known in the art and can be used during conventional biochemical purification methods to determine the presence of these enzymes.

[0237] In some embodiments, reverse transcriptases of the present invention may be modified to contain an affinity tag in order to facilitate the purification of the reverse transcriptase. Suitable affinity tags are well known to those skilled in the art and include, but are not limited to, repeated sequences of amino acids such as six histidines, epitopes such as the hemagglutinin epitope and the myc epitope, and other amino acid sequences that permit the simplified purification of the reverse transcriptase.

[0238] The invention further relates to fusion proteins comprising (1) a protein, or fragment thereof, having one or more activity associated with a reverse transcriptase and (2) a tag (e.g., an affinity tag). In particular embodiments, the invention includes a reverse transcriptase (e.g., a thermostable reverse transcriptase) described herein having one or more (e.g., one, two, three, four, five, six, seven, eight, etc.) tags. These tags may be located, for example, (1) at the N-terminus, (2) at the C-terminus, or (3) at both the N-terminus and C-terminus of the protein, or a fragment thereof having one or more activities associated with a reverse

transcriptase. A tag may also be located internally (e.g., between regions of amino acid sequence derived from a reverse transcriptase and/or attached to an amino acid side chain). For example, Ferguson et al., *Protein Sci.* 7:1636-1638 (1998), describe a siderophore receptor, FhuA, from *Escherichia coli* into which an affinity tag (i.e., a hexahistidine sequence) was inserted. This tag was shown to function in purification protocols employing metal chelate affinity chromatography. Additional fusion proteins with internal tags are described in U.S. Pat. No. 6,143,524, the entire disclosure of which is incorporated herein by reference.

[**0239**] Tags used to prepare compositions of the invention may vary in length but will typically be from about 5 to about 500, from about 5 to about 100, from about 10 to about 100, from about 15 to about 100, from about 20 to about 100, from about 25 to about 100, from about 30 to about 100 from about 35 to about 100, from about 40 to about 100, from about 45 to about 100, from about 50 to about 100, from about 55 to about 100, from about 60 to about 100, from about 65 to about 100, from about 70 to about 100, from about 75 to about 100, from about 80 to about 100, from about 85 to about 100, from about 90 to about 100, from about 95 to about 100, from about 5 to about 80, from about 10 to about 80, from about 20 to about 80, from about 30 to about 80, from about 40 to about 80, from about 50 to about 80, from about 60 to about 80, from about 70 to about 80, from about 5 to about 60, from about 10 to about 60, from about 20 to about 60, from about 30 to about 60, from about 40 to about 60, from about 50 to about 60, from about 5 to about 40, from about 10 to about 40, from about 20 to about 40, from about 30 to about 40, from about 5 to about 30, from about 10 to about 30, from about 20 to about 30, from about 5 to about 25, from about 10 to about 25, or from about 15 to about 25 amino acid residues in length.

[**0240**] Tags used to prepare compositions of the invention include those which contribute to the thermostability of the fusion protein. Thus, these tags may be at least partly responsible, for example, for a particular protein (e.g., a protein having one or more activities of a reverse transcriptase activity) having increased thermostability. Examples of tags that enhance the thermostability of a reverse transcriptase (i.e., M-MLV reverse transcriptase) include, but are not limited to, tags having the following amino acid sequences: MGGSHHHHHHGMASMTGGQQMGRDLYDDDDKH, which corresponds to amino acids 1-32 of the sequence set forth in SEQ ID NO:2 and Table 3, and MASGTGGQQMGRDLYDDDDKH, (SEQ ID NO:3). Fragments of these tags may also be used in accordance with the invention (preferably those having 3 or more, 5 or more, 10 or more, or 15 or more amino acids) Thus, the invention includes, in part, reverse transcriptases, or fragments thereof that comprise tags and demonstrate enhanced thermostability. Using well known methods, one of skill in the art can attach one or more of above-mentioned tags to one or more RT enzymes, or fragments thereof having reverse transcriptase activity, to produce a thermostable RT enzyme or fragment thereof. Suitable RT enzymes include, but are not limited to, retroviral reverse transcriptase, retrotransposon reverse transcriptase, hepatitis B reverse transcriptase, cauliflower mosaic virus reverse transcriptase, bacterial reverse transcriptase, Tth DNA polymerase, Taq DNA polymerase (Saiki, R. K., et al., *Science* 239:487-491 (1988); U.S. Pat. Nos. 4,889,818 and 4,965,188), Tne DNA polymerase (PCT Publication No. WO 96/10640), Tma DNA polymerase (U.S. Pat. No. 5,374,553) and mutants, fragments, variants or derivatives thereof (see, e.g., commonly owned U.S. Pat. Nos. 5,948,614 and 6,015,668, which are incorporated by reference herein in their entireties). Reverse transcriptases for use in the invention also include retroviral reverse transcriptases such as M-MLV reverse transcriptase, AMV reverse transcriptase, RSV reverse transcriptase, RAV reverse transcriptase, MAV reverse transcriptase, and generally ASLV reverse transcriptases.

[**0241**] Tags used in the practice of the invention may serve any number of purposes and a number of tags may be added to impart one or more different functions to the reverse transcriptase of the invention. For example, tags may (1) contribute to protein-protein interactions both internally within a protein and with other protein molecules, (2) make the protein amenable to particular purification methods, (3) enable one to identify whether the protein is present in a composition; or (4) give the protein other functional characteristics.

[**0242**] Examples of tags which may be used in the practice of the invention include metal binding domains (e.g., a poly-histidine segments such as a three, four, five, six, or seven histidine region), immunoglobulin binding domains (e.g., (1) Protein A; (2) Protein G; (3) T cell, B cell, and/or Fc receptors; and/or (4) complement protein antibody-binding domain); sugar binding domains (e.g., a maltose binding domain, chitin-binding domain); and detectable domains (e.g., at least a portion of beta-galactosidase). Fusion proteins may contain one or more tags such as those described above. Typically, fusion proteins that contain more than one tag will contain these tags at one terminus or both termini (i.e., the N-terminus and the C-terminus) of the reverse transcriptase, although one or more tags may be located internally instead of or in addition to those present at termini. Further, more than one tag may be present at one terminus, internally and/or at both termini of the reverse transcriptase. For example, three consecutive tags could be linked end-to-end at the N-terminus of the reverse transcriptase. The invention further include compositions and reaction mixture which contain the above fusion proteins, as well as methods for preparing these fusion proteins, nucleic acid molecules (e.g., vectors) which encode these fusion proteins and recombinant host cells which contain these nucleic acid molecules. The invention also includes methods for using these fusion proteins as described elsewhere herein (e.g., methods for reverse transcribing nucleic acid molecules).

[**0243**] Tags which enable one to identify whether the fusion protein is present in a composition include, for example, tags which can be used to identify the protein in an electrophoretic gel. A number of such tags are known in the art and include epitopes and antibody binding domains which can be used for Western blots.

[**0244**] The amino acid composition of the tags for use in the present invention may vary. In some embodiments, a tag may contain from about 1% to about 5% amino acids that have a positive charge at physiological pH, e.g., lysine, arginine, and histidine, or from about 5% to about 10% amino acids that have a positive charge at physiological pH, or from about 10% to about 20% amino acids that have a positive charge at physiological pH, or from about 10% to

about 30% amino acids that have a positive charge at physiological pH, or from about 10% to about 50% amino acids that have a positive charge at physiological pH, or from about 10% to about 75% amino acids that have a positive charge at physiological pH. In some embodiments, a tag may contain from about 1% to about 5% amino acids that have a negative charge at physiological pH, e.g., aspartic acid and glutamic acid, or from about 5% to about 10% amino acids that have a negative charge at physiological pH, or from about 10% to about 20% amino acids that have a negative charge at physiological pH, or from about 10% to about 30% amino acids that have a negative charge at physiological pH, or from about 10% to about 50% amino acids that have a negative charge at physiological pH, or from about 10% to about 75% amino acids that have a negative charge at physiological pH. In some embodiments, a tag may comprise a sequence of amino acids that contains two or more contiguous charged amino acids that may be the same or different and may be of the same or different charge. For example, a tag may contain a series (e.g., two, three, four, five, six, ten etc.) of positively charged amino acids that may be the same or different. A tag may contain a series (e.g., two, three, four, five, six, ten etc.) of negatively charged amino acids that may be the same or different. In some embodiments, a tag may contain a series (e.g., two, three, four, five, six, ten etc.) of alternating positively charged and negatively charged amino acids that may be the same or different (e.g., positive, negative, positive, negative, etc.). Any of the above-described series of amino acids (e.g., positively charged, negatively charged or alternating charge) may comprise one or more neutral polar or non-polar amino acids (e.g., two, three, four, five, six, ten etc.) spaced between the charged amino acids. Such neutral amino acids may be evenly distributed through out the series of charged amino acids (e.g., charged, neutral, charged, neutral) or may be unevenly distributed throughout the series (e.g., charged, a plurality of neutral, charged, neutral, a plurality of charged, etc.). In some embodiments, tags to be attached to the polypeptides of the invention may have an overall charge at physiological pH (e.g., positive charge or negative charge). The size of the overall charge may vary, for example, the tag may contain a net plus one, two, three, four, five, etc. or may possess a net negative one, two, three, four, five, etc. The present invention also relates to reverse transcriptases (e.g., thermostable reverse transcriptases) comprising one or more of the above-described tag sequences, vectors encoding such reverse transcriptases, host cells reaction mixture, compositions and reaction mixtures comprising such reverse transcriptases, as well as kits comprising containers containing such reverse transcriptases.

[0245] In some embodiments, it may be desirable to remove all or a portion of a tag sequence from a fusion protein comprising a tag sequence and a sequence having reverse transcriptase (RT) activity. In embodiments of this type, one or more amino acids forming a cleavage site, e.g., for a protease enzyme, may be incorporated into the primary sequence of the fusion protein. The cleavage site may be located such that cleavage at the site may remove all or a portion of the tag sequence from the fusion protein. In some embodiments, the cleavage site may be located between the tag sequence and the sequence having RT activity such that all of the tag sequence is removed by cleavage with a protease enzyme that recognizes the cleavage site. Examples of suitable cleavage sites include, but are not limited to, the Factor Xa cleavage site having the sequence Ile-Glu-Gly-Arg (SEQ ID NO:4), which is recognized and cleaved by blood coagulation factor Xa, and the thrombin cleavage site having the sequence Leu-Val-Pro-Arg (SEQ ID NO:5), which is recognized and cleaved by thrombin. Other suitable cleavage sites are known to those skilled in the art and may be used in conjunction with the present invention.

[0246] The reverse transcriptases of the invention preferably have specific activities (e.g., RNA-directed DNA polymerase activity and/or RNase H activity) greater than about 5 units/mg, more preferably greater than about 50 units/mg, still more preferably greater than about 100 units/mg, 250 units/mg, 500 units/mg, 1000 units/mg, 5000 units/mg or 10,000 units/mg, and most preferably greater than about 15,000 units/mg, greater than about 16,000 units/mg, greater than about 17,000 units/mg, greater than about 18,000 units/mg, greater than about 19,000 units/mg and greater than about 20,000 units/mg. In some embodiments, the reverse transcriptases of the present invention may have specific activities greater than about 50,000 units/mg, greater than about 100,000 units/mg, greater than about 150,000 units/mg, greater than about 200,000 units/mg, greater than about 250,000 units/mg and greater than about 300,000 units/mg. Preferred ranges of specific activities for the reverse transcriptases of the invention include a specific activity from about 5 units/mg to about 750,000 units/mg, a specific activity from about 5 units/mg to about 500,000 units/mg, from about 5 units/mg to about 300,000 units/mg, a specific activity of from about 50 units/mg to about 750,000 units/mg, a specific activity from about 100 units/mg to about 750,000 units/mg, a specific activity from about 250 units/mg to about 750,000 units/mg, a specific activity from about 500 units/mg to about 750,000 units/mg, a specific activity from about 1000 units/mg to about 750,000 units/mg, a specific activity from about 5000 units/mg to about 750,000 units/mg, a specific activity from about 10,000 units/mg to about 750,000 units/mg, a specific activity from about 25,000 units/mg to about 750,000 units/mg, a specific activity from about 100 units/mg to about 500 units/mg, a specific activity from about 100 units/mg to about 400 units/mg, and a specific activity from about 200 units/mg to about 500 units/mg. Other preferred ranges of specific activities include a specific activity of from about 200,000 units/mg to about 350,000 units/mg, a specific activity from about 225,000 units/mg to about 300,000 units/mg, a specific activity from about 250,000 units/mg to about 300,000 units/mg, a specific activity of from about 200,000 units/mg to about 750,000 units/mg, a specific activity of from about 200,000 units/mg to about 500,000 units/mg, a specific activity of from about 200,000 units/mg to about 400,000 units/mg, a specific activity of from about 250,000 units/mg to about 750,000 units/mg, a specific activity of from about 250,000 units/mg to about 500,000 units/mg, and a specific activity of from about 250,000 units/mg to about 400,000 units/mg. Preferably, the lower end of the specific activity range may vary from 50, 100, 200, 300, 400, 500, 700, 900, 1,000, 5,000, 10,000, 20,000, 30,000, 35,000, 40,000, 45,000, 50,000, 55,000, 60,000, 65,000, 70,000, 75,000, and 80,000 units/mg, while the upper end of the range may vary from 750,000, 650,000, 600,000, 550,000, 500,000, 450,000, 400,000, 350,000, 300,000, 250,000, 200,000, 150,000, 140,000, 130,000, 120,000, 110,000, 100,000, and 90,000 units/mg. Specific activity may be determined using enzyme assays and protein assays

well known in the art. DNA polymerase assays and RNase H assays are described, for example, in U.S. Pat. No. 5,244,797 and WO 98/47912. In some embodiments of the present invention, the specific activity of the thermostable reverse transcriptase prepared in accordance with the present invention may be higher than the specific activity of a non-thermostable reverse transcriptase. In some embodiments, the specific activity of the thermostable reverse transcriptase may be 5%, 10,%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or more higher than the specific activity of a corresponding non-thermostable reverse transcriptase. In some preferred embodiments, the specific activity of the thermostable reverse transcriptase according to the present invention may be 30% or more higher than the specific activity of a corresponding non-thermostable reverse transcriptase. In accordance with the invention, specific activity is a measurement of the enzymatic activity (in units) of the protein or enzyme relative to the total amount of protein or enzyme used in a reaction. The measurement of a specific activity may be determined by standard techniques well-known to one of ordinary skill in the art.

[0247] The reverse transcriptases of the invention may be used to make nucleic acid molecules from one or more templates. Such methods comprise mixing one or more nucleic acid templates (e.g., mRNA, and more preferably a population of mRNA molecules) with one or more of the reverse transcriptases of the invention and incubating the mixture under conditions sufficient to make one or more nucleic acid molecules complementary to all or a portion of the one or more nucleic acid templates.

[0248] The invention also relates to methods for the amplification of one or more nucleic acid molecules comprising mixing one or more nucleic acid templates with one of the reverse transcriptases of the invention, and incubating the mixture under conditions sufficient to amplify one or more nucleic acid molecules complementary to all or a portion of the one or more nucleic acid templates. Such amplification methods may further comprise the use of one or more DNA polymerases and may be employed as in standard RT-PCR reactions.

[0249] The invention also concerns methods for the sequencing of one or more nucleic acid molecules comprising (a) mixing one or more nucleic acid molecules to be sequenced with one or more primer nucleic acid molecules, one or more reverse transcriptases of the invention, one or more nucleotides and one or more terminating agents; (b) incubating the mixture under conditions sufficient to synthesize a population of nucleic acid molecules complementary to all or a portion of the one or more nucleic acid molecules to be sequenced; and (c) separating the population of nucleic acid molecules to determine the nucleotide sequence of all or a portion of the one or more nucleic acid molecules to be sequenced.

[0250] The invention also concerns nucleic acid molecules produced by such methods (which may be full-length cDNA molecules), vectors (particularly expression vectors) comprising these nucleic acid molecules and host cells comprising these vectors and nucleic acid molecules.

[0251] Sources of DNA Polymerase

[0252] A variety of DNA polymerases are useful in accordance with the present invention. Such polymerases include, but are not limited to, *Thermus thermophilus* (Tth) DNA polyrnerase, *Thermus aquaticus* (Taq) DNA polymerase, *Thermotoga neapolitana* (Tne) DNA polymerase, *Thermotoga maritima* (Tma) DNA polymerase, *Thermococcus litoralis* (Tli or VENT™) DNA polymerase, *Thermococcus kodakaraensis* KOD1 DNA Polymerase, *Pyrococcus furiosis* (Pfu) DNA polymerase, *Pyrococcus* species GB-D (DEEPVENT™) DNA polymerase, *Pyrococcus woosii* (Pwo) DNA polymerase, *Bacillus sterothermophilus* (Bst) DNA polymerase, *Bacillus caldophilus* (Bca) DNA polymerase, *Sulfolobus acidocaldarius* (Sac) DNA polymerase, *Thermoplasma acidophilum* (Tac) DNA polymerase, *Thermus flavus* (Tfl/Tub) DNA polymerase, *Thermus ruber* (Tru) DNA polymerase, *Thermus brockianus* (DYNAZYME™) DNA polymerase, *Methanobacterium thermoautotrophicum* (Mth) DNA polymerase, *Mycobacterium* spp. DNA polymerase (Mtb, Mlep), and mutants, variants and derivatives thereof.

[0253] DNA polymerases used in accordance with the invention may be any enzyme that can synthesize a DNA molecule from a nucleic acid template, typically in the 5' to 3' direction. Such polymerases may be mesophilic or thermophilic, but are preferably thermophilic. Mesophilic polymerases include T5 DNA polymerase, T7 DNA polymerase, Klenow fragment DNA polymerase, DNA polymerase III, and the like. Preferred DNA polymerases are thermostable DNA polymerases such as Taq, Tne, Tma, Pfu, VENT™, DEEPVENT™, Tth and mutants, variants and derivatives thereof (U.S. Pat. No. 5,436,149; U.S. Pat. No. 5,512,462; PCT Publication No. WO 92/06188; PCT Publication No. WO 92/06200; PCT Publication No. WO 96/10640; Barnes, W. M., *Gene* 112:29-35 (1992); Lawyer, F. C., et al., *PCR Meth. Appl.* 2:275-287 (1993); Flaman, J.-M., et al., *Nucl. Acids Res.* 22(15):3259-3260 (1994)). For amplification of long nucleic acid molecules (e.g., nucleic acid molecules longer than about 3-5 Kb in length), at least two DNA polymerases (one substantially lacking 3' exonuclease activity and the other having 3' exonuclease activity) are typically used. See U.S. Pat. No. 5,436,149; U.S. Pat. No. 5,512,462; Barnes, W. M., *Gene* 112:29-35 (1992); PCT Publication No. WO 98/06736; and commonly owned, co-pending U.S. patent application Ser. No. 08/801,720, filed Feb. 14, 1997, the disclosures of all of which are incorporated herein in their entireties. Examples of DNA polymerases substantially lacking in 3' exonuclease activity include, but are not limited to, Taq, Tne(exo$^-$), Tma, Pfu(exo$^-$), Pwo and Tth DNA polymerases, and mutants, variants and derivatives thereof. Nonlimiting examples of DNA polymerases having 3' exonuclease activity include Pfu, DEEPVENT™ and Tli/VENT™ and mutants, variants and derivatives thereof.

[0254] Formulation of Compositions and Reaction Mixtures

[0255] To form the compositions of the present invention, one or more reverse transcriptases are preferably admixed in a buffered salt solution. One or more DNA polymerases and/or one or more nucleotides, and/or one or more primers may optionally be added to make the compositions of the invention. More preferably, the enzymes are provided at working concentrations in stable buffered salt solutions. The terms "stable" and "stability" as used herein generally mean the retention by a composition, such as an enzyme composition, of at least 70%, preferably at least 80%, and most preferably at least 90%, of the original enzymatic activity (in

units) after the enzyme or composition containing the enzyme has been stored for about one week at a temperature of about 4° C., about two to six months at a temperature of about −20° C., and about six months or longer at a temperature of about −80° C. As used herein, the term "working concentration" means the concentration of an enzyme that is at or near the optimal concentration used in a solution to perform a particular function (such as reverse transcription of nucleic acids).

[0256] The water used in forming the compositions of the present invention is preferably distilled, deionized and sterile filtered (through a 0.1-0.2 micrometer filter), and is free of contamination by DNase and RNase enzymes. Such water is available commercially, for example from Sigma Chemical Company (Saint Louis, Mo.), or may be made as needed according to methods well known to those skilled in the art.

[0257] In addition to the enzyme components, the present compositions preferably comprise one or more buffers and cofactors necessary for synthesis of a nucleic acid molecule such as a cDNA molecule. Particularly preferred buffers for use in forming the present compositions are the acetate, sulfate, hydrochloride, phosphate or free acid forms of Tris-(hydroxymethyl)aminomethane (TRIS®), although alternative buffers of the same approximate ionic strength and pKa as TRIS® may be used with equivalent results. In addition to the buffer salts, cofactor salts such as those of potassium (preferably potassium chloride or potassium acetate) and magnesium (preferably magnesium chloride or magnesium acetate) are included in the compositions. Addition of one or more carbohydrates and/or sugars to the compositions and/or synthesis reaction mixtures may also be advantageous, to support enhanced stability of the compositions and/or reaction mixtures upon storage. Preferred such carbohydrates or sugars for inclusion in the compositions and/or synthesis reaction mixtures of the invention include, but are not limited to, sucrose, trehalose, glycerol, and the like. Furthermore, such carbohydrates and/or sugars may be added to the storage buffers for the enzymes used in the production of the enzyme compositions and kits of the invention. Such carbohydrates and/or sugars are commercially available from a number of sources, including Sigma (St. Louis, Mo.).

[0258] It is often preferable to first dissolve the buffer salts, cofactor salts and carbohydrates or sugars at working concentrations in water and to adjust the pH of the solution prior to addition of the enzymes. In this way, the pH-sensitive enzymes will be less subject to acid- or alkaline-mediated inactivation during formulation of the present compositions.

[0259] To formulate the buffered salts solution, a buffer salt which is preferably a salt of Tris(hydroxymethyl)aminomethane (TRIS®), and most preferably the hydrochloride salt thereof, is combined with a sufficient quantity of water to yield a solution having a TRIS® concentration of 5-150 millimolar, preferably 10-60 millimolar, and most preferably about 20-60 millimolar. To this solution, a salt of magnesium (preferably either the chloride or acetate salt thereof) or other divalent cation, may be added to provide a working concentration thereof of 1-10 millimolar, preferably 1.5-8.0 millimolar, and most preferably about 3-7.5 millimolar. A salt of potassium (preferably a chloride or acetate salt of potassium), or other monovalent cation (e.g., Na), may also be added to the solution, at a working concentration of 10-100 millimolar and most preferably about 75 millimolar. A reducing agent, such as dithiothreitol, may be added to the solution, preferably at a final concentration of about 1-100 mM, more preferably a concentration of about 5-50 mM or about 7.5-20 mM, and most preferably at a concentration of about 10 mM. Preferred concentrations of carbohydrates and/or sugars for inclusion in the compositions of the invention range from about 5% (w/v) to about 30% (w/v), from about 7.5% (w/v) to about 25% (w/v), from about 10% (w/v) to about 25% (w/v), from about 10% (w/v) to about 20% (w/v), and preferably from about 10% (w/v) to about 15% (w/v). A small amount of a salt of ethylenediaminetetraacetate (EDTA), such as disodium EDTA, may also be added (preferably about 0.1 millimolar), although inclusion of EDTA does not appear to be essential to the function or stability of the compositions of the present invention. After addition of all buffers and salts, this buffered salt solution is mixed well until all salts are dissolved, and the pH is adjusted using methods known in the art to a pH value of from about 7.4 to about 9.2, preferably from about 8.0 to about 9.0, and most preferably to about 8.4.

[0260] To these buffered salt solutions, the enzymes (reverse transcriptases and/or DNA polymerases) are added to produce the compositions of the present invention. M-MLV reverse transcriptases are preferably added at a working concentration in the solution of from about 1,000 to about 50,000 units per milliliter, from about 2,000 to about 30,000 units per milliliter, from about 2,500 to about 25,000 units per milliliter, from about 3,000 to about 22,500 units per milliliter, from about 4,000 to about 20,000 units per milliliter, and most preferably at a working concentration of from about 5,000 to about 20,000 units per milliliter. In some embodiments, a reverse transcriptases of the invention (e.g., an M-MLV reverse transcriptase) may be added at a working concentration described above in a first strand reaction mixture (e.g., a reaction to reverse transcribe an mRNA molecule) and/or in a couple RT/PCR. A suitable concentration of a reverse transcriptase of the invention for these reactions may be from about 5,000 units/ml to about 50,000 units/ml, from about 5,000 units/ml to about 40,000 units/ml, from about 5,000 units/ml to about 30,000 units/ml, or from about 5,000 units/ml to about 20,000 units/ml of reverse transcriptase. A reaction may be conducted in a volume of 20 μl to 50 μl and such a reaction may contain 50 units, 100, units, 200 units, 300 units, 400 units or more of a reverse transcriptase of the invention. Those skilled in the art will appreciate that adding additional reverse transcriptase may allow increased synthesis of the first strand (e.g., the DNA strand complementary to the mRNA strand) at the expense of increased enzyme usage. The skilled artisan can determine without undue experimentation the amount of a reverse transcriptase of the invention to add to a reaction in order to produce a desired amount of product at an acceptable expense.

[0261] AMV reverse transcriptases, RSV reverse transcriptases and HIV reverse transcriptases, including those of the invention described above, are preferably added at a working concentration in the solution of from about 100 to about 5000 units per milliliter, from about 125 to about 4000 units per milliliter, from about 150 to about 3000 units per milliliter, from about 200 to about 2500 units per milliliter, from about 225 to about 2000 units per milliliter, and most preferably at a working concentration of from about 250 to

about 1000 units per milliliter. The enzymes in the thermophilic DNA polymerase group (Taq, Tne, Tma, Pfu, VENT, DEEPVENT, Tth and mutants, variants and derivatives thereof) are preferably added at a working concentration in the solution of from about 100 to about 1000 units per milliliter, from about 125 to about 750 units per milliliter, from about 150 to about 700 units per milliliter, from about 200 to about 650 units per milliliter, from about 225 to about 550 units per milliliter, and most preferably at a working concentration of from about 250 to about 500 units per milliliter. The enzymes may be added to the solution in any order, or may be added simultaneously.

[0262] The compositions of the invention may further comprise one or more nucleotides, which are preferably deoxynucleoside triphosphates (dNTPs) or dideoxynucleoside triphosphates (ddNTPs). The dNTP components of the present compositions serve as the "building blocks" for newly synthesized nucleic acids, being incorporated therein by the action of the polymerases, and the ddNTPs may be used in sequencing methods according to the invention. Examples of nucleotides suitable for use in the present compositions include, but are not limited to, dUTP, dATP, dTTP, dCTP, dGTP, dITP, 7-deaza-dGTP, α-thio-dATP, α-thio-dTTP, α-thio-dGTP, α-thio-dCTP, ddUTP, ddATP, ddTTP, ddCTP, ddGTP, ddITP, 7-deaza-ddGTP, α-thio-ddATP, α-thio-ddTTP, α-thio-ddGTP, α-thio-ddCTP or derivatives thereof, all of which are available commercially from sources including Invitrogen Corporation (Carlsbad, Calif.), New England BioLabs (Beverly, Mass.) and Sigma Chemical Company (Saint Louis, Mo.). The nucleotides may be unlabeled, or they may be detectably labeled by coupling them by methods known in the art with radioisotopes (e.g., ^{3}H, ^{14}C, ^{32}P or ^{35}S), vitamins (e.g., biotin), fluorescent moieties (e.g., fluorescein, rhodamine, Texas Red, or phycoerythrin), chemiluminescent labels (e.g., using the PHOTO-GENE™ or ACES™ chemiluminescence systems, available commercially from Invitrogen Corporation (Carlsbad, Calif.)), dioxigenin and the like. Labeled nucleotides may also be obtained commercially, for example from Invitrogen Corporation (Carlsbad, Calif.) or Sigma Chemical Company (Saint Louis, Mo.). In the present compositions, the nucleotides are added to give a working concentration of each nucleotide of about 10-4000 micromolar, about 50-2000 micromolar, about 100-1500 micromolar, or about 200-1200 micromolar, and most preferably a concentration of about 1000 micromolar.

[0263] To reduce component deterioration, storage of the reagent compositions is preferably at about 4° C. for up to one day, or most preferably at −20° C. for up to one year.

[0264] In another aspect, the compositions and reverse transcriptases of the invention may be prepared and stored in dry form in the presence of one or more carbohydrates, sugars, or synthetic polymers. Preferred carbohydrates, sugars or polymers for the preparation of dried compositions or reverse transcriptases include, but are not limited to, sucrose, trehalose, and polyvinylpyrrolidone (PVP) or combinations thereof. See, e.g., U.S. Pat. Nos. 5,098,893, 4,891,319, and 5,556,771, the disclosures of which are entirely incorporated herein by reference. Such dried compositions and enzymes may be stored at various temperatures for extended times without significant deterioration of enzymes or components of the compositions of the invention. Preferably, the dried reverse transcriptases or compositions are stored at 4° C. or at −20° C.

[0265] The invention further includes reaction solutions for reverse transcribing nucleic acid molecules, as well as reverse transcription methods employing such reaction solutions and product nucleic acid molecules produced using such methods. In many instances, reaction solutions of the invention will contain one or more of the following components: (1) one or more buffering agent (e.g., sodium phosphate, sodium acetate, 2-(N-moropholino)-ethanesulfonic acid (MES), tris-(hydroxymethyl)aminomethane (Tris), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPS), citrate, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), acetate, 3-(N-morpholino-)prpoanesulfonic acid (MOPS), N-tris(hydroxymethyl)methyl-3-aminopropanesulfonio acid (TAPS), etc.), (2) one or more monovalent cationic salt (e.g., NaCl, KCl, etc.), (3) one or more divalent cationic salt (e.g., $MnCl_2$, $MgCl_2$, $MgSO_4$, $CaCl_2$, etc.), (4) one or more reducing agent (e.g., dithiothreitol, β-mercaptoethanol, etc.), (5) one or more ioninc or non-ionic detergent (e.g., TRITON X-100™, NONIDET P40™, sodium dodecyl sulphate, etc.), (6) one or more DNA polymerase inhibitor (e.g., Actinomycin D, etc.), (7) nucleotides (e.g., dNTPs, such as dGTP, dATP, dCTP, dTTP, etc.), (8) RNA to be reverse transcribed and/or amplified, (9) one or more RNase inhibitor (e.g., RNASE-OUT™, Invitrogen Corporation, Carlsbad, Calif., catalog number 10777-019 etc.), (10) a reverse transcriptase (e.g., a reverse transcriptase of the invention, and/or (11) one or more diluent (e.g., water). Other components and/or constituents (e.g., primers, DNA polymerases, etc.) may also be present in reaction solutions.

[0266] The concentration of the buffering agent in the reaction solutions of the invention will vary with the particular buffering agent used. Typically, the working concentration (i.e., the concentration in the reaction mixture) of the buffering agent will be from about 5 mM to about 500 mM (e.g., about 10 mM, about 15 mM, about 20 mM, about 25 mM, about 30 mM, about 35 mM, about 40 mM, about 45 mM, about 50 mM, about 55 mM, about 60 mM, about 65 mM, about 70 mM, about 75 mM, about 80 mM, about 85 mM, about 90 mM, about 95 mM, about 100 mM, from about 5 mM to about 500 mM, from about 10 mM to about 500 mM, from about 20 mM to about 500 mM, from about 25 mM to about 500 mM, from about 30 mM to about 500 mM, from about 40 mM to about 500 mM, from about 50 mM to about 500 mM, from about 75 mM to about 500 mM, from about 100 mM to about 500 mM, from about 25 mM to about 50 mM, from about 25 mM to about 75 mM, from about 25 mM to about 100 mM, from about 25 mM to about 200 mM, from about 25 mM to about 300 mM, etc.). When Tris (e.g., Tris-HCl) is used, the Tris working concentration will typically be from about 5 mM to about 100 mM, from about 5 mM to about 75 mM, from about 10: mM to about 75 mM, from about 10 mM to about 60 mM, from about 10 mM to about 50 mM, from about 25 mM to about 50 mM, etc.

[0267] The final pH of solutions of the invention will generally be set and maintained by buffering agents present in reaction solutions of the invention. The pH of reaction solutions of the invention, and hence reaction mixtures of the invention, will vary with the particular use and the

buffering agent present but will often be from about pH 5.5 to about pH 9.0 (e.g., about pH 6.0, about pH 6.5, about pH 7.0, about pH 7.1, about pH 7.2, about pH 7.3, about pH 7.4, about pH 7.5, about pH 7.6, about pH 7.7, about pH 7.8, about pH 7.9, about pH 8.0, about pH 8.1, about pH 8.2, about pH 8.3, about pH 8.4, about pH 8.5, about pH 8.6, about pH 8.7, about pH 8.8, about pH 8.9, about pH 9.0, from about pH 6.0 to about pH 8.5, from about pH 6.5 to about pH 8.5, from about pH 7.0 to about pH 8.5, from about pH 7.5 to about pH 8.5, from about pH 6.0 to about pH 8.0, from about pH 6.0 to about pH 7.7, from about pH 6.0 to about pH 7.5, from about pH 6.0 to about pH 7.0, from about pH 7.2 to about pH 7.7, from about pH 7.3 to about pH 7.7, from about pH 7.4 to about pH 7.6, from about pH 7.0 to about pH 7.4, from about pH 7.6 to about pH 8.0, from about pH 7.6 to about pH 8.5, from about pH 7.7 to about pH 8.5, from about pH 7.9 to about pH 8.5, from about pH 8.0 to about pH 8.5, from about pH 8.2 to about pH 8.5, from about pH 8.3 to about pH 8.5, from about pH 8.4 to about pH 8.5, from about pH 8.4 to about pH 9.0, from about pH 8.5 to about pH 9.0, etc.)

[0268] As indicated, one or more monovalent cationic salts (e.g., NaCl, KCl, etc.) may be included in reaction solutions of the invention. In many instances, salts used in reaction solutions of the invention will dissociate in solution to generate at least one species which is monovalent (e.g., Na+, K+, etc.) When included in reaction solutions of the invention, salts will often be present either individually or in a combined concentration of from about 0.5 mM to about 500 mM (e.g., about 1 mM, about 2 mM, about 3 mM, about 5 mM, about 10 mM, about 12 mM, about 15 mM, about 17 mM, about 20 mM, about 22 mM, about 23 mM, about 24 mM, about 25 mM, about 27 mM, about 30 mM, about 35 mM, about 40 mM, about 45 mM, about 50 mM, about 55 mM, about 60 mM, about 64 mM, about 65 mM, about 70 mM, about 75 mM, about 80 mM, about 85 mM, about 90 mM, about 95 mM, about 100 mM, about 120 mM, about 140 mM, about 150 mM, about 175 mM, about 200 mM, about 225 mM, about 250 mM, about 275 mM, about 300 mM, about 325 mM, about 350 mM, about 375 mM, about 400 mM, from about 1 mM to about 500 mM, from about 5 mM to about 500 mM, from about 10 mM to about 500 mM, from about 20 mM to about 500 mM, from about 30 mM to about 500 mM, from about 40 mM to about 500 mM, from about 50 mM to about 500 mM, from about 60 mM to about 500 mM, from about 65 mM to about 500 mM, from about 75 mM to about 500 mM, from about 85 mM to about 500 mM, from about 90 mM to about 500 mM, from about 100 mM to about 500 mM, from about 125 mM to about 500 mM, from about 150 mM to about 500 mM, from about 200 mM to about 500 mM, from about 10 mM to about 100 mM, from about 10 mM to about 75 mM, from about 10 mM to about 50 mM, from about 20 mM to about 200 mM, from about 20 mM to about 150 mM, from about 20 mM to about 125 mM, from about 20 mM to about 100 mM, from about 20 mM to about 80 mM, from about 20 mM to about 75 mM, from about 20 mM to about 60 mM, from about 20 mM to about 50 mM, from about 30 mM to about 500 mM, from about 30 mM to about 100 mM, from about 30 mM to about 70 mM, from about 30 mM to about 50 mM, etc.).

[0269] As indicated, one or more divalent cationic salts (e.g., $MnCl_2$, $MgCl_2$, $MgSO_4$, $CaCl_2$, etc.) may be included in reaction solutions of the invention. In many instances, salts used in reaction solutions of the invention will dissociate in solution to generate at least one species which is monovalent (e.g., Mg^{++}, Mn^{++}, Ca^{++}, etc.) When included in reaction solutions of the invention, salts will often be present either individually or in a combined concentration of from about 0.5 mM to about 500 mM (e.g., about 1 mM, about 2 mM, about 3 mM, about 4 mM, about 5 mM, about 6 mM, about 7 mM, about 8 mM, about 9 mM, about 10 mM, about 12 mM, about 15 mM, about 17 mM, about 20 mM, about 22 mM, about 23 mM, about 24 mM, about 25 mM, about 27 mM, about 30 mM, about 35 mM, about 40 mM, about 45 mM, about 50 mM, about 55 mM, about 60 mM, about 64 mM, about 65 mM, about 70 mM, about 75 mM, about 80 mM, about 85 mM, about 90 mM, about 95 mM, about 100 mM, about 120 mM, about 140 mM, about 150 mM, about 175 mM, about 200 mM, about 225 mM, about 250 mM, about 275 mM, about 300 mM, about 325 mM, about 350 mM, about 375 mM, about 400 mM, from about 1 mM to about 500 mM, from about 5 mM to about 500 mM, from about 10 mM to about 500 mM, from about 20 mM to about 500 mM, from about 30 mM to about 500 mM, from about 40 mM to about 500 mM, from about 50 mM to about 500 mM, from about 60 mM to about 500 mM, from about 65 mM to about 500 mM, from about 75 mM to about 500 mM, from about 85 mM to about 500 mM, from about 90 mM to about 500 mM, from about 100 mM to about 500 mM, from about 125 mM to about 500 mM, from about 150 mM to about 500 mM, from about 200 mM to about 500 mM, from about 10 mM to about 100 mM, from about 10 mM to about 75 mM, from about 10 mM to about 50 mM, from about 20 mM to about 200 mM, from about 20 mM to about 150 mM, from about 20 mM to about 125 mM, from about 20 mM to about 100 mM, from about 20 mM to about 80 mM, from about 20 mM to about 75 mM, from about 20 mM to about 60 mM, from about 20 mM to about 50 mM, from about 30 mM to about 500 mM, from about 30 mM to about 100 mM, from about 30 mM to about 70 mM, from about 30 mM to about 50 mM, etc.).

[0270] When included in reaction solutions of the invention, reducing agents (e.g., dithiothreitol, β-mercaptoethanol, etc.) will often be present either individually or in a combined concentration of from about 0.1 mM to about 50 mM (e.g., about 0.2 mM, about 0.3 mM, about 0.5 mM, about 0.7 mM, about 0.9 mM, about 1 mM, about 2 mM, about 3 mM, about 4 mM, about 5 mM, about 6 mM, about 10 mM, about 12 mM, about 15 mM, about 17 mM, about 20 mM, about 22 mM, about 23 mM, about 24 mM, about 25 mM, about 27 mM, about 30 mM, about 35 mM, about 40 mM, about 45 mM, about 50 mM, from about 0.1 mM to about 50 mM, from about 0.5 mM to about 50 mM, from about 1 mM to about 50 mM, from about 2 mM to about 50 mM, from about 3 mM to about 50 mM, from about 0.5 mM to about 20 mM, from about 0.5 mM to about 10 mM, from about 0.5 mM to about 5 mM, from about 0.5 mM to about 2.5 mM, from about 1 mM to about 20 mM, from about 1 mM to about 10 mM, from about 1 mM to about 5 mM, from about 1 mM to about 3.4 mM, from about 0.5 mM to about 3.0 mM, from about 1 mM to about 3.0 mM, from about 1.5 mM to about 3.0 mM, from about 2 mM to about 3.0 mM, from about 0.5 mM to about 2.5 mM, from about 1 mM to about 2.5 mM, from about 1.5 mM to about 2.5 mM, from about 2 mM to about 3.0 mM, from about 2.5 mM to about 3.0 mM, from about 0.5 mM to about 2 mM, from about 0.5 mM to about 1.5 mM, from about 0.5 mM to about 1.1 mM, from about 5.0 mM to about 10 mM, from about 5.0 mM to

about 15 mM, from about 5.0 mM to about 20 mM, from about 10 mM to about 15 mM, from about 10 mM to about 20 mM, etc.).

[0271] Reaction solutions of the invention may also contain one or more ioninc or non-ionic detergent (e.g., TRITON X-100υ, NONIDET P40™, sodium dodecyl sulphate, etc.). When included in reaction solutions of the invention, detergents will often be present either individually or in a combined concentration of from about 0.01% to about 5.0% (e.g., about 0.01%, about 0.02%, about 0.03%, about 0.04%, about 0.05%, about 0.06%, about 0.07%, about 0.08%, about 0.09%, about 0.1%, about 0.15%, about 0.2%, about 0.3%, about 0.5%, about 0.7%, about 0.9%, about 1%, about 2%, about 3%, about 4%, about 5%, from about 0.01% to about 5.0%, from about 0.01% to about 4.0%, from about 0.01% to about 3.0%, from about 0.01% to about 2.0%, from about 0.01% to about 1.0%, from about 0.05% to about 5.0%, from about 0.05% to about 3.0%, from about 0.05% to about 2.0%, from about 0.05% to about 1.0%, from about 0.1% to about 5.0%, from about 0.1% to about 4.0%, from about 0.1% to about 3.0%, from about 0.1% to about 2.0%, from about 0.1% to about 1.0%, from about 0.1% to about 0.5%, etc.). For example, reaction solutions of the invention may contain TRITON X-100™ at a concentration of from about 0.01% to about 2.0%, from about 0.03% to about 1.0%, from about 0.04% to about 1.0%, from about 0.05% to about 0.5%, from about 0.04% to about 0.6%, from about 0.04% to about 0.3%, etc.

[0272] Reaction solutions of the invention may also contain one or more DNA polymerase inhibitor (e.g., Actinomycin D, etc.). When included in reaction solutions of the invention, such inhibitors will often be present either individually or in a combined concentration of from about 0.1 μg/ml to about 100 μg/ml (e.g., about 0.1 μg/ml, about 0.2 μg/ml, about 0.3 μg/ml, about 0.4 μg/ml, about 0.5 μg/ml, about 0.6 μg/ml, about 0.7 μg/ml, about 0.8 μg/ml, about 0.9 μg/ml, about 1.0 μg/ml, about 1.1 μg/ml, about 1.3 μg/ml, about 1.5 μg/ml, about 1.7 μg/ml, about 2.0 μg/ml, about 2.5 μg/ml, about 3.5 μg/ml, about 5.0 μg/ml, about 7.5 μg/ml, about 10 μg/ml, about 15 μg/ml, about 20 μg/ml, about 25 μg/ml, about 30 μg/ml, about 35 μg/ml, about 40 μg/ml, about 50 μg/ml, about 60 μg/ml, about 70 μg/ml, about 80 μg/ml, about 90 μg/ml, about 100 μg/ml, from about 0.5 μg/ml to about 30 μg/ml, from about 0.75 μg/ml to about 30 μg/ml, from about 1.0 μg/ml to about 30 μg/ml, from about 2.0 μg/ml to about 30 μg/ml, from about 3.0 μg/ml to about 30 μg/ml, from about 4.0 μg/ml to about 30 μg/ml, from about 5.0 μg/ml to about 30 μg/ml, from about 7.5 μg/ml to about 30 μg/ml, from about 10 μg/ml to about 30 μg/ml, from about 15 μg/ml to about 30 μg/ml, from about 0.5 μg/ml to about 20 μg/ml, from about 0.5 μg/ml to about 10 μg/ml, from about 0.5 μg/ml to about 5 μg/ml, from about 0.5 μg/ml to about 2 μg/ml, from about 0.5 μg/ml to about 1 μg/ml, from about 1 μg/ml to about 10 μg/ml, from about 1 μg/ml to about 5 μg/ml, from about 1 μg/ml to about 2 μg/ml, from about 1 μg/ml to about 100 μg/ml, from about 10 μg/ml to about 100 μg/ml, from about 20 μg/ml to about 100 μg/ml, from about 40 μg/ml to about 100 μg/ml, from about 30 μg/ml to about 80 μg/ml, from about 30 μg/ml to about 70 μg/ml, from about 40 μg/ml to about 60 μg/ml, from about 40 μg/ml to about 70 μg/ml, from about 40 μg/ml to about 80 μg/ml, etc.).

[0273] In many instances, nucleotides (e.g., dNTPs, such as dGTP, dATP, dCTP, dTTP, etc.) will be present in reaction mixtures of the invention. Typically, individually nucleotides will be present in concentrations of from about 0.05 mM to about 50 mM (e.g., about 0.07 mM, about 0.1 mM, about 0.15 mM, about 0.18 mM, about 0.2 mM, about 0.3 mM, about 0.5 mM, about 0.7 mM, about 0.9 mM, about 1 mM, about 2 mM, about 3 mM, about 4 mM, about 5 mM, about 6 mM, about 10 mM, about 12 mM, about 15 mM, about 17 mM, about 20 mM, about 22 mM, about 23 mM, about 24 mM, about 25 mM, about 27 mM, about 30 mM, about 35 mM, about 40 mM, about 45 mM, about 50 mM, from about 0.1 mM to about 50 mM, from about 0.5 mM to about 50 mM, from about 1 mM to about 50 mM, from about 2 mM to about 50 mM, from about 3 mM to about 50 mM, from about 0.5 mM to about 20 mM, from about 0.5 mM to about 10 mM, from about 0.5 mM to about 5 mM, from about 0.5 mM to about 2.5 mM, from about 1 mM to about 20 mM, from about 1 mM to about 10 mM, from about 1 mM to about 5 mM, from about 1 mM to about 3.4 mM, from about 0.5 mM to about 3.0 mM, from about 1 mM to about 3.0 mM, from about 1.5 mM to about 3.0 mM, from about 2 mM to about 3.0 mM, from about 0.5 mM to about 2.5 mM, from about 1 mM to about 2.5 mM, from about 1.5 mM to about 2.5 mM, from about 2 mM to about 3.0 mM, from about 2.5 mM to about 3.0 mM, from about 0.5 mM to about 2 mM, from about 0.5 mM to about 1.5 mM, from about 0.5 mM to about 1.1 mM, from about 5.0 mM to about 10 mM, from about 5.0 mM to about 15 mM, from about 5.0 mM to about 20 mM, from about 10 mM to about 15 mM, from about 10 mM to about 20 mM, etc.). The combined nucleotide concentration, when more than one nucleotides is present, can be determined by adding the concentrations of the individual nucleotides together. When more than one nucleotide is present in reaction solutions of the invention, the individual nucleotides may not be present in equimolar amounts. Thus, a reaction solution may contain, for example, 1 mM dGTP, 1 mM dATP, 0.5 mM dCTP, and 1 mM dTTP.

[0274] RNA will typically be present in reaction solutions of the invention. In most instances, RNA will be added to the reaction solution shortly prior to reverse transcription. Thus, reaction solutions may be provided without RNA. This will typically be the case when reaction solutions are provided in kits. RNA, when present in reaction solutions will often be present in a concentration of 1 picogram to 100 μg/20 μl reaction mixture (e.g., about 1 picogram/20 μl, about 10 picograms/20 μl, about 50 picograms/20 μl, about 100 picograms/20 μl, about 200 picograms/20 μl, about 10 picograms/20 μl, about 500 picograms/20 μl, about 800 picograms/20 μl, about 1.0 nanogram/20 μl, about 5.0 nanograms/20 μl, about 10 nanograms/20 μl, about 25 nanograms/20 μl, about 50 nanograms/20 μl, about 75 nanograms/20 μl, about 100 nanograms/20 μl, about 150 nanograms/20 μl, about 250 nanograms/20 μl, about 400 nanograms/20 μl, about 500 nanograms/20 μl, about 750 nanograms/20 μl, about 1.0 μg/20 μl, about 5.0 μg/20 μl, about 10 μg/20 μl, about 20 μg/20 μl, about 30 μg/20 μl, about 40 μg/20 μl, about 50 μg/20 μl, about 70 μg/20 μl, about 85 μg/20 μl, about 100 μg/20 μl, from about 10 picograms/20 μl to about 100 μg/20 μl, from about 10 picograms/20 μl to about 100 μg/20 μl, from about 100 picograms/20 μl to about 100 μg/20 μl, from about 1.0 nanograms/20 μl to about 100 μg/20 μl, from about 100

nanograms/20 μl to about 100 μg/20 μl, from about 10 picograms/20 μl to about 10 μg/20 μl, from about 10 picograms/20 μl to about 5 μg/20 μl, from about 100 nanograms/20 μl to about 5 μg/20 μl, from about 1 μg/20 μl to about 10 μg/20 μl, from about 1 μg/20 μl to about 5 μg/20 μl, from about 100 nanograms/20 μl to about 1 μg/20 μl, from about 500 nanograms/20 μl to about 5 μg/20 μl, etc.). As one skilled in the art would recognize, different reverse transcription reactions may be performed in volumes other than 20 μl. In such instances, the total amount of RNA present will vary with the volume used. Thus, the above amounts are provided as examples of the amount of RNA/20 μl of reaction solution.

[0275] Reverse transcriptases (e.g., reverse transcriptases of the invention) may also be present in reaction solutions. When present, reverse transcriptases, will often be present in a concentration which results in about 0.01 to about 1,000 units of reverse transcriptase activity/μl (e.g, about 0.01 unit/μl, about 0.05 unit/μl, about 0.1 unit/μl, about 0.2 unit/μl, about 0.3 unit/μl, about 0.4 unit/μl, about 0.5 unit/μl, about 0.7 unit/μl, about 1.0 unit/μl, about 1.5 unit/μl, about 2.0 unit/μl, about 2.5 unit/μl, about 5.0 unit/μl, about 7.5 unit/μl, about 10 unit/μl, about 20 unit/μl, about 25 unit/μl, about 50 unit/μl, about 100 unit/μl, about 150 unit/μl, about 200 unit/μl, about 250 unit/μl, about 350 unit/μl, about 500 unit/μl, about 750 unit/μl, about 1,000 unit/μl, from about 0.1 unit/μl to about 1,000 unit/μl, from about 0.2 unit/μl to about 1,000 unit/μl, from about 1.0 unit/μl to about 1,000 unit/μl, from about 5.0 unit/μl to about 1,000 unit/μl, from about 10 unit/μl to about 1,000 unit/μl, from about 20 unit/μl to about 1,000 unit/μl, from about 50 unit/μl to about 1,000 unit/μl, from about 100 unit/μl to about 1,000 unit/μl, from about 200 unit/μl to about 1,000 unit/μl, from about 400 unit/μl to about 1,000 unit/μl, from about 500 unit/μl to about 1,000 unit/μl, from about 0.1 unit/μl to about 300 unit/μl, from about 0.1 unit/μl to about 200 unit/μl, from about 0.1 unit/μl to about 100 unit/μl, from about 0.1 unit/μl to about 50 unit/μl, from about 0.1 unit/μl to about 10 unit/μl, from about 0.1 unit/μl to about 5.0 unit/μl, from about 0.1 unit/μl to about 1.0 unit/μl, from about 0.2 unit/μl to about 0.5 unit/μl, etc.

[0276] Reaction solutions of the invention may be prepared as concentrated solutions (e.g., 5× solutions) which are diluted to a working concentration for final use. With respect to a 5× reaction solution, a 5:1 dilution is required to bring such a 5× solution to a working concentration. Reaction solutions of the invention may be prepared, for examples, as a 2×, a 3×, a 4×, a 5×, a 6×, a 7×, a 8×, a 9×, a 10×, etc. solutions. One major limitation on the fold concentration of such solutions is that, when compounds reach particular concentrations in solution, precipitation occurs. Thus, concentrated reaction solutions will generally be prepared such that the concentrations of the various components are low enough so that precipitation of buffer components will not occur. As one skilled in the art would recognize, the upper limit of concentration which is feasible for each solution will vary with the particular solution and the components present.

[0277] In many instances, reaction solutions of the invention will be provided in sterile form. Sterilization may be performed on the individual components of reaction solutions prior to mixing or on reaction solutions after they are prepared. Sterilization of such solutions may be performed by any suitable means including autoclaving or ultrafiltration.

[0278] Labeling Nucleic Acids

[0279] In general, the invention provides, in part, compositions for use in reverse transcription of a nucleic acid molecule to produce labeled nucleic acid molecules. Such compositions may comprise one or more reverse transcriptases (e.g., single subunit and/or multi-subunit RTs). The enzymes in these compositions are preferably present in working concentrations and have RNase H activity or are reduced or substantially reduced or lacking in RNase H activity, although mixtures of enzymes, some having RNase H activity and some reduced or substantially reduced or lacking in RNase H activity, may be used in the compositions of the invention. Preferred reverse transcriptases include M-MLV reverse transcriptase, RSV reverse transcriptase, AMV reverse transcriptase, RAV reverse transcriptase, MAV reverse transcriptase and HIV reverse transcriptase or other ASLV reverse transcriptases.

[0280] The invention is also directed to methods for reverse transcription of one or more nucleic acid molecules comprising mixing one or more nucleic acid templates, which is preferably RNA or messenger RNA (mRNA) and more preferably a population of mRNA molecules, with one or more polypeptides having reverse transcriptase activity and incubating the mixture under conditions sufficient to make one or more labeled nucleic acid molecules complementary to all or a portion of the one or more templates. To make the nucleic acid molecule or molecules complementary to the one or more templates, at least one primer (e.g., an oligo(dT) primer) and one or more nucleotides (a portion of which are preferably labeled, most preferably fluorescently labeled) are used for nucleic acid synthesis. Nucleic acid templates suitable for reverse transcription according to this aspect of the invention include any nucleic acid molecule, particularly those derived from a prokaryotic or eukaryotic cell. Such cells may include normal cells, diseased cells, transformed cells, established cells, progenitor cells, precursor cells, fetal cells, embryonic cells, bacterial cells, yeast cells, animal cells (including human cells), avian cells, plant cells and the like, or tissue isolated from a plant or an animal (e.g., human, cow, pig, mouse, sheep, horse, monkey, canine, feline, rat, rabbit, bird, fish, insect, etc.). Such nucleic acid molecules may also be isolated from viruses. In some embodiments, methods of the invention result in the direct labeling of a nucleic acid molecule by incorporation of a labeled nucleotide (e.g., a nucleotide containing a fluorescent label). In other methods, nucleic acid molecules are indirectly labeled by first, incorporating a nucleotide analog containing a reactive functionality to produce a nucleic acid containing one or more reactive functionalities. The nucleic acid containing reactive functionalities may subsequently be reacted with a molecule containing a label that reacts with the functionality to attach the label to the nucleic acid molecule. The reaction may be result in covalent attachment of all or a portion of the label-containing molecule to the nucleic acid molecule (e.g., chemical coupling). In some embodiments, amine-modified NTPs (e.g., amino allyl-dUTP/UTP) are incorporated during reverse transcription. Amino allyl-NTPs are incorporated with similar efficiency as unmodified NTPs during enzymatic reactions such as reverse transcription. The amine

functionality is then coupled with a dye using standard techniques. Kits for indirect labeling of cDNA are commercially available from, for example, Ambion, Inc., Austin, Tex. In some embodiments, the label-containing molecule may be non-covalently bound to the reactive functionality. For example, the reactive functionality may be a biotin moiety and the label-containing molecule may be a labeled (e.g., fluorescently labeled) avidin or streptavidin molecule.

[0281] The invention also provides labeled nucleic acid molecules produced according to the above-described methods. Such labeled nucleic acid molecules may be single or double stranded and are useful as detection probes. Depending on the labeled nucleotide(s) used during synthesis, the labeled molecules may contain one or a number of labels. Where multiple labels are used, the molecules may comprise a number of the same or different labels. Thus, one type or multiple different labeled nucleotides may be used during synthesis of nucleic acid molecules to provide for the labeled nucleic acid molecules of the invention. Such labeled nucleic acid molecules will thus comprise one or more (e.g., two, three, four, five, six, seven, eight, nine, ten, etc.) labeled nucleotides (which may be the same or different).

[0282] Labeled nucleic acid molecules produced by methods of the invention may either (1) comprise a particular numbers of labeled nucleotides or (2) a particular percentage of the nucleotides present in the nucleic acid molecule may be labeled. In either instance, the concentration of labeled nucleotides present in the reaction mixture may be adjusted, with respect to un-labeled nucleotides, such that product nucleic acid molecules are produced which contain (1) a particular number of labeled nucleotides or (2) a particular percentage of label nucleotides as compared to un-labeled nucleotides. In particular instances, from about 0.1% to about 20%, from about 0.1% to about 15%, from about 0.1% to about 10%, from about 0.1% to about 5.0%, from about 0.1% to about 2.5%, from about 0.1% to about 1.5%, from about 0.1% to about 1.0%, from about 0.1% to about 0.5%, from about 2.0% to about 20%, from about 4.0% to about 20%, from about 0.5% to about 10%, from about 0.5% to about 5%, from about 0.5% to about 2.0%, or from about 0.5% to about 1.0% of the total nucleotides present in product nucleic acid molecules are labeled. As one skilled in the art would recognize, the actual number of labeled nucleotides, and thus the percentage of nucleotides which are labeled, present in individual molecules of a population will typically differ. In other words, different members of populations of nucleic acid molecules will typically contain different numbers of labeled nucleotides with the overall average of labeled nucleotides present in each product molecule varying with a number of factors (e.g., the ratio of labeled to un-labeled nucleotides present in the reaction mixture). In most instances, at least 85%, at least 90%, at least 95%, or at least 99% of the individual product nucleic acid molecules in the population will contain labeled nucleotides which fall within a range set out above.

[0283] In accordance with the invention, the amount of labeled product is preferably measured based on percent incorporation of the label of interest into synthesized product as may be determined by one skilled in the art, although other means of measuring the amount or efficiency of labeling of product will be recognized by one of ordinary skill in the art. The invention provides for enhanced or increased percent incorporation of labeled nucleotide during synthesis of a nucleic acid molecule from a template, preferably during synthesis of one or more cDNA molecules from RNA. According to the invention, such enhancement or increase in percent incorporation is preferably about equal to or greater than a 2-fold, a 5-fold, a 10-fold, a 15-fold, a 20-fold, a 25-fold, a 30-fold, a 40-fold or a 50-fold increase or enhancement in percent incorporation compared to a standard reverse transcriptase.

[0284] The invention also provides kits for use in accordance with the invention. Such kits comprise a carrier means, such as a box or carton, having in close confinement therein one or more container means, such as vials, tubes, bottles and the like, wherein the kit comprises, in the same or different containers, one or more reverse transcriptases. The kits of the invention may also comprise, in the same or different containers, one or more DNA polymerases, one or more primers, one or more suitable buffers and/or one or more nucleotides (such as deoxynucleoside triphosphates (dNTPs) and preferably labeled dNTPs (e.g., fluorescently labeled dNTPs)).

[0285] In some embodiments, the RTs used in the invention comprise two or more subunits (or derivatives, variants, fragments or mutants thereof) and preferably comprise two subunits (e.g., a dimer or heterodimer). Two subunit reverse transcriptases typically have an α and a β subunit forming a dimer, although any form or combination of subunits (and derivatives, variants or mutants of such subunits) may be used. Such combinations may include $\alpha\beta$, $\beta\beta$, $\alpha\alpha$ and the like. Preferred two subunit RTs for use in the invention include RSV RT, AMV RT, AEV RT, RAV RT, HIV RT and MAV RT, or other ASLV RTs, or mutants, variants or derivatives thereof. In a particular embodiment, AMV RT and/or RSV RT is used in accordance with the invention. Preferred single subunit RTs include M-MLV reverse transcriptase.

[0286] Production/Sources of cDNA Molecules

[0287] In accordance with the invention, cDNA molecules (single-stranded or double-stranded) may be prepared from a variety of nucleic acid template molecules. Preferred nucleic acid molecules for use in the present invention include single-stranded or double-stranded DNA and RNA molecules, as well as double-stranded DNA:RNA hybrids. More preferred nucleic acid molecules include messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA) molecules, although mRNA molecules are the preferred template according to the invention.

[0288] The nucleic acid molecules that are used to prepare cDNA molecules according to the methods of the present invention may be prepared synthetically according to standard organic chemical synthesis methods that will be familiar to one of ordinary skill. More preferably, the nucleic acid molecules may be obtained from natural sources, such as a variety of cells, tissues, organs or organisms. Cells that may be used as sources of nucleic acid molecules may be prokaryotic (bacterial cells, including but not limited to those of species of the genera *Escherichia, Bacillus, Serratia, Salmonella, Staphylococcus, Streptococcus, Clostridium, Chlamydia, Neisseria, Treponema, Mycoplasma, Borrelia, Legionella, Pseudomonas, Mycobacterium, Helicobacter, Erwinia, Agrobacterium, Rhizobium, Xanthomonas* and *Streptomyces*) or eukaryotic (including

fungi (especially yeasts), plants, protozoans and other parasites, and animals including insects (particularly *Drosophila* spp. cells), nematodes (particularly *Caenorhabditis elegans* cells), and mammals (particularly human cells)).

[0289] Mammalian somatic cells that may be used as sources of nucleic acids include blood cells (reticulocytes and leukocytes), endothelial cells, epithelial cells, neuronal cells (from the central or peripheral nervous systems), muscle cells (including myocytes and myoblasts from skeletal, smooth or cardiac muscle), connective tissue cells (including fibroblasts, adipocytes, chondrocytes, chondroblasts, osteocytes and osteoblasts) and other stromal cells (e.g., macrophages, dendritic cells, Schwann cells). Mammalian germ cells (spermatocytes and oocytes) may also be used as sources of nucleic acids for use in the invention, as may the progenitors, precursors and stem cells that give rise to the above somatic and germ cells. Also suitable for use as nucleic acid sources are mammalian tissues or organs such as those derived from brain, kidney, liver, pancreas, blood, bone marrow, muscle, nervous, skin, genitourinary, circulatory, lymphoid, gastrointestinal and connective tissue sources, as well as those derived from a mammalian (including human) embryo or fetus.

[0290] Any of the above prokaryotic or eukaryotic cells, tissues and organs may be normal, diseased, transformed, established, progenitors, precursors, fetal or embryonic. Diseased cells may, for example, include those involved in infectious diseases (caused by bacteria, fungi or yeast, viruses (including AIDS, HIV, HTLV, herpes, hepatitis and the like) or parasites), in genetic or biochemical pathologies (e.g., cystic fibrosis, hemophilia, Alzheimer's disease, muscular dystrophy or multiple sclerosis) or in cancerous processes. Transformed or established animal cell lines may include, for example, COS cells, CHO cells, VERO cells, BHK cells, HeLa cells; HepG2 cells, K562 cells, 293 cells, L929 cells, F9 cells, and the like. Other cells, cell lines, tissues, organs and organisms suitable as sources of nucleic acids for use in the present invention will be apparent to one of ordinary skill in the art.

[0291] Once the starting cells, tissues, organs or other samples are obtained, nucleic acid molecules (such as mRNA) may be isolated therefrom by methods that are well-known in the art (See, e.g., Maniatis, T., et al., *Cell* 15:687-701 (1978); Okayama, H., and Berg, P., *Mol. Cell. Biol.* 2:161-170 (1982); Gubler, U., and Hoffman, B. J., *Gene* 25:263-269 (1983)). The nucleic acid molecules thus isolated may then be used to prepare cDNA molecules and cDNA libraries in accordance with the present invention.

[0292] In the practice of the invention, cDNA molecules or cDNA libraries are produced by mixing one or more nucleic acid molecules obtained as described above, which is preferably one or more mRNA molecules such as a population of mRNA molecules, with a polypeptide having reverse transcriptase activity of the present invention, or with one or more of the compositions of the invention, under conditions favoring the reverse transcription of the nucleic acid molecule by the action of the enzymes or the compositions to form one or more cDNA molecules (single-stranded or double-stranded). Thus, the method of the invention comprises (a) mixing one or more nucleic acid templates (preferably one or more RNA or mRNA templates, such as a population of mRNA molecules) with one or more reverse transcriptases of the invention and (b) incubating the mixture under conditions sufficient to make one or more nucleic acid molecules complementary to all or a portion of the one or more templates. Such methods may include the use of one or more DNA polymerases, one or more nucleotides, one or more primers, one or more buffers, and the like. The invention may be used in conjunction with methods of cDNA synthesis such as those described in the Examples below, or others that are well-known in the art (see, e.g., Gubler, U., and Hoffman, B. J., *Gene* 25:263-269 (1983); Krug, M. S., and Berger, S. L., *Meth. Enzymol.* 152:316-325 (1987); Sambrook, J., et al., *Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press, pp. 8.60-8.63 (1989); PCT Publication No. WO 99/15702; PCT Publication No. WO 98/47912; and PCT Publication No. WO 98/51699), to produce cDNA molecules or libraries.

[0293] Other methods of cDNA synthesis which may advantageously use the present invention will be readily apparent to one of ordinary skill in the art.

[0294] Having obtained cDNA molecules or libraries according to the present methods, these cDNAs may be isolated for further analysis or manipulation. Detailed methodologies for purification of cDNAs are taught in the GENETRAPPER™ manual (Invitrogen Corporation (Carlsbad, Calif.)), which is incorporated herein by reference in its entirety, although alternative standard techniques of cDNA isolation that are known in the art (see, e.g., Sambrook, J., et al., *Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press, pp. 8.60-8.63 (1989)) may also be used.

[0295] In other aspects of the invention, the invention may be used in methods for amplifying and sequencing nucleic acid molecules. Nucleic acid amplification methods according to this aspect of the invention may be one-step (e.g., one-step RT-PCR) or two-step (e.g., two-step RT-PCR) reactions. According to the invention, one-step RT-PCR type reactions may be accomplished in one tube thereby lowering the possibility of contamination. Such one-step reactions comprise (a) mixing a nucleic acid template (e.g., mRNA) with one or more reverse transcriptases of the present invention and with one or more DNA polymerases and (b) incubating the mixture under conditions sufficient to amplify a nucleic acid molecule complementary to all or a portion of the template. Such amplification may be accomplished by the reverse transcriptase activity alone or in combination with the DNA polymerase activity. Two-step RT-PCR reactions may be accomplished in two separate steps. Such a method comprises (a) mixing a nucleic acid template (e.g., mRNA) with a reverse transcriptase of the present invention, (b) incubating the mixture under conditions sufficient to make a nucleic acid molecule (e.g., a DNA molecule) complementary to all or a portion of the template, (c) mixing the nucleic acid molecule with one or more DNA polymerases, and (d) incubating the mixture of step (c) under conditions sufficient to amplify the nucleic acid molecule. For amplification of long nucleic acid molecules (i.e., greater than about 3-5 Kb in length) a combination of DNA polymerases may be used, such as one DNA polymerase having 3' exonuclease activity and another DNA polymerase being substantially reduced in 3' exonuclease activity.

[0296] Nucleic acid sequencing methods according to this aspect of the invention may comprise both cycle sequencing

(sequencing in combination with amplification) and standard sequencing reactions. The sequencing method of the invention thus comprises (a) mixing a nucleic acid molecule to be sequenced with one or more primers, one or more reverse transcriptases of the invention, one or more nucleotides and one or more terminating agents, (b) incubating the mixture under conditions sufficient to synthesize a population of nucleic acid molecules complementary to all or a portion of the molecule to be sequenced, and (c) separating the population to determine the nucleotide sequence of all or a portion of the molecule to be sequenced. According to the invention, one or more DNA polymerases (preferably thermostable DNA polymerases) may be used in combination with or separate from the reverse transcriptases of the invention.

[0297] Amplification methods which may be used in accordance with the present invention include PCR (U.S. Pat. Nos. 4,683,195 and 4,683,202), Strand Displacement Amplification (SDA; U.S. Pat. No. 5,455,166; EP 0 684 315), and Nucleic Acid Sequence-Based Amplification (NASBA; U.S. Pat. No. 5,409,818; EP 0 329 822), as well as more complex PCR-based nucleic acid fingerprinting techniques such as _Random_amplified Polymorphic DNA (RAPD) analysis (Williams, J. G. K., et al, *Nucl. Acids Res.* 18(22):6531-6535, 1990), Arbitrarily Primed PCR (AP-PCR; Welsh, J., and McClelland, M., *Nucl. Acids Res.* 18(24):7213-7218, 1990), DNA Amplification Fingerprinting (DAF; Caetano-Anollés et al., *Bio/Technology* 9:553-557, 1991), microsatellite PCR or Directed Amplification of Minisatellite-region DNA (DAMD; Heath, D. D., et al., *Nucl. Acids Res.* 21(24): 5782-5785, 1993), and Amplification Fragment Length Polymorphism (AFLP) analysis (EP 0 534 858; Vos, P., et al., *Nucl. Acids Res.* 23(21):4407-4414, 1995; Lin, J. J., and Kuo, J., *FOCUS* 17(2):66-70, 1995). Nucleic acid sequencing techniques which may employ the present compositions include dideoxy sequencing methods such as those disclosed in U.S. Pat. Nos. 4,962,022 and 5,498,523. In a particularly preferred aspects, the invention may be used in methods of amplifying or sequencing a nucleic acid molecule comprising one or more polymerase chain reactions (PCRs), such as any of the PCR-based methods described above.

[0298] Kits

[0299] In another embodiment, the present invention may be assembled into kits, which may be used in reverse transcription or amplification of a nucleic acid molecule, or into kits for use in sequencing of a nucleic acid molecule. Kits according to this aspect of the invention comprise a carrier means, such as a box, carton, tube or the like, having in close confinement therein one or more container means, such as vials, tubes, ampoules, bottles and the like, wherein a first container means contains one or more polypeptides of the present invention having reverse transcriptase activity. When more than one polypeptide having reverse transcriptase activity is used, they may be in a single container as mixtures of two or more polypeptides, or in separate containers. The kits of the invention may also comprise (in the same or separate containers) one or more DNA polymerases, a suitable buffer, one or more nucleotides and/or one or more primers. The kits of the invention may also comprise one or more hosts or cells including those that are competent to take up nucleic acids (e.g., DNA molecules including vectors). Preferred hosts may include chemically competent or electrocompetent bacteria such as *E. coli* (including DH5, DH5α, DH10B, HB101, Top 10, and other K-12 strains as well as *E. coli* B and *E. coli* W strains).

[0300] In a specific aspect of the invention, the kits of the invention (e.g., reverse transcription and amplification kits) may comprise one or more components (in mixtures or separately) including one or more polypeptides having reverse transcriptase activity of the invention, one or more nucleotides (one or more of which may be labeled, e.g., fluorescently labeled) used for synthesis of a nucleic acid molecule, and/or one or more primers (e.g., oligo(dT) for reverse transcription). Such kits (including the reverse transcription and amplification kits) may further comprise one or more DNA polymerases. Sequencing kits of the invention may comprise one or more polypeptides having reverse transcriptase activity of the invention, and optionally one or more DNA polymerases, one or more terminating agents (e.g., dideoxynucleoside triphosphate molecules) used for sequencing of a nucleic acid molecule, one or more nucleotides and/or one or more primers. Preferred polypeptides having reverse transcriptase activity, DNA polymerases, nucleotides, primers and other components suitable for use in the reverse transcription, amplification and sequencing kits of the invention include those described above. The kits encompassed by this aspect of the present invention may further comprise additional reagents and compounds necessary for carrying out standard nucleic acid reverse transcription, amplification or sequencing protocols. Such polypeptides having reverse transcriptase activity of the invention, DNA polymerases, nucleotides, primers, and additional reagents, components or compounds may be contained in one or more containers, and may be contained in such containers in a mixture of two or more of the above-noted components or may be contained in the kits of the invention in separate containers. Such kits may also comprise instructions (e.g., for performing the methods of the invention such as for labeling nucleic acid molecules in accordance with the invention).

[0301] Use of Nucleic Acid Molecules

[0302] The nucleic acid molecules or cDNA libraries prepared by the methods of the present invention may be further characterized, for example by cloning and sequencing (i.e., determining the nucleotide sequence of the nucleic acid molecule), by the sequencing methods of the invention or by others that are standard in the art (see, e.g., U.S. Pat. Nos. 4,962,022 and 5,498,523, which are directed to methods of DNA sequencing). Alternatively, these nucleic acid molecules may be used for the manufacture of various materials in industrial processes, such as hybridization probes by methods that are well-known in the art. Production of hybridization probes from cDNAs will, for example, provide the ability for those in the medical field to examine a patient's cells or tissues for the presence of a particular genetic marker such as a marker of cancer, of an infectious or genetic disease, or a marker of embryonic development. Furthermore, such hybridization probes can be used to isolate DNA fragments from genomic DNA or cDNA libraries prepared from a different cell, tissue or organism for further characterization.

[0303] The nucleic acid molecules of the present invention may also be used to prepare compositions for use in recombinant DNA methodologies. Accordingly, the present inven-

tion relates to recombinant vectors which comprise the cDNA or amplified nucleic acid molecules of the present invention, to host cells which are genetically engineered with the recombinant vectors, to methods for the production of a recombinant polypeptide using these vectors and host cells, and to recombinant polypeptides produced using these methods.

[0304] Recombinant vectors may be produced according to this aspect of the invention by inserting, using methods that are well-known in the art, one or more of the cDNA molecules or amplified nucleic acid molecules prepared according to the present methods into a vector. The vector used in this aspect of the invention may be, for example, a phage or a plasmid, and is preferably a plasmid. Preferred are vectors comprising cis-acting control regions to the nucleic acid encoding the polypeptide of interest. Appropriate trans-acting factors may be supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

[0305] In certain preferred embodiments in this regard, the vectors provide for specific expression (and are therefore termed "expression vectors"), which may be inducible and/or cell type-specific. Particularly preferred among such vectors are those inducible by environmental factors that are easy to manipulate, such as temperature and nutrient additives.

[0306] Expression vectors useful in the present invention include chromosomal-, episomal- and virus-derived vectors, e.g., vectors derived from bacterial plasmids or bacteriophages, and vectors derived from combinations thereof, such as cosmids and phagemids, and will preferably include at least one selectable marker such as a tetracycline or ampicillin resistance gene for culturing in a bacterial host cell. Prior to insertion into such an expression vector, the cDNA or amplified nucleic acid molecules of the invention should be operatively linked to an appropriate promoter, such as the phage lambda P_L promoter, the *E. coli* lac, trp and tac promoters. Other suitable promoters will be known to the skilled artisan.

[0307] Among vectors preferred for use in the present invention include pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; pcDNA3 available from Invitrogen; pGEX, pTrxfus, pTrc99a, pET-5, pET-9, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia; and pSPORT1, pSPORT2 and pSV•SPORT1, available from Invitrogen Corporation (Carlsbad, Calif.). Other suitable vectors will be readily apparent to the skilled artisan.

[0308] The invention also provides methods of producing a recombinant host cell comprising the cDNA molecules, amplified nucleic acid molecules or recombinant vectors of the invention, as well as host cells produced by such methods. Representative host cells (prokaryotic or eukaryotic) that may be produced according to the invention include, but are not limited to, bacterial cells, yeast cells, plant cells and animal cells. Preferred bacterial host cells include *Escherichia coli* cells (most particularly *E. coli* strains DH10B and Stb12, which are available commercially (Invitrogen Corporation (Carlsbad, Calif.)), *Bacillus subtilis* cells, *Bacillus megaterium* cells, *Streptomyces* spp. cells, *Erwinia* spp. cells, *Klebsiella* spp. cells and *Salmonella typhimurium* cells. Preferred animal host cells include insect cells (most particularly *Spodoptera frugiperda* Sf9 and Sf21 cells and *Trichoplusa* HigH-Five cells) and mammalian cells (most particularly CHO, COS, VERO, BHK and human cells). Such host cells may be prepared by well-known transformation, electroporation or transfection techniques that will be familiar to one of ordinary skill in the art.

[0309] In addition, the invention provides methods for producing a recombinant polypeptide, and polypeptides produced by these methods. According to this aspect of the invention, a recombinant polypeptide may be produced by culturing any of the above recombinant host cells under conditions favoring production of a polypeptide therefrom, and isolation of the polypeptide. Methods for culturing recombinant host cells, and for production and isolation of polypeptides therefrom, are well-known to one of ordinary skill in the art.

[0310] It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

EXAMPLES

Example 1

Preparation of Mutant Reverse Transcriptases

[0311] Plasmid pBAD was obtained from Invitrogen Corporation, Carlsbad, Calif. and the coding sequence of M-MLV reverse transcriptase was inserted to produce plasmid pBAD-6-His-M-MLV H– (F1). Plasmid pBAD-6-His-M-MLV H– (F1) was used as both a cloning vector and as a target for PCR mutagenesis (**FIG. 1**). pBAD-6-His-M-MLV H– (F1) replicates in *E. coli* and confers ampicillin resistance to transformed cells. The M-MLV reverse transcriptase gene is expressed from the ara BAD promoter which is induced by the presence of arabinose. The promoter is repressed by the product of the araC gene, which is present on the plasmid. The host used, *E. coli* strain DH10B, is an araD mutant and cannot metabolize arabinose, making arabinose a gratuitous inducer in DH10B cells transformed with pBAD-6-His-M-MLV H– (F1). The plasmid contains a 6 histidine containing leader sequence in frame with the coding sequence of the M-MLV reverse transcriptase gene. The gene starting at nucleotide 2598 and ending at nucleotide 4628 (Shinnick, et al., (1981) *Nature* 293, 543-548.) was cloned under control of an araD promoter into plasmid pBAD/HisA (Invitrogen). The M-MLV gene was further modified by site-directed mutagenesis without changing amino acid coding to include several unique restriction endonuclease sites that divided the gene into five segments (**FIG. 2**). The amino end of the protein contained a His_6 tag to simplify purification that included the following amino acids: MGGSHHHHHHGMASMTGGQQMGRDLYDDDDKH corresponding to amino acids 1-32 of SEQ ID NO:2. The carboxy end of the protein contained the additional amino acids NSRLIN, corresponding to amino acids

711-716 of SEQ ID NO:2, present as the result of subcloning from pRT601. In addition, the M-MLV RT gene was mutated (D524G, E562Q, D583N) to eliminate RNase H activity. The final construct was designated pBAD-HSS2 (**FIG. 1**), and the gene and gene product were designated His_6 H− RT. In addition to this construct, other constructs having different N-terminal sequences are contemplated in the present invention. For example, a construct beginning at methionine 12 of SEQ ID NO:6 and Table 3 and containing a mutation changing methionine 15 to glycine (M15G) to produce a protein with an N-terminal sequence MASGTGGQQMGRDLYDDDDKH (SEQ ID NO:1) followed by the remaining sequence of M-MLV RT from Table 3 has been produced as well as a construct beginning with methionine 33 of SEQ ID NO:6 and Table 3.

[**0312**] With reference to the sequence of this plasmid provided in Table 3 (SEQ ID NOS:1 and 2), nucleotides 1-96 encode the leader sequence and nucleotides 97-99 encode a methionine. Those skilled in the art will appreciate that the wild-type M-MLV reverse transcriptase is derived by proteolysis from a precursor polyprotein and thus the wild-type M-MLV reverse transcriptase does not start with a methionine. Therefore, amino acid number 1 of the M-MLV reverse transcriptase is the threonine (amino acid 34 in SEQ ID NO:2 and Table 3) following the methionine encoded by nucleotides 97-99 (amino acid 33 in SEQ ID NO:2 and Table 3).

[**0313**] The sequence of the M-MLV reverse transcriptase gene in pBAD-6-His-M-MLV H− (F1) which was used in these experiments was derived from the sequence of plasmid pRT601. pRT601 is described in Kotewicz, et al., (1988) *Nuc. Acids Res.* 16, 265-277, Gerard, et al., (1986) *DNA* 5, 271-279, U.S. Pat. Nos. 5,668,005 and 5,017,492, which are incorporated herein by reference in their entireties.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 46

<210> SEQ ID NO 1
<211> LENGTH: 2151
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Mutant Reverse Transcriptase Derived from
Moloney Murine Leukemia Virus
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(2148)

<400> SEQUENCE: 1

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Met Gly Gly Ser His His His His His His Gly Met Ala Ser Met Thr
1               5                   10                  15

ggt gga cag caa atg ggt cgg gat ctg tac gac gat gac gat aag cat       96
Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys His
            20                  25                  30

atg acc cta aat ata gaa gat gag tat cgg cta cat gag acc tca aaa      144
Met Thr Leu Asn Ile Glu Asp Glu Tyr Arg Leu His Glu Thr Ser Lys
        35                  40                  45

gag cca gat gtt tct cta ggg tcc aca tgg ctg tct gat ttt cct cag      192
Glu Pro Asp Val Ser Leu Gly Ser Thr Trp Leu Ser Asp Phe Pro Gln
    50                  55                  60

gcc tgg gcg gaa acc ggg ggc atg gga ctg gca gtt cgc caa gct cct      240
Ala Trp Ala Glu Thr Gly Gly Met Gly Leu Ala Val Arg Gln Ala Pro
65                  70                  75                  80

ctg atc ata ctt ctg aaa gca acc tct acc ccc gtg tcc ata aaa caa      288
Leu Ile Ile Leu Leu Lys Ala Thr Ser Thr Pro Val Ser Ile Lys Gln
                85                  90                  95

tac ccc atg tca caa gaa gcc aga ctg ggg atc aag ccc cac ata cag      336
Tyr Pro Met Ser Gln Glu Ala Arg Leu Gly Ile Lys Pro His Ile Gln
            100                 105                 110

aga ctg ttg gac cag gga ata ctg gta ccc tgc cag tcc ccc tgg aac      384
Arg Leu Leu Asp Gln Gly Ile Leu Val Pro Cys Gln Ser Pro Trp Asn
        115                 120                 125

acg ccc ctg cta ccc gtc aag aaa ccc ggg act aat gat tac agg cct      432
Thr Pro Leu Leu Pro Val Lys Lys Pro Gly Thr Asn Asp Tyr Arg Pro
    130                 135                 140
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-continued

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gtc caa gat ctg aga gag gtc aac aaa cgc gta gaa gac atc cac ccc      480
Val Gln Asp Leu Arg Glu Val Asn Lys Arg Val Glu Asp Ile His Pro
145                 150                 155                 160

acc gta ccc aac ccc tac aac ctc ttg agt ggg ctc cca ccg tcc cac      528
Thr Val Pro Asn Pro Tyr Asn Leu Leu Ser Gly Leu Pro Pro Ser His
                165                 170                 175

cag tgg tac act gtt cta gac tta aaa gat gcc ttt ttc tgc ctg aga      576
Gln Trp Tyr Thr Val Leu Asp Leu Lys Asp Ala Phe Phe Cys Leu Arg
            180                 185                 190

ctc cac ccg acg tct cag cct ctc ttc gcc ttt gaa tgg aga gac cca      624
Leu His Pro Thr Ser Gln Pro Leu Phe Ala Phe Glu Trp Arg Asp Pro
        195                 200                 205

gag atg gga atc tct ggc caa cta acc tgg acc aga ctc cca cag gga      672
Glu Met Gly Ile Ser Gly Gln Leu Thr Trp Thr Arg Leu Pro Gln Gly
    210                 215                 220

ttc aaa aac agt ccc acc ctg ttt gat gag gca ctg cgc aga gac cta      720
Phe Lys Asn Ser Pro Thr Leu Phe Asp Glu Ala Leu Arg Arg Asp Leu
225                 230                 235                 240

gca gac ttc cgg atc cag cac cca gac ttg atc ctg cta cag tac gta      768
Ala Asp Phe Arg Ile Gln His Pro Asp Leu Ile Leu Leu Gln Tyr Val
                245                 250                 255

gat gac tta ctg ctg gcc gcc act tct gag ctc gac tgc caa caa ggt      816
Asp Asp Leu Leu Leu Ala Ala Thr Ser Glu Leu Asp Cys Gln Gln Gly
            260                 265                 270

act cgg gcc ctg tta caa acc cta gga gac ctc ggg tat cgg gcc tcg      864
Thr Arg Ala Leu Leu Gln Thr Leu Gly Asp Leu Gly Tyr Arg Ala Ser
        275                 280                 285

gcc aag aaa gcc caa att tgc cag aaa cag gtc aag tat ctg ggg tat      912
Ala Lys Lys Ala Gln Ile Cys Gln Lys Gln Val Lys Tyr Leu Gly Tyr
    290                 295                 300

ctt cta aaa gag ggt cag aga tgg ctg act gag gcc aga aaa gag act      960
Leu Leu Lys Glu Gly Gln Arg Trp Leu Thr Glu Ala Arg Lys Glu Thr
305                 310                 315                 320

gtg atg ggg cag cct act ccg aag acc ccg cgg caa cta agg gag ttc     1008
Val Met Gly Gln Pro Thr Pro Lys Thr Pro Arg Gln Leu Arg Glu Phe
                325                 330                 335

cta ggg acg gca ggc ttc tgt cgc ctc tgg atc cct ggg ttt gca gaa     1056
Leu Gly Thr Ala Gly Phe Cys Arg Leu Trp Ile Pro Gly Phe Ala Glu
            340                 345                 350

atg gca gcc ccc ttg tac cct ctc acc aaa acg ggg act ctg ttt aat     1104
Met Ala Ala Pro Leu Tyr Pro Leu Thr Lys Thr Gly Thr Leu Phe Asn
        355                 360                 365

tgg ggc cca gac caa caa aag gcc tat caa gaa atc aag caa gct ctt     1152
Trp Gly Pro Asp Gln Gln Lys Ala Tyr Gln Glu Ile Lys Gln Ala Leu
    370                 375                 380

cta act gcc cca gcc ctg ggg ttg cca gat ttg act aag ccc ttt gaa     1200
Leu Thr Ala Pro Ala Leu Gly Leu Pro Asp Leu Thr Lys Pro Phe Glu
385                 390                 395                 400

ctc ttt gtc gac gag aag cag ggc tac gcc aaa ggt gtc cta acg caa     1248
Leu Phe Val Asp Glu Lys Gln Gly Tyr Ala Lys Gly Val Leu Thr Gln
                405                 410                 415

aaa ctg gga cct tgg cgt cgg ccg gtg gcc tac ctg tcc aaa aag cta     1296
Lys Leu Gly Pro Trp Arg Arg Pro Val Ala Tyr Leu Ser Lys Lys Leu
            420                 425                 430

gac cca gta gca gct ggg tgg ccc cct tgc cta cgg atg gta gca gcc     1344
Asp Pro Val Ala Ala Gly Trp Pro Pro Cys Leu Arg Met Val Ala Ala
        435                 440                 445
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att gcc gta ctg aca aag gat gca ggc aag cta acc atg gga cag cca     1392
Ile Ala Val Leu Thr Lys Asp Ala Gly Lys Leu Thr Met Gly Gln Pro
    450                 455                 460

cta gtc att ctg gcc ccc cat gca gta gag gca cta gtc aaa caa ccc     1440
Leu Val Ile Leu Ala Pro His Ala Val Glu Ala Leu Val Lys Gln Pro
465                 470                 475                 480

ccc gat cga tgg ctt tcc aac gcc cgg atg act cac tat cag gcc ttg     1488
Pro Asp Arg Trp Leu Ser Asn Ala Arg Met Thr His Tyr Gln Ala Leu
                485                 490                 495

ctt ttg gac acg gac cgg gtc cag ttc gga ccg gtg gta gcc ctg aac     1536
Leu Leu Asp Thr Asp Arg Val Gln Phe Gly Pro Val Val Ala Leu Asn
            500                 505                 510

ccg gct aca ctg ctc cca ctg cct gag gaa ggg ctg cag cac aac tgc     1584
Pro Ala Thr Leu Leu Pro Leu Pro Glu Glu Gly Leu Gln His Asn Cys
        515                 520                 525

ctt gat atc ctg gcc gaa gcc cac gga acc cga ccc gac cta acg gac     1632
Leu Asp Ile Leu Ala Glu Ala His Gly Thr Arg Pro Asp Leu Thr Asp
    530                 535                 540

cag ccg ctc cca gac gcc gac cac acc tgg tac acg ggt gga tcc agt     1680
Gln Pro Leu Pro Asp Ala Asp His Thr Trp Tyr Thr Gly Gly Ser Ser
545                 550                 555                 560

ctc ttg caa gag gga cag cgt aag gcg gga gct gcg gtg acc acc gag     1728
Leu Leu Gln Glu Gly Gln Arg Lys Ala Gly Ala Ala Val Thr Thr Glu
                565                 570                 575

acc gag gta atc tgg gct aaa gcc ctg cca gcc ggg aca tcc gct cag     1776
Thr Glu Val Ile Trp Ala Lys Ala Leu Pro Ala Gly Thr Ser Ala Gln
            580                 585                 590

cgg gct cag ctg ata gca ctc acc cag gcc cta agg atg gca gaa ggt     1824
Arg Ala Gln Leu Ile Ala Leu Thr Gln Ala Leu Arg Met Ala Glu Gly
        595                 600                 605

aag aag cta aat gtt tat acg aat tcc cgt tat gct ttt gct act gcc     1872
Lys Lys Leu Asn Val Tyr Thr Asn Ser Arg Tyr Ala Phe Ala Thr Ala
    610                 615                 620

cat atc cat gga gaa ata tac aga agg cgt ggg ttg ctc aca tca gaa     1920
His Ile His Gly Glu Ile Tyr Arg Arg Arg Gly Leu Leu Thr Ser Glu
625                 630                 635                 640

ggc aaa gag atc aaa aat aag gac gag ata ttg gcc cta cta aaa gcc     1968
Gly Lys Glu Ile Lys Asn Lys Asp Glu Ile Leu Ala Leu Leu Lys Ala
                645                 650                 655

ctc ttt ctg ccc aaa aga ctt agc ata atc cat tgt cca gga cat caa     2016
Leu Phe Leu Pro Lys Arg Leu Ser Ile Ile His Cys Pro Gly His Gln
            660                 665                 670

aag gga cac agc gcc gag gct aga ggc aac cgg atg gct gac caa gcg     2064
Lys Gly His Ser Ala Glu Ala Arg Gly Asn Arg Met Ala Asp Gln Ala
        675                 680                 685

gcc cga aag gca gcc atc aca gag aat cca gac acc tct acc ctc ctc     2112
Ala Arg Lys Ala Ala Ile Thr Glu Asn Pro Asp Thr Ser Thr Leu Leu
    690                 695                 700

ata gaa aat tca tca ccc aat tcc cgc tta att aat taa                2151
Ile Glu Asn Ser Ser Pro Asn Ser Arg Leu Ile Asn
705                 710                 715
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<210> SEQ ID NO 2
<211> LENGTH: 716
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Mutant Reverse Transcriptase Derived from

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Moloney Murine Leukemia Virus

<400> SEQUENCE: 2

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Met Gly Gly Ser His His His His His His Gly Met Ala Ser Met Thr
1               5                  10                  15

Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys His
            20                  25                  30

Met Thr Leu Asn Ile Glu Asp Glu Tyr Arg Leu His Glu Thr Ser Lys
        35                  40                  45

Glu Pro Asp Val Ser Leu Gly Ser Thr Trp Leu Ser Asp Phe Pro Gln
    50                  55                  60

Ala Trp Ala Glu Thr Gly Gly Met Gly Leu Ala Val Arg Gln Ala Pro
65                  70                  75                  80

Leu Ile Ile Leu Leu Lys Ala Thr Ser Thr Pro Val Ser Ile Lys Gln
                85                  90                  95

Tyr Pro Met Ser Gln Glu Ala Arg Leu Gly Ile Lys Pro His Ile Gln
            100                 105                 110

Arg Leu Leu Asp Gln Gly Ile Leu Val Pro Cys Gln Ser Pro Trp Asn
        115                 120                 125

Thr Pro Leu Leu Pro Val Lys Lys Pro Gly Thr Asn Asp Tyr Arg Pro
    130                 135                 140

Val Gln Asp Leu Arg Glu Val Asn Lys Arg Val Glu Asp Ile His Pro
145                 150                 155                 160

Thr Val Pro Asn Pro Tyr Asn Leu Leu Ser Gly Leu Pro Pro Ser His
                165                 170                 175

Gln Trp Tyr Thr Val Leu Asp Leu Lys Asp Ala Phe Phe Cys Leu Arg
            180                 185                 190

Leu His Pro Thr Ser Gln Pro Leu Phe Ala Phe Glu Trp Arg Asp Pro
        195                 200                 205

Glu Met Gly Ile Ser Gly Gln Leu Thr Trp Thr Arg Leu Pro Gln Gly
    210                 215                 220

Phe Lys Asn Ser Pro Thr Leu Phe Asp Glu Ala Leu Arg Arg Asp Leu
225                 230                 235                 240

Ala Asp Phe Arg Ile Gln His Pro Asp Leu Ile Leu Leu Gln Tyr Val
                245                 250                 255

Asp Asp Leu Leu Leu Ala Ala Thr Ser Glu Leu Asp Cys Gln Gln Gly
            260                 265                 270

Thr Arg Ala Leu Leu Gln Thr Leu Gly Asp Leu Gly Tyr Arg Ala Ser
        275                 280                 285

Ala Lys Lys Ala Gln Ile Cys Gln Lys Gln Val Lys Tyr Leu Gly Tyr
    290                 295                 300

Leu Leu Lys Glu Gly Gln Arg Trp Leu Thr Glu Ala Arg Lys Glu Thr
305                 310                 315                 320

Val Met Gly Gln Pro Thr Pro Lys Thr Pro Arg Gln Leu Arg Glu Phe
                325                 330                 335

Leu Gly Thr Ala Gly Phe Cys Arg Leu Trp Ile Pro Gly Phe Ala Glu
            340                 345                 350

Met Ala Ala Pro Leu Tyr Pro Leu Thr Lys Thr Gly Thr Leu Phe Asn
        355                 360                 365

Trp Gly Pro Asp Gln Gln Lys Ala Tyr Gln Glu Ile Lys Gln Ala Leu
    370                 375                 380
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Leu Thr Ala Pro Ala Leu Gly Leu Pro Asp Leu Thr Lys Pro Phe Glu
385                 390                 395                 400

Leu Phe Val Asp Glu Lys Gln Gly Tyr Ala Lys Gly Val Leu Thr Gln
                405                 410                 415

Lys Leu Gly Pro Trp Arg Arg Pro Val Ala Tyr Leu Ser Lys Lys Leu
            420                 425                 430

Asp Pro Val Ala Ala Gly Trp Pro Pro Cys Leu Arg Met Val Ala Ala
        435                 440                 445

Ile Ala Val Leu Thr Lys Asp Ala Gly Lys Leu Thr Met Gly Gln Pro
    450                 455                 460

Leu Val Ile Leu Ala Pro His Ala Val Glu Ala Leu Val Lys Gln Pro
465                 470                 475                 480

Pro Asp Arg Trp Leu Ser Asn Ala Arg Met Thr His Tyr Gln Ala Leu
                485                 490                 495

Leu Leu Asp Thr Asp Arg Val Gln Phe Gly Pro Val Val Ala Leu Asn
            500                 505                 510

Pro Ala Thr Leu Leu Pro Leu Pro Glu Glu Gly Leu Gln His Asn Cys
        515                 520                 525

Leu Asp Ile Leu Ala Glu Ala His Gly Thr Arg Pro Asp Leu Thr Asp
    530                 535                 540

Gln Pro Leu Pro Asp Ala Asp His Thr Trp Tyr Thr Gly Gly Ser Ser
545                 550                 555                 560

Leu Leu Gln Glu Gly Gln Arg Lys Ala Gly Ala Ala Val Thr Thr Glu
                565                 570                 575

Thr Glu Val Ile Trp Ala Lys Ala Leu Pro Ala Gly Thr Ser Ala Gln
            580                 585                 590

Arg Ala Gln Leu Ile Ala Leu Thr Gln Ala Leu Arg Met Ala Glu Gly
        595                 600                 605

Lys Lys Leu Asn Val Tyr Thr Asn Ser Arg Tyr Ala Phe Ala Thr Ala
    610                 615                 620

His Ile His Gly Glu Ile Tyr Arg Arg Arg Gly Leu Leu Thr Ser Glu
625                 630                 635                 640

Gly Lys Glu Ile Lys Asn Lys Asp Glu Ile Leu Ala Leu Leu Lys Ala
                645                 650                 655

Leu Phe Leu Pro Lys Arg Leu Ser Ile Ile His Cys Pro Gly His Gln
            660                 665                 670

Lys Gly His Ser Ala Glu Ala Arg Gly Asn Arg Met Ala Asp Gln Ala
        675                 680                 685

Ala Arg Lys Ala Ala Ile Thr Glu Asn Pro Asp Thr Ser Thr Leu Leu
    690                 695                 700

Ile Glu Asn Ser Ser Pro Asn Ser Arg Leu Ile Asn
705                 710                 715


<210> SEQ ID NO 3
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: N-terminal tag sequence

<400> SEQUENCE: 3

Met Ala Ser Gly Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp
1               5                   10                  15
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Asp Asp Asp Lys His
20

<210> SEQ ID NO 4
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Factor Xa cleavage site

<400> SEQUENCE: 4

Ile Glu Gly Arg
1

<210> SEQ ID NO 5
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Thrombin cleavage site

<400> SEQUENCE: 5

Leu Val Pro Arg
1

<210> SEQ ID NO 6
<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: 47mer DNA template

<400> SEQUENCE: 6

gagttacagt gtttttgttc cagtctgtag cagtgtgtga atggaag 47

<210> SEQ ID NO 7
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: 18mer DNA primer

<400> SEQUENCE: 7

cttccattca cacactgc 18

<210> SEQ ID NO 8
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Sequencing primer

<400> SEQUENCE: 8

gaagatcgca ctccagccag c 21

<210> SEQ ID NO 9
<211> LENGTH: 304
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Mutations introduced by SuperScript II into
LacZ Alpha DNA
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (139)..(139)

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<223> OTHER INFORMATION: May be an inserted cytosine, may not be present
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (212)..(212)
<223> OTHER INFORMATION: May be an inserted thymine, may not be present
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (234)..(234)
<223> OTHER INFORMATION: May be 1, 3 or 4 inserted adenines, may not be present
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (277)..(277)
<223> OTHER INFORMATION: May be an inserted cytosine or adenine, may not be present
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (281)..(281)
<223> OTHER INFORMATION: May be 1, 3 or 5 inserted thymines, may not be present
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (285)..(285)
<223> OTHER INFORMATION: May be an inserted cytosine, may not be present
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (304)..(304)
<223> OTHER INFORMATION: May be deleted

<400> SEQUENCE: 9

agcgcaaygc aattaatgtg agttagctya ctcattaggc accccaggyy ytacacttta 60

tgcttccggc tcgyrtgttg tgtggaattg tgagcggata acaattycac acmsgaaaca 120

gctaysacca tgattacgns caagcytgca tgcctgcagg tcgactctag aggatccccg 180

ggtaccgagc tcgaattyac tggycgtcgt tntwacaacg tcgtgwctgg gaanaaccct 240

ggcgttaccy macytaatcg ccytgcagya ymtcycncyt nttcngccrg ctgkcgtaat 300

agcg 304

<210> SEQ ID NO 10
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: 18 mer DNA primer

<400> SEQUENCE: 10

gaacaaaaac actgtaactc 20

<210> SEQ ID NO 11
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: N-terminal tag sequence

<400> SEQUENCE: 11

Met Ala Ser Gly Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp
1 5 10 15

Asp Asp Asp Lys
20

<210> SEQ ID NO 12
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 12

gctcgtcgtc gacaacggct c 21

<210> SEQ ID NO 13
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 13

caaacatgat ctgggtcatc ttctc 25

<210> SEQ ID NO 14
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 14

gtgaaggtcg gagtcaacgg attt 24

<210> SEQ ID NO 15
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 15

cacagtcttc tgggtggcag tgat 24

<210> SEQ ID NO 16
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 16

atggcgatcg tcgaaccgga 20

<210> SEQ ID NO 17
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 17

cactgtctta atatgaatgg gacctactga g 31

<210> SEQ ID NO 18
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 18

gagccaagca gacaagcaaa gcaagc 26

<210> SEQ ID NO 19
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 19

tgttttaatt caatcccacg cccctgt 27

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<210> SEQ ID NO 20
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 20

aaggctggcg gattactgcc 20

<210> SEQ ID NO 21
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 21

gatgctgctg gtgatgtact c 21

<210> SEQ ID NO 22
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 22

gaggagggcg aggtggacgg c 21

<210> SEQ ID NO 23
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 23

gaactaacac acagcgatgg gtgggaa 27

<210> SEQ ID NO 24
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 24

ggagtttatc atcaccgcgg aaatactgag ag 32

<210> SEQ ID NO 25
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 25

tatttcactg acaggcaata ccgtccaagg 30

<210> SEQ ID NO 26
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 26

cgccaaattt ctcccctgaa 20

<210> SEQ ID NO 27
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 27

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ccgtagtgct gggcaatgtt c 21

<210> SEQ ID NO 28
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 28

gctgcagctt catatgatca gttgtta 27

<210> SEQ ID NO 29
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 29

aatggcgctt aggactttgg 20

<210> SEQ ID NO 30
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 30

tggaggctgg gaacgtgaag gaaa 24

<210> SEQ ID NO 31
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 31

acaggaatga ccgagggtaa tcttggc 27

<210> SEQ ID NO 32
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Rattus sp.

<400> SEQUENCE: 32

gcggcgctgg aggagaa 17

<210> SEQ ID NO 33
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Rattus sp.

<400> SEQUENCE: 33

aggtggcggc tcaaacacaa ag 22

<210> SEQ ID NO 34
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 34

cgggtggtaa ctggctgctg tgga 24

<210> SEQ ID NO 35
<211> LENGTH: 24

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<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 35

tggaggctgg gaacgtgaag gaaa 24

<210> SEQ ID NO 36
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 36

gcggacctcc cgcttctgcc gga 23

<210> SEQ ID NO 37
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 37

ccaagtaaaa cagcatcgga acaccagg 28

<210> SEQ ID NO 38
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 38

aaagtcgatc agcagttgcc agggg 25

<210> SEQ ID NO 39
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 39

cccacagtca ccgccgctta cct 23

<210> SEQ ID NO 40
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 40

cttcatccct ccccaacccc aatct 25

<210> SEQ ID NO 41
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 41

gaggagggcg aggtggacgg c 21

<210> SEQ ID NO 42
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 42

gaactaacac acagcgatgg gtgggaa 27

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<210> SEQ ID NO 43
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 43

cgccaaattt ctcccctgaa 20

<210> SEQ ID NO 44
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 44

ccgtagtgct gggcaatgtt c 21

<210> SEQ ID NO 45
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 45

gctcgtcgtc gacaacggct c 21

<210> SEQ ID NO 46
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 46

accacatgat ctgggtcatc ttctc 25

What is claimed is:

1. A reverse transcriptase which has been modified or mutated to increase or enhance thermostability.

2. The reverse transcriptase of claim 1, wherein the reverse transcriptase has one or more modifications or mutations at positions corresponding to amino acids selected from the group consisting of:

(a) leucine 52 of M-MLV reverse transcriptase;

(b) tyrosine 64 of M-MLV reverse transcriptase;

(c) lysine 152 of M-MLV reverse transcriptase;

(d) histidine 204 of M-MLV reverse transcriptase;

(e) methionine 289 of M-MLV reverse transcriptase;

(f) threonine 306 of M-MLV reverse transcriptase; and

(g) phenylalanine 309 of M-MLV reverse transcriptase.

3. The reverse transcriptase of claim 2, which is M-MLV reverse transcriptase.

4. The reverse transcriptase of claim 3, wherein leucine 52 is replaced with proline.

5. The reverse transcriptase of claim 3, wherein tyrosine 64 is replaced with arginine.

6. The reverse transcriptase of claim 3, wherein lysine 152 is replaced with methionine.

7. The reverse transcriptase of claim 3, wherein histidine 204 is replaced with arginine.

8. The reverse transcriptase of claim 3, wherein methionine 289 is replaced with leucine.

9. The reverse transcriptase of claim 3, wherein threonine 306 is replaced with either lysine or arginine.

10. The reverse transcriptase of claim 3, wherein the reverse transcriptase has a mutation or modification at amino acids histidine 204 and threonine 306.

11. The reverse transcriptase of claim 10, wherein histidine 204 is replaced with arginine and threonine 306 is replaced with either lysine or arginine.

12. The reverse transcriptase of claim 1, which retains at least 50% of reverse transcriptase activity after heating to 50° C. for 5 minutes.

13. The reverse transcriptase of claim 1, which retains at least 70% of reverse transcriptase activity after heating to 50° C. for 5 minutes.

14. The reverse transcriptase of claim 1, which retains at least 85% of reverse transcriptase activity after heating to 50° C. for 5 minutes.

15. The reverse transcriptase of claim 1, which retains at least 95% of reverse transcriptase activity after heating to 50° C. for 5 minutes.

16. The reverse transcriptase of claim 1, wherein the reverse transcriptase has one or more properties selected from the group consisting of:

(a) reduced or substantially reduced RNase H activity;

(b) reduced or substantially reduced terminal deoxynucleotidyl transferase activity; and

(c) increased fidelity.

17. The reverse transcriptase of claim 16, wherein the reverse transcriptase has reduced or substantially reduced RNase H activity.

18. The reverse transcriptase of claim 16, wherein the reverse transcriptase has reduced or substantially reduced terminal deoxynucleotidyl transferase activity.

19. The reverse transcriptase of claim 18, wherein the reverse transcriptase has one or more one or more modifications or mutations at positions corresponding to amino acids selected from the group consisting of:

(a) tyrosine 133 of M-MLV reverse transcriptase;

(b) threonine 197 of M-MLV reverse transcriptase; and

(c) phenylalanine 309 of M-MLV reverse transcriptase.

20. The reverse transcriptase of claim 19, which is M-MLV reverse transcriptase.

21. The reverse transcriptase of claim 20, wherein tyrosine 133 is replaced with alanine.

22. The reverse transcriptase of claim 20, wherein threonine 197 is replaced with glutamic acid.

23. The reverse transcriptase of claim 20, wherein phenylalanine 309 is replaced with asparagine.

24. The reverse transcriptase of claim 16, wherein the reverse transcriptase has increased fidelity.

25. The reverse transcriptase of claim 24, wherein the reverse transcriptase has one or more one or more modifications or mutations at positions corresponding to amino acids selected from the group consisting of:

(a) tyrosine 64 of M-MLV reverse transcriptase;

(b) arginine 116 of M-MLV reverse transcriptase;

(c) glutamine 190 of M-MLV reverse transcriptase; and

(d) valine 223 of M-MLV reverse transcriptase.

26. The reverse transcriptase of claim 1, wherein the reverse transcriptase is selected from the group consisting of M-MLV, RSV, AMV, and HIV reverse transcriptases.

27. The reverse transcriptase of claim 26, wherein the reverse transcriptase is selected from the group consisting of M-MLV RNase H− reverse transcriptase, RSV RNase H− reverse transcriptase, AMV RNase H− reverse transcriptase, RAV RNase H− reverse transcriptase, and HIV RNase H− reverse transcriptase.

28. The reverse transcriptase of claim 26, wherein the reverse transcriptase is an M-MLV reverse transcriptase.

29. The reverse transcriptase of claim 28, wherein aspartic acid 524 is replaced with glycine, glutamic acid 562 is replaced with glutamine, and aspartic acid 583 is replaced with asparagine.

30. A vector comprising nucleic acid encoding the reverse transcriptase of claim 1.

31. The vector of claim 30, wherein the nucleic acid is operably linked to a promoter.

32. The vector of claim 31, wherein the promoter is selected from the group consisting of a X-PL promoter, a tac promoter, a trp promoter, an ara BAD promoter and a trc promoter.

33. A host cell comprising the vector of claim 30.

34. A method of producing a reverse transcriptase, the method comprising:

(a) culturing the host cell of claim 33;

(b) expressing the nucleic acid; and

(c) isolating the reverse transcriptase from the host cell.

35. The method of claim 34, wherein the host cell is an *Escherichia coli*.

36. A method for reverse transcription of one or more nucleic acid molecules comprising:

(a) mixing one or more nucleic acid templates with one or more reverse transcriptases of claim 1; and

(b) incubating the mixture of (a) under conditions sufficient to make one or more first nucleic acid molecules complementary to all or a portion of the one or more templates.

37. The method of claim 36, wherein the nucleic acid template is a messenger RNA molecule or a population of mRNA molecules.

38. The method of claim 37, the method further comprising the step of incubating the one or more first nucleic acid molecules under conditions sufficient to make one or more second nucleic acid molecules complementary to all or a portion of the one or more first nucleic acid molecules.

39. A cDNA molecule made according to the method of claim 36.

40. A cDNA molecule made according to the method of claim 38.

41. A method for amplifying one or more nucleic acid molecules, the method comprising:

(a) mixing one or more nucleic acid templates with one or more reverse transcriptases of claim 1 and one or more DNA polymerases; and

(b) incubating the mixture of (a) under conditions sufficient to amplify one or more nucleic acid molecules complementary to all or a portion of the one or more templates.

42. A method for sequencing one or more nucleic acid molecules, the method comprising:

(a) mixing one or more nucleic acid molecules to be sequenced with one or more primers, one or more reverse transcriptases of claim 1, one or more nucleotides and one or more terminating agents;

(b) incubating the mixture of (a) under conditions sufficient to synthesize a population of molecules complementary to all or a portion of the one or more molecules to be sequenced; and

(c) separating the population to determine the nucleotide sequence of all or a portion of the one or more molecules to be sequenced.

43. A method for sequencing a nucleic acid molecule, the method comprising:

(a) mixing a nucleic acid molecules to be sequenced with one or more primers, one or more reverse transcriptases of claim 1, one or more nucleotides and one or more terminating agents;

(b) incubating the mixture of (a) under conditions sufficient to synthesize a population of molecules complementary to all or a portion of the molecule to be sequenced; and

(c) separating members of the population to determine the nucleotide sequence of all or a portion of the molecule to be sequenced.

44. A kit for use in reverse transcription, amplification or sequencing of a nucleic acid molecule, the kit comprising one or more reverse transcriptases of claim 1.

45. The kit of claim 44, the kit further comprising one or more components selected from the group consisting of one or more nucleotides, one or more DNA polymerases, a suitable buffer, one or more primers and one or more terminating agents.

46. The kit of claim 45, wherein the terminating agent is a dideoxynucleotide.

47. The kit of claim 44, wherein the reverse transcriptase is an M-MLV reverse transcriptase.

48. The kit of claim 47, wherein the reverse transcriptase has one or more modifications or mutations at positions corresponding to. amino acids selected from the group consisting of:

(a) leucine 52 of M-MLV reverse transcriptase;

(b) tyrosine 64 of M-MLV reverse transcriptase;

(c) lysine 152 of M-MLV reverse transcriptase;

(d) arginine 204 of M-MLV reverse transcriptase;

(e) methionine 289 of M-MLV reverse transcriptase;

(f) threonine 306 of M-MLV reverse transcriptase; and

(g) phenylalanine 309 of M-MLV reverse transcriptase.

49. A modified or mutated reverse transcriptase having an RNA-dependent DNA polymerase activity that has a half-life of greater than 10 minutes at 50° C.

50. The reverse transcriptase of claim 49, which is a retroviral reverse transcriptase.

51. The reverse transcriptase of claim 50 which is selected from a group consisting of M-MLV reverse transcriptase, ASV reverse transcriptase, HIV reverse transcriptase, Avian Sarcoma-Leukosis Virus (ASLV) reverse transcriptase, Rous Sarcoma Virus (RSV) reverse transcriptase, Avian Myeloblastosis Virus (AMV) reverse transcriptase, Avian Erythroblastosis Virus (AEV) Helper Virus MCAV reverse transcriptase, Avian Myelocytomatosis Virus MC29 Helper Virus MCAV reverse transcriptase, Avian Reticuloendotheliosis Virus (REV-T) Helper Virus REV-A reverse transcriptase, Avian Sarcoma Virus UR2 Helper Virus UR2AV reverse transcriptase, Avian Sarcoma Virus Y73 Helper Virus YAV reverse transcriptase, Rous Associated Virus (RAV) reverse transcriptase, Myeloblastosis Associated Virus (MAV) reverse transcriptase, and fragments of any of the above having reverse transcriptase activity.

52. The reverse transcriptase of claim 49, wherein the half-life is greater than about 100 minutes.

53. The reverse transcriptase of claim 49, wherein the half-life is greater than about 200 minutes.

54. A modified or mutated reverse transcriptase having a reverse transcriptase activity that has a half-life of greater than about 2 minutes at 55° C.

55. The reverse transcriptase of claim 54, which is a retroviral reverse transcriptase.

56. The reverse transcriptase of claim 55, which is selected from a group consisting of M-MLV reverse transcriptase, ASV reverse transcriptase, HIV reverse transcriptase, Avian Sarcoma-Leukosis Virus (ASLV) reverse transcriptase, Rous Sarcoma Virus (RSV) reverse transcriptase, Avian Myeloblastosis Virus (AMV) reverse transcriptase, Avian Erythroblastosis Virus (AEV) Helper Virus MCAV reverse transcriptase, Avian Myelocytomatosis Virus MC29 Helper Virus MCAV reverse transcriptase, Avian Reticuloendotheliosis Virus (REV-T) Helper Virus REV-A reverse transcriptase, Avian Sarcoma Virus UR2 Helper Virus UR2AV reverse transcriptase, Avian Sarcoma Virus Y73 Helper Virus YAV reverse transcriptase, Rous Associated Virus (RAV) reverse transcriptase, Myeloblastosis Associated Virus (MAV) reverse transcriptase, and fragments of any of the above having reverse transcriptase activity.

57. The reverse transcriptase of claim 54, wherein the half-life is greater than about 5 minutes.

58. A reverse transcriptase that synthesizes an amount of full length product, wherein the amount of full length product synthesized at 50° C. is at least 50% or more of the amount of full length product it synthesizes at 40° C.

59. The reverse transcriptase of claim 58, wherein the amount of full length product synthesized at 50° C. is at least 70% or more of the amount of full length product it synthesizes at 40° C.

60. The reverse transcriptase of claim 58, wherein the amount of full length product synthesized at 50° C. is at least 80% or more of the amount of full length product it synthesizes at 40° C.

61. The reverse transcriptase of claim 58, wherein the amount of full length product synthesized at 50° C. is at least 90% or more of the amount of full length product it synthesizes at 40° C.

62. A reverse transcriptase that synthesizes an amount of full length product, wherein the amount of full length product synthesized at 52.5° C is at least 20% or more of the amount of full length product it synthesizes at 40° C.

63. The reverse transcriptase of claim 62, wherein the amount of full length product synthesized at 52.5° C. is at least 40% or more of the amount of full length product it synthesizes at 40° C.

64. The reverse transcriptase of claim 62, wherein the amount of full length product synthesized at 52.5° C. is at least 50% or more of the amount of full length product it synthesizes at 40° C.

65. The reverse transcriptase of claim 62, wherein the amount of full length product synthesized at 52.5° C. is at least 60% or more of the amount of full length product it synthesizes at 40° C.

66. A reverse transcriptase that synthesizes an amount of full length product, wherein the amount of full length product synthesized at 55° C. is at least 1% or more of the amount of full length product it synthesizes at 40° C.

67. The reverse transcriptase of claim 66, wherein the amount of full length product synthesized at 55° C. is at least 5% or more of the amount of full length product it synthesizes at 40° C.

68. The reverse transcriptase of claim 66, wherein the amount of full length product synthesized at 55° C. is at least 10% or more of the amount of full length product it synthesizes at 40° C.

69. The reverse transcriptase of claim 66, wherein the amount of full length product synthesized at 55° C. is at least 20% or more of the amount of full length product it synthesizes at 40° C.

70. The reverse transcriptase of any one of claims **58-69**, which is a modified or mutated reverse transcriptase.

71. The reverse transcriptase of claim 70, which comprises one or more modifications or mutations corresponding to amino acids selected from the group consisting of:

(a) leucine 52 of M-MLV reverse transcriptase;

(b) tyrosine 64 of M-MLV reverse transcriptase;

(c) lysine 152 of M-MLV reverse transcriptase;

(d) histidine 204 of M-MLV reverse transcriptase;

(e) methionine 289 of M-MLV reverse transcriptase;

(f) threonine 306 of M-MLV reverse transcriptase; and

(g) phenylalanine 309 of M-MLV reverse transcriptase.

72. The reverse transcriptase of claim 71, which comprises modifications or mutations corresponding to H204R, M289L, T306K, and F309N of M-MLV reverse transcriptase.

73. A modified or mutated thermostable reverse transcriptase, wherein the reverse transcriptase has an increase in thermostability of greater than about 1.5 fold at 50° C. compared to a corresponding unmodified or un-mutated reverse transcriptase.

74. The reverse transcriptase of claim 73, wherein the increase in thermostability is measured by comparing an amount of full length product synthesized by the mutated reverse transcriptase to an amount synthesized by the unmodified or un-mutated reverse transcriptase.

75. A modified or mutated thermostable reverse transcriptase, wherein the reverse transcriptase has an increase in thermostability of greater than about 2 fold at 52.5° C. compared to a corresponding unmodified or un-mutated reverse transcriptase.

76. The reverse transcriptase of claim 75, wherein the reverse transcriptase has an increase in thermostability of greater than about 5 fold at 52.5° C. compared to a corresponding unmodified or un-mutated reverse transcriptase.

77. The reverse transcriptase of claim 75, wherein the reverse transcriptase has an increase in thermostability of greater than about 8 fold at 52.5° C. compared to a corresponding unmodified or un-mutated reverse transcriptase.

78. The reverse transcriptase according to claim 75, wherein the increase in thermostability is measured by comparing an amount of full length product synthesized by the mutated reverse transcriptase to an amount synthesized by the unmodified or un-mutated reverse transcriptase.

79. A mutated thermostable reverse transcriptase, wherein the reverse transcriptase has an increase in thermostability of greater than about 5 fold at 55° C. compared to a corresponding unmodified or un-mutated reverse transcriptase.

80. The reverse transcriptase of claim 79, wherein the reverse transcriptase has an increase in thermostability of greater than about 10 fold at 55° C. compared to a corresponding unmodified or un-mutated reverse transcriptase.

81. The reverse transcriptase of claim 79, wherein the reverse transcriptase has an increase in thermostability of greater than about 50 fold at 55° C. compared to a corresponding unmodified or un-mutated reverse transcriptase.

82. The reverse transcriptase of claim 79, wherein the increase in thermostability is measured by comparing an amount of full length product synthesized by the mutated reverse transcriptase to an amount synthesized by the unmodified or un-mutated reverse transcriptase.

83. A composition comprising a reverse transcriptase which has been modified or mutated to increase or enhance thermostability.

84. The composition of claim 83, wherein the reverse transcriptase has one or more modifications or mutations at positions corresponding to amino acids selected from the group consisting of:

(a) leucine 52 of M-MLV reverse transcriptase;

(b) tyrosine 64 of M-MLV reverse transcriptase;

(c) lysine 152 of M-MLV reverse transcriptase;

(d) histidine 204 of M-MLV reverse transcriptase;

(e) methionine 289 of M-MLV reverse transcriptase;

(f) threonine 306 of M-MLV reverse transcriptase; and

(g) phenylalanine 309 of M-MLV reverse transcriptase.

85. The composition of claim 83, wherein the reverse transcriptase comprises modifications or mutations corresponding to H204R, M289L, T306K, and F309N of M-MLV reverse transcriptase.

86. The composition of claim 83, further comprising a DNA polymerase.

87. The composition of claim 83, further comprising an mRNA molecule.

88. The composition of claim 83, further comprising one or more nucleoside triphosphates.

89. The composition of claim 88, wherein at least one nucleotide comprises a label.

90. The composition of claim 89, wherein at least one label is a fluorescent label.

91. The composition of claim 83, further comprising Mg^{2+} and not containing Mn^{2+}.

92. A method of preparing a labeled nucleic acid, comprising:

(a) hybridizing a primer to a first nucleic acid template molecule to form a complex; and

(b) incubating the complex in the presence of a polypeptide of the invention and one or more deoxyribonucleoside triphosphates under conditions sufficient to synthesize a second nucleic acid molecule complementary to all or a portion of the template, wherein the second nucleic acid molecule comprises at least one modified nucleotide and/or at least one labeled nucleotide.

93. The method of claim 92, wherein the template comprises about 20 μg of total RNA or 1 μg poly(A)RNA.

94. The method of claim 92, wherein the conditions comprise incubation at a temperature of from about 42° C. to about 60° C.

95. The method of claim 94, wherein the conditions comprise incubation at a temperature of from about 50° C. to about 55° C.

96. A labeled nucleic acid molecule produced by the method of claim 92.

97. A method of detecting a target nucleic acid sequence, comprising contacting a sample comprising the target sequence with the nucleic acid molecule of claim 96, and detecting the nucleic acid molecule of claim 96.

98. The method of claim 97, wherein from about 1 pg to about 100 pg of target sequence is present in the sample.

99. The method of claim 98, wherein about 10 pg of target sequence is present in the sample.

100. A method according to claim 92, wherein conditions comprise at least one modified nucleotide.

101. The method of claim 101, wherein the template comprises about 5 μg of total RNA or about 0.4 μg poly(A)RNA.

102. The method of claim 100, wherein the conditions comprise incubation at a temperature of from about 42° C. to about 60° C.

103. The method of claim 100, wherein the conditions comprise incubation at a temperature of from about 50° C. to about 55° C.

104. The method of claim 100, further comprising reacting the second nucleic acid molecule with a dye containing molecule to produce a labeled nucleic acid molecule.

105. A labeled nucleic acid molecule produced by the method of claim 105.

106. A method of detecting a target nucleic acid sequence, comprising

contacting a sample comprising the target sequence with the nucleic acid molecule of claim 105, and

detecting the nucleic acid molecule of claim 105.

107. The method of claim 106, wherein from about 0.1 pg to about 100 pg of target sequence is present in the sample.

108. The method of claim 107, wherein about 2 pg of target sequence is present in the sample.

109. The method of claim 104, wherein conditions result in uniform dye incorporation for both Cy3 and Cy5.

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