

sequence, resulting in a substantial increase in amplification during a set period of time. That is, a second primer nucleic acid is hybridized to a second target sequence, that is substantially complementary to the first target sequence, to form a second hybridization complex. The addition of the enzyme, followed by disassociation of the second hybridization complex, results in the generation of a number of newly synthesized second strands.

[0164] In this way, a number of target molecules are made, and transferred to a detection module, described below. As is more fully outlined below, these reactions (that is, the products of these reactions) can be detected in a number of ways. In general, either direct or indirect detection of the target products can be done. "Direct" detection as used in this context, as for the other amplification strategies outlined herein, requires the incorporation of a label, in this case an electron transfer moiety (ETM), into the target sequence, with detection proceeding according to either "mechanism-1" or "mechanism-2", outlined below. In this embodiment, the ETM(s) may be incorporated in three ways: (1) the primers comprise the ETM(s), for example attached to the base, a ribose, a phosphate, or to analogous structures in a nucleic acid analog; (2) modified nucleosides are used that are modified at either the base or the ribose (or to analogous structures in a nucleic acid analog) with the ETM(s); these ETM modified nucleosides are then converted to the triphosphate form and are incorporated into the newly synthesized strand by a polymerase; or (3) a "tail" of ETMs can be added, as outlined below. Either of these methods result in a newly synthesized strand that comprises ETMs, that can be directly detected as outlined below.

[0165] Alternatively, indirect detection proceeds as a sandwich assay, with the newly synthesized strands containing few or no ETMs. Detection then proceeds via the use of label probes that comprise the ETM(s); these label probes will hybridize either directly to the newly synthesized strand or to intermediate probes such as amplification probes, as is more fully outlined below. In this case, it is the ETMs on the label probes that are used for detection as outlined below.

[0166] In a preferred embodiment, the target amplification technique is nucleic acid sequence based amplification (NASBA). NASBA is generally described in U.S. Pat. No. 5,409,818 and "Profiting from Gene-based Diagnostics", CTB International Publishing Inc., N.J., 1996, both of which are expressly incorporated by reference in their entirety.

[0167] In general, NASBA may be described as follows. A single stranded target nucleic acid, usually an RNA target sequence (sometimes referred to herein as "the first target sequence" or "the first template"), is contacted with a first NASBA primer. A "NASBA primer" generally has a length of 25-100 nucleotides, with NASBA primers of approximately 50-75 nucleotides being preferred. The first NASBA primer is preferably a DNA primer that has at its 3' end a sequence that is substantially complementary to the 3' end of the first template. The first NASBA primer has an RNA polymerase promoter at its 5' end. The first NASBA primer is then hybridized to the first template to form a first hybridization complex. The NASBA reaction mixture also includes a reverse transcriptase enzyme (an "NASBA reverse transcriptase") and a mixture of the four dNTPs, such that the first NASBA primer is modified, i.e. extended,

to form a modified first primer, comprising a hybridization complex of RNA (the first template) and DNA (the newly synthesized strand).

[0168] By "reverse transcriptase" or "RNA-directed DNA polymerase" herein is meant an enzyme capable of synthesizing DNA from a DNA primer and an RNA template. Suitable RNA-directed DNA polymerases include, but are not limited to, avian myeloblastosis virus reverse transcriptase ("AMV RT") and the Moloney murine leukemia virus RT.

[0169] In addition to the components listed above, the NASBA reaction also includes an RNA degrading enzyme, also sometimes referred to herein as a ribonuclease, that will hydrolyze RNA of an RNA:DNA hybrid without hydrolyzing single- or double-stranded RNA or DNA. Suitable ribonucleases include, but are not limited to, RNase H from *E. coli* and calf thymus.

[0170] The ribonuclease degrades the first RNA template in the hybridization complex, resulting in a disassociation of the hybridization complex leaving a first single stranded newly synthesized DNA strand, sometimes referred to herein as "the second template".

[0171] In addition, the NASBA reaction also includes a second NASBA primer, generally comprising DNA (although as for all the probes herein, including primers, nucleic acid analogs may also be used). This second NASBA primer has a sequence at its 3' end that is substantially complementary to the 3' end of the second template, and also contains an antisense sequence for a functional promoter and the antisense sequence of a transcription initiation site. Thus, this primer sequence, when used as a template for synthesis of the third DNA template, contains sufficient information to allow specific and efficient binding of an RNA polymerase and initiation of transcription at the desired site. Preferred embodiments utilizes the antisense promoter and transcription initiation site are that of the T7 RNA polymerase, although other RNA polymerase promoters and initiation sites can be used as well, as outlined below.

[0172] The second primer hybridizes to the second template, and a DNA polymerase, also termed a "DNA-directed DNA polymerase", also present in the reaction, synthesizes a third template (a second newly synthesized DNA strand), resulting in second hybridization complex comprising two newly synthesized DNA strands.

[0173] Finally, the inclusion of an RNA polymerase and the required four ribonucleoside triphosphates (ribonucleotides or NTPs) results in the synthesis of an RNA strand (a third newly synthesized strand that is essentially the same as the first template). The RNA polymerase, sometimes referred to herein as a "DNA-directed RNA polymerase", recognizes the promoter and specifically initiates RNA synthesis at the initiation site. In addition, the RNA polymerase preferably synthesizes several copies of RNA per DNA duplex. Preferred RNA polymerases include, but are not limited to, T7 RNA polymerase, and other bacteriophage RNA polymerases including those of phage T3, phage  $\phi$ II, Salmonella phage sp6, or Pseudomonas phage gh-1.

[0174] Accordingly, the NASBA reaction requires, in no particular order, a first NASBA primer, a second NASBA primer comprising an antisense sequence of an RNA polymerase promoter, an RNA polymerase that recognizes the