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(54) Title: POLYNUCLEOTIDE AMPLIFICATION METHOD

(57) Abstract: Methods useful for improving results obtained with enzyme-based polynucleotide amplification reactions. More particularly, the invented methods are useful for: (1) promoting amplification of template-specific products such that the amount of amplicon produced reflects the pre-amplification amount of analyte, even in reactions primed with low levels of analyte polynucleotide; (2) facilitating biological specimen processing such that the amount of amplicon produced in subsequent amplification reactions will be substantially independent of the efficiency of analyte polynucleotide isolation from the specimen; and (3) controlling the amount of analyte amplicon produced in the amplification reaction.

POLYNUCLEOTIDE AMPLIFICATION METHOD

This application claims priority of provisional patent application Ser. No. 60/145,432 filed on Jul. 23, 1999.

Field of the Invention

The present invention relates to methods and compositions useful for improving the precision and quantitative capacity of polynucleotide amplification reactions commonly performed in molecular genetics laboratories.

Background of the Invention

Enzyme-based procedures for amplifying polynucleotides are now established tools for diagnostic, environmental and forensic testing. The market for DNA probe diagnostics in clinical laboratories now represents several hundred million dollars each year. The clinical diagnostics-probe business is expected to grow with viral screening and viral load determination representing major areas of active market expansion. Given the commercial value of this technology, great efforts have been invested in research and development of improved amplification procedures (see *Genetic Engineering News* **17**:6 (1997)).

Recently developed techniques for amplifying analyte polynucleotides have provided useful alternatives to methods based on the original Polymerase Chain Reaction (PCR) protocol. According to one technique, DNA amplification reactions are performed on solid-phase substrates made alternatively of glass, plastic, a semiconductor chip or a fiber-optic array. Labeled target DNA is synthesized as a molecular bridge between pairs of oligonucleotide primers immobilized to the solid substrate such that the amplification products remain attached to the solid substrate. U.S. Patent No. 5,399,491 discloses a different technique wherein a target polynucleotide is amplified autocatalytically under conditions of substantially constant temperature, ionic strength and pH. This method, termed Transcription Mediated Amplification (TMA), allows for the synthesis of multiple RNA copies of target sequence. New methods likely to emerge in the future will continue to expand the range of applications that can be addressed by polynucleotide amplification techniques.

Quantitative amplification assays represent one subset of assays that impose stringent requirements on all aspects of the procedure, including template isolation and standardizing amplification efficiency. Approaches that employ internal standards that participate in amplification reactions are intended to normalize reaction efficiency, but fail to account for

variable levels of analyte polynucleotide input into the reaction. Related methods that simultaneously amplify an analyte polynucleotide and control polynucleotides derived from constitutively expressed housekeeping genes also are imperfect because multiple primer sets are required to carry out the amplification reaction.

One example of methods based on the use of internal standards in quantitative PCR amplifications is disclosed in U.S. Patent No. 5,219,727. According to the method disclosed in this patent, the internal standard is included in the amplification reaction and is designed so that it will amplify at a similar efficiency as the target polynucleotide. Like methods that co-amplify constitutively expressed gene products for use as internal standards, the method disclosed in U.S. Patent No. 5,219,727 requires detecting and quantifying the amplicon derived from the internal standard in order to quantify the analyte polynucleotide. Thus, several steps still are required to quantitate analyte polynucleotides when an internal standard must be detected and quantitated

The fact that amplified polynucleotides ("amplicons") in conventional PCR and TMA procedures are synthesized as molecules free in solution represents another source of inaccuracy for analyte detection. These amplicons can easily be transferred between samples to produce false-positive results in the contaminated reactions. Standard precautions for minimizing false-positive results due to contamination by carried-over DNA templates include ultraviolet irradiation of pipetting devices, the use of disposable glass- and plastic-ware, use of separate laboratories or laboratory areas for conducting amplification reactions, and avoiding the formation of aerosols. One elaborate approach for ensuring that PCR products cannot be re-amplified in subsequent reactions involves a series of steps using specialized reagents to degrade the products from previous PCR amplifications. However, this procedure is somewhat complicated and involves first substituting dUTP for dTTP in the PCR mixture and then pre-treating all subsequent PCR mixtures with a uracil N-glycosylase (UNG) enzyme prior to PCR amplification. Products from previous PCR amplifications are then eliminated by excising uracil residues using UNG, and degrading the resulting abasic polynucleotide (Longo, et al., *Gene* **93**:125 (1990)). Clearly, these methods do not lend themselves to high throughput assays.

Accordingly, there exists a continuing need for techniques that can be used to enhance the precision of polynucleotide amplification procedures. Further, there exists a need for

techniques that can be used to diminish the incidence of false-positive results arising from positive carry-over contamination. The present invention addresses both of these needs.

Summary of the Invention

A first aspect of the invention relates to a method for quantifying analyte polynucleotides that are present in a test sample. The method includes steps for: (1) obtaining a test sample that contains an unknown amount of an analyte polynucleotide; (2) combining a predetermined amount of this test sample with a predetermined amount of a pseudo target; (3) co-amplifying in a polynucleotide amplification reaction the analyte polynucleotide and the pseudo target to produce a collection of amplification products that includes both an analyte amplicon if the sample contained the analyte polynucleotide and a pseudo target amplicon; and (4) quantifying the analyte amplicon without relying on information regarding the amount of pseudo target amplicon produced in the reaction, whereby the quantity of analyte amplicon is related in a dose-dependent manner to the unknown amount analyte polynucleotide that was present in the original test sample. Optionally there can be an added step for detecting the pseudo target amplicon. This optional step may be useful, for example, as a positive control for the amplification reaction. In certain preferred embodiments of the invention, the step for quantifying the analyte amplicon involves first hybridizing the collection of amplification products from the co-amplifying step with a labeled probe that is specific for the analyte amplicon but not the pseudo target amplicon, and then detecting any labeled probe that specifically hybridized to the analyte amplicon. Of course, it is to be understood that the analyte amplicon-specific probe can be a probe that binds the analyte polynucleotide or a nucleic acid strand that is complementary thereto. In other embodiments, the polynucleotide amplification reaction in the co-amplifying step can be any one of a Transcription Mediated Amplification (TMA) reaction, a NASBA reaction or a Polymerase Chain Reaction, with the TMA reaction representing a highly preferred embodiment of the invention. Regardless of the type of amplification reaction that is employed, the obtaining step can involve first collecting a biological specimen and then releasing nucleic acids contained in the specimen to result in the sample that contains the unknown amount of analyte polynucleotide. For all types of amplification reactions the amount of pseudo target in the combining step preferably is between 1×10^3 and 2×10^8 molecules, more preferably between 1×10^4 and 2×10^8 molecules, and still more preferably between 1×10^5 and 2×10^8 molecules. Optionally there can be included an

additional step for capturing the analyte polynucleotide onto a solid support prior to the co-amplifying step. In embodiments of the invented method that employ the additional capturing step the amount of pseudo target used in the combining step preferably ranges from between 1 x 10^3 and 2 x 10^8 molecules, more preferably between 1 x 10^4 and 2 x 10^8 molecules, and still more preferably between 1 x 10^5 and 2 x 10^8 molecules. An exemplary solid support is a bead that is derivatized with a synthetic polynucleotide. The biological specimen used in the procedure can be a blood sample or a plasma sample, and the nucleic acids contained in the specimen can include viral nucleic acids. In one embodiment of the invention, the analyte polynucleotide used in the procedure is a nucleic acid that is released from HIV virions. When the polynucleotide amplification reaction used in the invented method is the TMA reaction, there can be included in the method a further step for isolating the analyte polynucleotide and the pseudo target after the combining step and before the co-amplifying step. In embodiments of the invented method wherein the Transcription Mediated Amplification reaction is employed, the amount of the pseudo target used in the reaction preferably is between 1 x 10^3 and 2 x 10^8 molecules, more preferably between 1 x 10^4 and 2 x 10^8 molecules, and still more preferably between 1 x 10^5 and 2 x 10^8 molecules. When the step for quantitatively detecting involves hybridizing a labeled probe that is specific for the analyte amplicon, the labeled probe can be labeled with acridinium ester, in which case the step for quantitatively detecting may involve performing luminometry. In embodiments of the invention wherein the obtaining step involves collecting a biological specimen and releasing nucleic acids contained therein, the analyte polynucleotide can be a viral polynucleotide. In general, the invented method can involve the further step of consulting a standard curve that relates pre-amplification amounts of analyte polynucleotide and post-amplification amounts of analyte amplicon. This step for consulting a standard curve also is applicable when luminometry is employed to measure hybridization of probes labeled with acridinium ester, or when the amplification reaction is particularly a Transcription Mediated Amplification reaction. In still other preferred embodiments that employ the Transcription Mediated Amplification reaction, a paired set of oligonucleotide primers having the sequences of SEQ ID NO:1 and SEQ ID NO:2 can be used for conducting the reaction, and the pseudo target can have a polynucleotide sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:9.

Another aspect of the invention concerns a method for relating pre-amplification amounts of analyte polynucleotide and post-amplification amounts of analyte amplicon. This method includes steps for: (1) obtaining a plurality of control samples that include different predetermined amounts of an analyte polynucleotide; (2) combining each of the plurality of samples with a constant amount of a pseudo target to result in a plurality of mixed samples; (3) co-amplifying in a plurality of amplification reactions both the pseudo target and any of the analyte polynucleotide present in each of the plurality of mixed samples to produce amplification products, the amplification products including both a pseudo target amplicon for each of the plurality of mixed samples and an analyte amplicon for any of the plurality of mixed samples that contained the analyte polynucleotide; (4) quantifying the analyte amplicon for each of the plurality of amplification reactions without reference to the amount of pseudo target amplicon present in the collection of amplification products; and (5) preparing a standard curve having the different predetermined amounts of analyte polynucleotide plotted against the quantified amounts of analyte amplicon for each of the plurality of amplification reactions, thereby relating the pre-amplification amounts of analyte polynucleotide present in each of the plurality of control samples and the post-amplification amounts of analyte amplicon synthesized in each of the amplification reactions. Optionally there can be an added step for detecting the pseudo target amplicon. This optional step may be useful, for example, as a positive control for the amplification reaction. In a preferred embodiment the analyte polynucleotide is a viral polynucleotide, such as an HIV polynucleotide. Generally, the constant predetermined amount of pseudo target can range between 1×10^3 and 2×10^8 molecules, more preferably between 1×10^4 and 2×10^8 molecules, and still more preferably between 1×10^5 and 2×10^8 molecules. According to other embodiments of the invented method, the plurality of amplification reactions in the co-amplifying step can be any of a plurality of Transcription Mediated Amplification reactions, a plurality of NASBA reactions and a plurality of PCR reactions. In a collection of highly preferred embodiments, the amplification reactions in the co-amplifying step are Transcription Mediated Amplification reactions. Regardless of the type of amplification reactions that are employed, the quantifying step can involve first hybridizing the amplification products from the co-amplifying step with a labeled probe specific for the analyte amplicon but not the pseudo target amplicon and then quantitatively detecting any labeled probe that specifically hybridized. In certain instances, the labeled probe is labeled with acridinium ester.

In still other preferred embodiments wherein the quantifying step involves hybridization with a labeled analyte amplicon-specific probe, there can be an additional step for capturing the analyte polynucleotide onto a solid support prior to the co-amplifying step.

Yet another aspect of the invention relates to kits that can be used for performing polynucleotide amplification reactions using analyte polynucleotide templates. Exemplary kits can include: a pseudo target; at least one pair of oligonucleotide primers for co-amplifying the pseudo target and the analyte polynucleotide; reagents for carrying out the polynucleotide amplification reaction, including deoxynucleotide triphosphates and a DNA polymerizing enzyme; and printed instructions with directions for first carrying out the amplification reaction and then detecting only analyte amplicons produced in the amplification reaction. In one embodiment, the invented kit can also include a labeled probe for detecting any analyte amplicons produced in the amplification reaction. According to another embodiment, the invented kit further includes nucleotide triphosphates and an RNA polymerizing enzyme. The DNA polymerizing enzyme included in the kits can be a reverse transcriptase. In a highly preferred embodiment, no RNase H additional to that provided by the reverse transcriptase is used in the kit.

Yet another aspect of the invention relates to a qualitative method of determining whether a biological sample contains an analyte polynucleotide. This method includes steps for: (1) obtaining a biological sample to be tested for the presence of the analyte polynucleotide; (2) combining the biological sample with a pseudo target to result in a mixed sample; (3) isolating nucleic acids from the mixed sample, whereby there is obtained a collection of molecules that include the pseudo target and any of the analyte polynucleotide present in the biological sample; (4) conducting a polynucleotide amplification reaction to co-amplify the pseudo target and any of the analyte polynucleotide contained in the collection of molecules to produce amplification products, whereby pseudo target amplicons are formed, and whereby analyte amplicons are formed if the collection of molecules included the analyte polynucleotide; (5) detecting in the amplification products any of the analyte amplicons without detecting the pseudo target amplicons; and (6) determining that the biological sample contains the analyte polynucleotide if the analyte amplicons are detected among the amplification products. In certain embodiments, the amplification reaction is any of a Transcription Mediated Amplification reaction, a NASBA reaction and a PCR reaction. In certain highly preferred embodiments, the amplification

reaction is a Transcription Mediated Amplification reaction. When the Transcription Mediated Amplification reaction is employed, the obtaining step can involve drawing blood. Regardless of the type of amplification reaction that is employed, the detecting step can involve first hybridizing a labeled polynucleotide probe having binding specificity for the analyte amplicons and then measuring the extent of specific binding of the labeled polynucleotide probe. When the detecting step involves hybridizing a labeled analyte amplicon-specific probe, the isolating step can involve immobilizing the pseudo target and the analyte polynucleotide to a solid support. According to another preferred embodiment, the detecting step involves detecting by luminometry. In still yet another preferred embodiment, the analyte polynucleotide is from HIV virions. When this is the case, the pseudo target can have a sequence that is either SEQ ID NO:4 or SEQ ID NO:9.

Definitions

As used herein, the following terms have the following meanings unless expressly stated to the contrary.

A "polynucleotide" may be either RNA or DNA unless specified otherwise.

An "oligonucleotide" is a polynucleotide molecule having a length of from 10 to 100 nucleotides, or more preferably 10 to 50 nucleotides. Ordinarily, oligonucleotides will be synthesized by organic chemical methods and will be single-stranded unless specified otherwise. Oligonucleotides may be labeled with a detectable label.

An "amplicon" is a polynucleotide product generated in an amplification reaction.

An "analyte amplicon" is a polynucleotide product of an amplification reaction wherein an analyte polynucleotide served as the template for synthesis of polynucleotide copies or amplification products.

An "analyte polynucleotide" is a target polynucleotide that is to be replicated by a nucleic acid amplification process such as the TMA protocol, but is structurally distinguishable from a pseudo target polynucleotide. The two polynucleotides may be distinguishable, for example, by virtue of the presence or absence of a restriction enzyme cleavage site or an internal sequence difference that is distinguishable by a hybridization probe.

A "target polynucleotide" has a target sequence to be replicated, may be either single-stranded or double-stranded, and may include sequences in addition to the target sequence, which additional sequences may not be amplified.

A "target sequence" refers to the particular nucleotide sequence of the target polynucleotide which is to be amplified. The target sequence includes the complexing sequences to which oligonucleotide primers useful in the amplification reaction can hybridize prior to extension by a DNA polymerase. Where the target polynucleotide is originally single stranded, the term "target sequence" will also refer to the sequence complementary to the target polynucleotide. Where the target polynucleotide is originally double-stranded, the term "target sequence" refers to both the (+) and (-) strands that are complementary to each other.

A "pseudo target" is a polynucleotide that can be co-amplified with the analyte polynucleotide in a single amplification reaction. The pseudo target and the analyte polynucleotide may be amplified using the same set of oligonucleotide primers. However, it is also possible for the pseudo target and the analyte polynucleotide to co-amplify using independent primer sets. The pseudo target and the analyte polynucleotide will be nonidentical molecules so that the analyte polynucleotide and the pseudo target can be distinguished from each other.

A "pseudo target amplicon" is a polynucleotide product of an amplification reaction wherein a pseudo target served as the template for synthesis of polynucleotide copies or amplification products.

A "polynucleotide amplification reaction" is a template-dependent *in vitro* enzyme-catalyzed reaction for increasing the number of target polynucleotides.

In the context of the invention, "quantitatively detecting" or "quantifying" refers to a process for determining the extent of polynucleotide or amplicon production.

A "labeled probe" is a nucleotide polymer that harbors a detectable moiety and that can combine with a complementary single-stranded target nucleic acid sequence to form a double-stranded hybrid. The term also includes analogs of naturally occurring nucleotides and particularly includes analogs having a methoxy group at the 2' position of the ribose (OMe). The detectable moiety may be attached to the end(s) of the probe or may be positioned internally within the sequence of the probe. In general, labeled probes will be about 10 to about 100 nucleotides in length, but can be longer than 100 or shorter than 10 nucleotides,

A "detectable moiety" is a molecule attached to, or synthesized as part of, a labeled probe. This molecule should be uniquely detectable and will allow the probe to be detected as a

result. These detectable moieties are often radioisotopes, chemiluminescent molecules, enzymes, haptens, or even unique oligonucleotide sequences.

A "labeled probe specific for an analyte amplicon" is a labeled probe having a polynucleotide sequence complementary to a polynucleotide product synthesized in an amplification reaction wherein an analyte polynucleotide served as the template for synthesis of amplification products. Since an amplicon is a polynucleotide product generated in an amplification reaction, the labeled probe specific for the analyte amplicon can be complementary to any polynucleotide strand generated in the reaction. Thus, if an analyte polynucleotide is a single-stranded molecule that contains a target sequence, and if copies of the target sequence and its complement are generated in the amplification reaction, then the labeled probe specific for the analyte amplicon can be complementary to the target sequence or its complement.

"Co-amplifying" as used herein refers to the process of amplifying in a polynucleotide amplification reaction more than one species of target polynucleotide. For example, "co-amplifying an analyte polynucleotide and a pseudo target" is intended to refer to the process of simultaneously amplifying the two polynucleotides to result in the formation of analyte amplicons and pseudo target amplicons, respectively.

As used herein "obtaining" a sample that includes, or that may include, an analyte polynucleotide can mean either obtaining from a biological subject such as a human, or obtaining from a reagent depository, such as a commercial vendor. When a sample is obtained from an animal or a human it will be understood that any number of appropriate means familiar to those having ordinary skill in the art can be employed. For example, if a blood sample is obtained, it can be obtained either by drawing blood through venepuncture, but also can be obtained as a forensic sample.

As used herein, the phrase "without reference to the amount of pseudo target amplicon" means that quantitative information regarding the amount of pseudo target amplicon synthesized in an amplification reaction is not required to make a determination regarding another parameter in an amplification system. For example, the synthesized amount of analyte amplicon in an amplification system or the amount of analyte polynucleotide that would have led to the formation of that amount of amplicon can be determined according to the methods disclosed herein without quantitative information about the formation of pseudo target amplicons in the

same reaction. Indeed, it is not even necessary to detect the pseudo target amplicon for success of the quantitative method described herein. The present invention provides an approach for relating the pre-amplification amount of analyte polynucleotide and the post-amplification amount of analyte amplicon. This relationship can be established without relying on, or even having knowledge about, the amount of pseudo target amplicon that is co-amplified with the analyte amplicon in an amplification reaction. Thus, even if the pseudo target amplicon is detected or quantified in an experimental procedure, it is unnecessary to employ that information when relating the pre-amplification amount of an analyte polynucleotide and the post-amplification amount of a corresponding analyte amplicon.

As used herein, a "standard curve" is a representation that relates a pre-amplification amount of a polynucleotide and a post-amplification amount of a corresponding amplicon. For example, a standard curve can be a graph having known numbers of input template molecules on the x-axis, and either RLU values or pmols of amplicon product plotted on the y-axis. Standard curves typically are produced using control polynucleotide standards having known numbers of polynucleotide templates. Standard curves can be stored in electronic form or can be represented graphically.

A "biological specimen" is a sample of material derived from an organism.

Brief Description of the Drawings

Figure 1 is a schematic representation of electrophoretically separated TMA reaction products synthesized using different amounts of input target polynucleotide template. The lane marked "Neg" represents a reaction that did not include input template. The remaining lanes represent reactions conducted using increasing amounts of input target polynucleotide. The position on the gel of the specific amplification product derived from the target polynucleotide is marked by an arrow.

Figures 2a-2c schematically illustrate three different reaction conditions for a TMA reaction. When other variables such as enzyme, primer, and NTP concentrations are held constant, under the condition of low input levels of analyte polynucleotide (Figure 2a) the majority of the reaction product is a template-independent nonspecific product (NP) while the analyte amplicon or specific product (SP) represents only a minor component of the total reaction product. Under conditions of high input levels of analyte polynucleotide (Figure 2b), the analyte amplicon (SP) represents a majority of the total reaction product while the

nonspecific product (NP) is a minor component. Under conditions where the level of input analyte polynucleotide is low but the level of pseudo target is high (Figure 2c), pseudo target specific product (PTSP) formation is at the expense of nonspecific product formation.

Figures 3a-3b are schematic illustrations showing how inclusion of pseudo targets in idealized reactions that do not produce nonspecific amplification products can transform qualitative assays into quantitative assays. Figure 3a shows how low or high starting levels of analyte polynucleotide (TA) serve as templates for conversion of reactants (R) into similar amounts of analyte-specific products (SP). Figure 3b shows that including pseudo targets (PsT) in amplification reactions having low or high starting levels of analyte polynucleotide results in quantitative relationships between the levels of analyte-specific products synthesized in the reactions and the input levels of templates. The diagram shows that pseudo targets serve as templates in the reaction for the synthesis of pseudo target-specific products (PTSP) while analyte polynucleotides serve as templates for the synthesis of analyte-specific products.

Figures 4a-4d are idealized graphs illustrating how the dynamic range and precision of polynucleotide amplification reactions are improved when the reactions include pseudo targets. Figure 4a shows results be expected for a hypothetical amplification reaction that produces only analyte-specific amplicons. Figure 4b shows results expected for amplification reactions that spontaneously produce low levels of non-specific amplification products that are unrelated to the analyte polynucleotide. Figure 4c shows results expected for amplification reactions that spontaneously produce high levels of non-specific amplification products that are unrelated to the analyte polynucleotide. Figure 4d shows idealized results expected for reactions that include pseudo targets.

Figure 5 is a schematic diagram illustrating how variability in the efficiency of recovery of a collection of polynucleotides that includes an analyte polynucleotide and a pseudo target can yield similar quantities of amplicon following an amplification reaction. The analyte polynucleotide and the pseudo target are shown at the top of the diagram in a fixed starting ratio. Whether 100% or 50% of the polynucleotide sample is input into the amplification reaction. the final amounts of amplicon products are similar.

Figure 6 is a line graph showing how a pseudo target can be used to normalize amplicon synthesis in amplification reactions given different amounts of analyte polynucleotide. The

three conditions presented in the graph are: no pseudo target (♦); constant amount of pseudo target (■); and constant ratio of pseudo target and analyte polynucleotide (□).

Figure 7 is a line graph showing how a pseudo target can be used to control the production of analyte amplicons. The two conditions presented in the graph are: no pseudo target (●); and 2 x 10^6 copies of pseudo target per reaction (■).

Detailed Description of the Preferred Embodiment

Herein I disclose that polynucleotide amplification reactions that included a pseudo target advantageously exhibited improved precision with respect to the amount of analyte amplicon synthesized. Additionally, qualitative amplification reactions can be transformed into quantitative assays by including pseudo targets in the reactions and then quantitatively measuring the amount of analyte amplicon that was synthesized. Also disclosed is a new method of specimen processing which advantageously ensures the production of a pre-established ratio of pseudo target and analyte amplicons in a subsequent amplification reaction, regardless of the efficiency with which nucleic acids were isolated from the specimen. According to this method, pseudo targets are added to a biological specimen before nucleic acids are isolated from the specimen. Assays performed in a qualitative format employing pseudo target amplification, and that provide semi-quantitative information about the amount of analyte polynucleotide in a test sample are also described.

Introduction and Overview

An observation which led to the development of the invention concerned an inherent feature of the standard TMA reaction. More specifically, it was observed that enzymatic synthesis of nonspecific amplification products represented a substantial proportion of the reaction product when the reaction was initiated using only very low amounts of target polynucleotide. When visualized following electrophoresis, the nonspecific amplification products appeared as a smear that extended over a broad size range. This result is illustrated schematically in Figure 1.

Importantly, it was observed that TMA reactions carried out using increasing amounts of target polynucleotide templates resulted in diminished relative contributions of the nonspecific products. This result also is illustrated in Figure 1. Reactions that were initiated using higher concentrations of target polynucleotides resulted in the formation of larger amounts of specific products and only small amounts of nonspecific products. This inverse relationship led to

speculation that nonspecific reaction product formation could be suppressed by including an amplifiable template in the reaction mixture at the time the reaction was initiated.

While not wishing to be bound by any particular theory, this inverse relationship may have been particularly noticeable in autocatalytic reactions such as the TMA reaction because, unless interrupted prematurely, the nature of the reaction is to proceed to an end-point where the supply of available reactants is exhausted and no further synthesis can take place. A TMA reaction that proceeds indefinitely in the absence of a target polynucleotide will generate nonspecific products until the reactants are depleted and no additional synthesis occurs. A PCR reaction carried out indefinitely is also expected to proceed to a point where the reactants are exhausted and amplicon production ceases, and can also generate nonspecific amplification products (for example, see D. Persing in Diagnostic Molecular Microbiology; Ch 3, p. 58 (1993)).

As the method disclosed herein is ordinarily practiced, detection of the analyte amplicon is used to indicate the presence of analyte polynucleotides in a population of nucleic acid molecules. For example, a procedure for monitoring the serum level of human immunodeficiency virus (HIV) virions could involve amplifying a portion of the HIV genome and then detecting and quantitating that amplification product. If the procedure further included amplifying a pseudo target, then detection of the pseudo target amplicon would be an optional step that would not be required for success of the assay. Detection of the pseudo target amplicon could be used as a positive control procedure for indicating that an amplification reaction had occurred (i.e., an internal amplification control). However, quantitative characterization of the amount of analyte amplicon synthesized in an amplification reaction, or the quantity of analyte polynucleotide template that would have led to the formation of that amount of analyte amplicon, does not depend on knowledge of the amount of pseudo target amplicon synthesized in the amplification reaction. Thus, analyte amplicon can be quantified according to the methods disclosed herein without reference to the amount of pseudo target amplicon synthesized in an amplification reaction. A critical feature of the method disclosed herein is that the analyte amplicon must be distinguishable from the pseudo target amplicon. More specifically, it must be possible to detect analyte amplicon without also detecting pseudo target amplicon. In a preferred embodiment the analyte amplicon and the pseudo target

amplicon differentially bind at least one hybridization probe so that the two amplicon species can be detected independently.

One point particularly relevant to clinical procedures that employ amplification protocols relates to the variability of recovering polynucleotide templates from different biological specimens. For example, it is common to experience variability in the number of molecules of a given polynucleotide recovered from different tissue samples as the result of variable sample sizes and the complexity of different sample handling procedures. Nucleic acids can bind nonspecifically to glass, plastic and chromatography media such as cross-linked polyacrylamides and dextrans, thereby reducing the efficiency of sample recovery during extensive processing. Additionally, RNA recovered from a biological specimen may have degraded by a variable degree as the result of chemical or enzymatic hydrolysis. Enzymatic hydrolysis is particularly evident in biological samples which contain high concentrations of ribonuclease.

Quantitative Polynucleotide Amplification Assays

Incorporating a pseudo target into a polynucleotide amplification reaction not only can reduce amplification variability from sample to sample, but also can transform even a fully optimized qualitative assay into a quantitative assay. In the case of a polynucleotide amplification system where only specific amplification products are synthesized (meaning that target nonspecific products are not made) the amount of end product amplified from an initial target polynucleotide would be constant regardless of the starting amount of target polynucleotide included in the reaction. This is true when autocatalytic amplification reactions, such as the TMA reaction, proceed to the point where one of the reactants is depleted sufficiently so that the reaction terminates. The total amount of end product synthesized in this situation is largely determined by the initial concentrations of reactants included in the reaction. Such an optimized polynucleotide amplification system is qualitative but not quantitative when the reaction is carried out to the point where the concentration of one of the reactants becomes limiting. This is because the amount of end product produced in the reaction depends on the starting reactant concentrations and not on the starting amount of polynucleotide target.

When a pseudo target is included in an amplification reaction, such as a TMA reaction, the pseudo target preferably will be present at a higher copy number relative to the target polynucleotide. The amplification reaction stops when the amount of product amplified from

the target polynucleotide and the pseudo target is sufficiently great that one of the reactants has been depleted. Since the pseudo target ordinarily will represent the dominant amplification species, the extent of amplification of the target polynucleotide is determined by the starting amount of the pseudo target and not by the starting amount of the target polynucleotide. Therefore, by controlling the amount of pseudo target in the amplification reaction, the extent of target polynucleotide amplification can be controlled regardless of the starting amount of target polynucleotide in the reaction. While the combined amount of amplification products will be constant when the reactant concentrations are held fixed, the amount of analyte amplicon produced in the reaction will substantially reflect the starting proportion of the analyte polynucleotide relative to the starting level of pseudo target. In this way, a polynucleotide amplification assay can be made into a quantitative assay because the target polynucleotide will amplify by a pre-determined extent. This is illustrated schematically in Figures 2-4.

In general, the methods for converting qualitative polynucleotide amplification reactions into quantitative reactions by including a pseudo target polynucleotide in the reactions are applicable to all known polynucleotide amplification systems, including PCR, NASBA (nucleic acid sequence-based amplification), SDA (strand displacement amplification), and amplification methods using self-replicating polynucleotide molecules and replication enzymes like MDV-1 RNA and Q-beta enzyme. Methods for carrying out these various amplification techniques respectively can be found in U.S. Patent No. 4,965,188; published European patent application EP 0 525 882, U.S. Patent No. 5,455,166, U.S. Patent No. 5,472,840 and Lizardi et al., *BioTechnology* **6**:1197 (1988).

Quantitative Aspects of Amplification Reactions that Include Pseudo Targets

It is appreciated in the field of nucleic acid testing that polynucleotide amplification is an exponential process and that small differences in any of the variables that affect the reaction rate can lead to dramatic differences in the yield of analyte-specific amplicons. Disclosed herein is the novel finding that non-specific amplification products generated in amplification reactions can significantly contribute to total amplicon production and can consume reactants that otherwise would be used to synthesize analyte-specific amplification products. The contribution of non-specific products to the pool of amplification products is significant enough that small changes in the amounts of non-specific amplification products can profoundly influence the magnitude of analyte amplicon production. Thus, it was discovered during the development of

the present invention that reducing the amount of non-specific products formed in an amplification reaction advantageously improved the precision of analyte amplicon production and transformed qualitative amplification assays into quantitative assays.

The preferred approach for reducing formation of non-specific products requires including a pseudo target in the amplification reaction and then quantitatively detecting analyte amplicons that are synthesized in the reaction. In this way, analyte amplicon production can be related in a dose-dependent fashion to the amount of analyte polynucleotide present at the time the amplification reaction was initiated. Additionally, in accordance with the invented methods it is unnecessary to detect pseudo target amplicons in order to quantify the number of analyte polynucleotides present in a test sample.

Thus, herein there is disclosed a method of quantifying analyte polynucleotides that does not rely on the detection of amplification products arising from any internal standard. The development of this approach was made possible by recognizing the source of the problem which underlies variability in analyte amplicon production, and which can be controlled by including a pseudo target in the amplification reaction. Although the pseudo target serves as a template in the amplification reaction, detection of pseudo target amplicons is unnecessary for quantifying analyte polynucleotides. While it may seem counterintuitive that precision of an amplification reaction would be improved by adding into the reaction a template that competes with the analyte polynucleotide for reagents needed to synthesize amplicons. the results presented below clearly demonstrate the value of this procedure. Simply stated, the methods disclosed herein represent a procedure for controlling the otherwise highly variable production of non-specific amplification products by introducing into the system template polynucleotides that are amplified, but that are not necessarily detected or quantified.

Basis of Improved Precision and Quantitative Capacity of Amplification Reactions

Figures 3a-3b illustrate how pseudo targets can transform a qualitative polynucleotide amplification reaction into a quantitative assay. Figure 3a shows how an optimized reaction converts a pool of reactants (represented in the diagram by an octagon) into specific amplification products (SP) using target analyte (TA) polynucleotides as templates. In the absence of a pseudo target the assay is qualitative because the amount of analyte amplicon produced in the reaction is not quantitatively related to the starting level of target analyte polynucleotide. Whether the starting level of analyte polynucleotide is low or high does not

alter the amount of analyte amplicon synthesized in the reaction. Instead, the amount of analyte amplicon that can be produced in the reaction is defined by the starting reactant pool and not by the starting level of analyte polynucleotide. Thus, a constant amount of amplicon is produced when the amplification reaction is carried out to the point of reactant depletion. Conversely, Figure 3b shows how amplification reactions conducted in the presence of pseudo targets synthesize analyte-specific products in proportion to the amount of starting analyte polynucleotide when parallel reactions include a pseudo target. More particularly, when polynucleotide amplification reactions include a pseudo target, the final amount of analyte amplicon is related in a dose-dependent fashion to the starting level of analyte polynucleotide that served as a template in the reaction. Accordingly, it is only necessary to quantify the analyte amplicon (and not the pseudo target amplicon) to gain information about the starting level of analyte polynucleotide in the reaction. This method of quantifying polynucleotides advantageously circumvents the need for detecting amplicons other than the analyte amplicon, or for employing different probes to distinguish different amplicon species. Additionally, the same paired set of oligonucleotide primers can be used to amplify both the analyte polynucleotide and the pseudo target, since the two products of the amplification reaction will be distinguishable using an analyte-specific hybridization probe.

Figures 4a-4d illustrate how analyte amplicon synthesis quantitatively reflects the starting analyte polynucleotide level over an extended range when two competing amplification reactions occur simultaneously. Figure 4a shows results that would be expected for an idealized amplification reaction that takes place in the absence of non-specific product formation. A constant level of analyte amplicon formation is expected at all levels of input analyte polynucleotide. This case reflects the reaction illustrated in Figure 3a. Figure 4b shows results that would be expected for amplification reactions that spontaneously generate non-specific products at low levels. A narrow range of dose-dependency exists only at very low levels of input analyte polynucleotide. Figure 4c shows results that would be expected for amplification reactions that spontaneously generate high levels of non-specific amplification products. In this instance, amplification reactions conducted at any given level of input analyte polynucleotide produce amounts of amplicon falling within a range, as indicated on the Y-axis of the graph. The more significant contribution of the non-specific product formation distinguishes the results shown in Figures 4b and 4c. The breadth of the lines relating the input levels of analyte

polynucleotide and analyte amplicon synthesis reflects the low precision of analyte amplicon formation and is attributable to the fact that spontaneous formation of non-specific amplification products can be highly variable. Figure 4d shows results that would be expected for an idealized amplification reaction that includes a pseudo target. The amount of analyte amplicon produced in the reaction exhibits both improved precision and a dose-dependent relationship over a broad range of input analyte polynucleotide levels. This case reflects the idealized reaction illustrated in Figure 3b.

Thus, the precision and quantitative aspects of amplification reactions conducted according to the invented method are interrelated by the existence and controllability of competing reactions wherein analyte and non-analyte polynucleotides co-amplify and compete for reactants. Improved dynamic range results when a second amplification reaction competes with the analyte-specific reaction for reactants. Improved precision in the amount of analyte amplicon synthesis results when the second reaction is made highly controllable by including pseudo targets in the amplification reaction at a level of from 1×10^3 - 2×10^8 molecules per reaction, where a typical reaction has a volume of 100 µl.

Use of a Standard Curve — Quantifying Pre-Amplification Amounts of Analyte Polynucleotide

Since amplification reactions that include pseudo targets advantageously feature quantitative relationships between the number of analyte polynucleotides input into the reaction and the number of analyte amplicons synthesized, the number of analyte polynucleotides present in a test sample can be determined using a standard curve. More particularly, a plurality of amplification reactions containing constant amounts of pseudo target and known amounts of analyte polynucleotide standard can be run in parallel with an amplification reaction prepared using a test sample containing an unknown number of analyte polynucleotides. Alternatively, a standard curve can be prepared in advance so that it is unnecessary to prepare a curve each time an analytical procedure is carried out. Such a curve prepared in advance can even be stored electronically in a memory device of a testing instrument. Preferred amplification methods include Transcription Mediated Amplification reactions, NASBA reactions and Polymerase Chain Reactions. Transcription Mediated Amplification is highly preferred. The amounts of pseudo target used should be the same for each reaction and preferably fall in the range of from 10^3 to 2×10^8, from 10^4 to 2×10^8, from 10^5 to 2×10^8, or from 10^7 and 2×10^8 pseudo target molecules per reaction. Reactions that include pseudo targets can be carried out according the

methods described herein with the number of analyte amplicons synthesized in each reaction being quantified by standard hybridization and detection procedures. Although detection of pseudo target amplicons is unnecessary for quantifying pre-amplification amounts of analyte polynucleotide in the test sample, detection of pseudo target amplicons optionally can be used to confirm success of the amplification reactions. In this way detection of pseudo target amplicons serves as an internal amplification control. A standard curve having pre-amplification amounts of the analyte polynucleotide standard on a first axis and corresponding post-amplification amounts of analyte amplicon on a second axis is then prepared. The post-amplification amount of analyte amplicon measured for the test reaction is then located on the post-amplification axis of the standard curve. The corresponding value on the other axis of the curve represents the pre-amplification amount of analyte polynucleotide that was present in the test reaction. Thus, determining the number of molecules of analyte polynucleotide present in the test sample is accomplished by consulting the standard curve, or more particularly by comparing the quantitative results obtained for the test sample with the standard curve, a procedure that will be familiar to those having an ordinary level of skill in the art.

The procedures described herein can easily be used to quantify analyte polynucleotides present in a test sample. Indeed, if a plurality of pseudo target-containing control amplification reactions are initiated using known numbers of molecules of an analyte polynucleotide standard, and if a test reaction that includes the pseudo target is initiated using an unknown number of analyte polynucleotide molecules, then it becomes possible after quantifying the number of analyte amplicons in each reaction to determine the number of analyte polynucleotide molecules that must have been present in the test sample. For example, if standard reactions that respectively included 500, 1,000 and 1,500 molecules of analyte polynucleotide standard produced analyte amplicon signals of 1x, 2x and 3x following an analyte-specific probe-based hybridization procedure, and if the test sample produced an analyte amplicon signal corresponding to 1.5x, then the test sample must have contained 750 analyte polynucleotide molecules. In this exemplary case a linear relationship exists between the signal generated by the amplicons arising from the analyte polynucleotide standard in the range of from 500 to 1,500 molecules. The relationship between the number of analyte polynucleotide molecules input into the standard amplification reactions and the amplicon-specific signal strength is most conveniently established using a graph. Determining the number of analyte polynucleotide

molecules present in a test sample is simply a matter of determining from the standard graph the number of analyte polynucleotide molecules that correspond to a measured analyte amplicon signal strength. This illustrates how analyte polynucleotide standards can be used in connection with pseudo targets in polynucleotide amplification reactions to quantify pre-amplification amounts of analyte polynucleotide contained in test samples.

Structural Features of Useful Pseudo Target Polynucleotides

The following information can be used to design pseudo target polynucleotides for use in connection with the methods disclosed herein. Given this information, useful pseudo targets corresponding to any number of analyte polynucleotides that are to be detected and quantified can be made. Exemplary applications where pseudo targets may be used in connection with polynucleotide amplification procedures include, but are not limited to: (1) detecting a bacterial or viral pathogen; (2) quantitating polynucleotides where such quantitation is useful as an indicator of a disease process, such as HIV disease progression; and (3) numerous other applications including forensic analysis, environmental and food testing.

In one preferred embodiment of the invention the pseudo target and the analyte polynucleotide are amplifiable using the same set of two oligonucleotide primers. In this instance, a single oligonucleotide primer that will have a complementary binding site on the pseudo target also will have a complementary binding site on the analyte polynucleotide.

In another preferred embodiment the pseudo target and analyte polynucleotide, which are to serve as templates in an amplification reaction, amplify with substantially similar efficiencies. Thus, whether the amplification is carried out using TMA, PCR or some other procedure such as SDA (strand displacement amplification) or methods employing self-replicating polynucleotide molecules and replication enzymes like MDV-1 RNA and Q-beta enzymes, the pseudo target and analyte polynucleotides preferably will have similar amplification efficiencies.

One way to ensure that the pseudo target and analyte polynucleotide templates will have similar amplification efficiencies is to require that the two templates exhibit closely related, but nonidentical, polynucleotide sequences over the span of the sequence that is amplified in the procedure. For example, a pseudo target polynucleotide may be created by scrambling an internal portion of the sequence of an analyte polynucleotide, where the scrambled sequence corresponds to the portion of the analyte polynucleotide that serves as the part of the molecule

that is hybridized by a probe specific for the analyte polynucleotide. The length of the pseudo target polynucleotide is not critical to its operation in the practice of the methods disclosed herein.

It is essential that the pseudo target and the analyte polynucleotide are co-amplifiable in a single reaction, and that the resulting two amplicon species can be detected independently. More particularly, it is essential that the pseudo target and analyte polynucleotide amplification products have polynucleotide sequences that differ from each other so that the two products can be distinguished by length, by the ability to hybridize to a detection probe, or by other methods. Since the polynucleotide templates amplified in the amplification reactions ordinarily will contain a substantial number of nucleotide bases interposed between the regions homologous or complementary to the primer binding sites used to carry out the amplification reaction, these interposed sequences may serve as regions to which selected hybridization probes can bind. Criteria useful for selecting hybridization and detection probes will be familiar to those having an ordinary level of skill in the art. Probes useful in connection with the invention include labeled polynucleotides as well as oligonucleotides useful as primers in subsequent amplification reactions.

When the pseudo target is added to a biological specimen at a time before analyte polynucleotide is isolated from the specimen, for example as an aid to sample processing, it is important that the pseudo target and the analyte polynucleotide are recoverable from the specimen by the same sample processing procedure. For example, if the analyte polynucleotide is recoverable under strongly alkaline conditions that denature DNA and hydrolyze RNA, then it should also be true that the pseudo target is recoverable as a structurally intact molecule under the same conditions. Thus, if alkaline buffer conditions are used to isolate analyte polynucleotides in the presence of added pseudo target polynucleotides, then neither the analyte nor the pseudo target would be an RNA molecule that would be degraded during the isolation procedure. Similarly, if the pseudo target and the analyte polynucleotide are to be precipitated, for example by the addition of an alcohol such as ethanol, then the pseudo target and the analyte polynucleotide should precipitate with substantially similar efficiencies.

Relationship between Pseudo Target and Analyte Polynucleotide Sequences

Significantly, it is preferred but not essential for the pseudo target and the analyte polynucleotide to be co-amplifiable using the same set of two oligonucleotide primers. More

specifically, qualitative polynucleotide amplification assays for detecting an analyte polynucleotide using a paired set of analyte-specific primers can be transformed into quantitative assays by further including in the reaction a pseudo target and a set of primers for amplifying the pseudo target. In one embodiment of the invented method, the analyte polynucleotide and the pseudo target are co-amplifiable using the same two primers.

It is also possible to employ a "universal pseudo target" and a set of pseudo target-specific primers to produce quantitative amplification reactions. In one embodiment of the invention, the primers used for amplifying the universal pseudo target can be the same as the primers used for amplifying the analyte polynucleotide. The universal pseudo target need not be related to the structure of the analyte polynucleotide, and need not co-amplify with the analyte polynucleotide with similar amplification efficiency. Amplification reactions conducted using low or high starting levels of analyte polynucleotide will synthesize analyte amplicons in a fashion that is dose-dependent on the starting amounts of analyte polynucleotide present when the amplification reactions were initiated. Using this procedure, two or more amplification reactions can be assessed for the production of analyte amplicons, with the analyte amplicon levels being related to the starting levels of analyte polynucleotide in each sample in a dose-dependent manner.

While good results can be achieved using pseudo targets and associated primers that are unrelated to the analyte polynucleotide, it is preferable to employ pseudo targets that co-amplify with the analyte polynucleotide using the same set of primers. This preferred approach advantageously reduces variability in the composition of the reagent pool used as a resource for synthesizing amplicons in the amplification reaction. However, the illustrative Examples presented herein for describing the invention employ pseudo targets and exemplary analyte polynucleotides that co-amplify using common sets of oligonucleotide primers.

Choosing an Amount of Pseudo Target to be Included in an Amplification Reaction

In general, the positive benefits achieved by including pseudo targets in polynucleotide amplification reactions are achievable over a very broad range of pseudo target concentrations. More particularly, including pseudo targets in amplification reactions, such as TMA reactions, in amounts ranging from 1×10^3 - 2×10^8 molecules will result in: (1) higher precision of amplification, (2) reduced likelihood of positive carryover and (3) normalization of target recovery variability, all as disclosed herein. Within practical limits, higher starting levels of

pseudo target in an amplification reaction will result in greater improvement of the three above-referenced parameters.

Since pseudo target amplicons will be synthesized using nucleotide triphosphate reactants that otherwise could be used to synthesize analyte amplicons, the presence of a pseudo target in an amplification reaction will result in a reduction of the synthesis of analyte amplicons. This is because both analyte amplicons and pseudo target amplicons are synthesized from a limited pool of reactants. Accordingly, increasingly high starting levels of pseudo target will result in decreasing amounts of analyte amplicon produced in the amplification reaction. Thus, the upper limit of starting pseudo target concentration in an amplification reaction will be a practical matter dependent on the sensitivity of the procedure used for detecting the analyte amplicon.

The upper limit amount or concentration of a pseudo target that can be included in an amplification reaction, and that will yield levels of analyte amplicon adequate for detection, is most easily determined by routine experimentation. Again, it will be readily apparent to those having ordinary skill in the art that higher levels of pseudo target in an amplification reaction conducted to the point of reagent exhaustion will result in lower amounts of analyte amplicon produced in the reaction. This is because pseudo target amplicons are synthesized at the expense of analyte amplicons in the amplification reaction. Thus, amplification reactions that include very high levels of a pseudo target will result in the production of low levels of analyte amplicon. Highly sensitive assays for detecting analyte amplicons will be particularly useful for detecting these lower levels of analyte amplicon. Conversely, less sensitive assays that require larger amounts of analyte amplicon for a positive detection signal will be useful for detecting larger quantities of analyte amplicon that might result from amplification reactions that included only low starting levels of pseudo target, and that resulted in higher levels of analyte amplicon. Thus, the upper limit of the amount of pseudo target that can be used for conducting an amplification reaction will depend on the sensitivity of the assay that is ultimately to be used for detecting analyte amplicons, and not on the amplification reaction itself.

Since it is generally true that higher levels of input pseudo target provide enhanced reduction of nonspecific amplification products in reactions such as the TMA reaction, it follows that the amount of pseudo target included in an amplification reaction preferably should be as high as possible. The highest pseudo target level disclosed in the Examples which follow

was 2 x 10^8 molecules in a 100 µl reaction. Of course, the detection system used for detecting analyte amplicons must be sensitive enough to give a positive signal when analyte polynucleotides are present in the starting sample so that analyte amplicons are synthesized in the amplification reaction. In practice, a range of pseudo target concentrations can be tested to identify an optimal amount that gives good results in the amplification reaction as measured by detectability of analyte amplicons using a detection system having a given sensitivity for detecting analyte amplicons. Ordinarily, positive and negative controls will be included in this procedure to indicate the results that would be expected for amplification reactions that did or did not include analyte polynucleotides, respectively.

The amount of pseudo target selected for conducting an amplification reaction can be influenced by the magnitude of the amplification, the starting number of analyte polynucleotides in the reaction and on the sensitivity of the detection system used for detecting analyte amplicons. Standard TMA reactions typically amplify starting polynucleotide levels by 10^{12} - 10^{13} fold. An exemplary polynucleotide detection system can detect approximately 6 x 10^7 molecules in a hybridization assay. In order to detect 100 molecules of an analyte polynucleotide in a sample that served as the source of templates in an amplification reaction, it would be necessary to achieve an amplification of approximately 6 x 10^5 fold (6 x 10^7 divided by 100). To achieve at least 6 x 10^5 fold amplification of the 100 analyte polynucleotide molecules the amplification reaction should not include more than 1 x 10^7 pseudo target molecules. This is because 6 x 10^{12} (as an example value in the range of from 10^{12} - 10^{13} fold as indicated above) divided by 1 x 10^7 equals a 6 x 10^5 fold increase. Including a greater number of pseudo target molecules would reduce the fold amplification to less than the acceptable 6 x 10^5 value. If instead a PCR protocol leading to a 1 x 10^9 fold amplification were employed, the maximum acceptable amount of pseudo target in the amplification reaction would be 1 x 10^9 divided by 6 x 10^5, or 1.7 x 10^3 molecules. Thus, it should be clear that: (1) a broad range of pseudo target concentrations will be useful in the practice of the method disclosed herein, and (2) an optimal amount of pseudo target can be determined empirically when the number of analyte polynucleotides in a sample being tested in an amplification protocol is unknown.

Preferred amounts of pseudo target useful for conducting amplification reactions typically range from between 10^3 and 10^9 molecules per reaction, where a typical reaction is conducted in a 100 µl volume. For example, an assay for detecting HIV polynucleotides in a

serum sample isolated from an HIV-infected human can be conducted using between 10^3 and 2 x 10^8, between 10^4 and 2 x 10^8 or between 10^5 and 2 x 10^8, or between 10^7 and 2 x 10^8 pseudo target molecules per reaction. In a highly preferred embodiment, the amplification reaction is a TMA reaction and the HPA ("homogenous protection assay") method is used for detecting analyte amplicons produced in amplification reactions conducted using these amounts of pseudo target. As indicated in the Examples which follow, broad ranges of pseudo target concentrations have been tested and shown to give good results.

Kits for Performing the Invented Method of Polynucleotide Amplification

Kits useful for performing the polynucleotide amplification methods described herein will include: (1) a pseudo target, (2) oligonucleotide primers for co-amplifying the pseudo target and the analyte polynucleotide, (3) reagents for carrying out the polynucleotide amplification reaction, and (4) printed instructions for carrying out an amplification reaction and for specifically detecting only analyte amplicons produced in the reaction. Optionally the kit may include a labeled probe for detecting analyte amplicons. Reagents included with the kit will comprise deoxyribonucleotide triphosphates and a DNA polymerizing enzyme, which may be a reverse transcriptase. Nucleotide triphosphates and an RNA polymerase are optional reagents that can be included in the kit.

With this background, three particular aspects of the invention now are described in more detail.

I. Enhancing the Precision of Analyte Polynucleotide Amplification

Amplification techniques, both quantitative and qualitative, represent powerful tools for detecting and measuring even trace amounts of specific target polynucleotides. However, difficulty in obtaining uniform amplification efficiency among different reactions means that variability in the extent of amplification compromises the precision of quantitation and the ability to detect small amounts of target. I sought to develop methods that could minimize the formation of nonspecific products, enhance precision in the amount of analyte amplicon synthesis and maximize the ease with which quantitative amplification reactions could be performed.

Variability in the extent of amplification appears to be partly attributable to the enzymatic synthesis of nonspecific reaction products. The formation of these nonspecific products was most noticeable when reactions contained only very low starting levels of target

polynucleotides that served as amplification templates. At higher levels of target polynucleotide, the formation of nonspecific reaction products was less significant and represented only a small proportion of the total product of the reaction. Thus, an object of the present invention was to simulate reaction conditions that were characteristic of high target polynucleotide concentrations even when starting levels of analyte polynucleotides in the reaction mixtures were very low. More particularly, it was desirable to simulate favorable reaction conditions that would minimize the formation of nonspecific products, as represented in Figure 2.

Amplification reactions supplemented with pseudo target polynucleotides that co-amplified with the analyte polynucleotide provided the desired reaction conditions to achieve these objectives. This allowed the amplification reaction to behave as if high levels of analyte were present even when the true analyte level in the amplification reaction was low. As indicated by experimental results presented below, adding greater than 10^5 copies of the pseudo target polynucleotide improved amplification precision as measured by an improved coefficient of variability (CV%) of relative light units (RLUs), where RLUs represent a measurable indicator of the quantity of hybridized probe. In the present context, CV% is a statistical value that is calculated by dividing the standard deviation (SD) for a collection of data by the net average for that collection and then multiplying the result by 100. Lower CV% values reflect less spread among data points and are taken as indicators of higher experimental precision. Thus, the method described herein provides a way to improve the precision with which the analyte polynucleotide is amplified while reducing the formation of variably sized nonspecific reaction products.

The methods disclosed herein additionally provide a mechanism for standardizing the results of polynucleotide amplification reactions regardless of the starting level of analyte polynucleotide as long as the analyte polynucleotide was present at a copy number lower than the copy number of the pseudo target. In an exemplary procedure described below, analyte polynucleotide was present in a set of amplification reaction mixtures at starting levels of from 10^1-10^5 molecules while the pseudo target was present in all mixtures at 10^6 molecules. This meant that all the amplification reactions were initiated from substantially 10^6 polynucleotide templates because the contribution of the analyte polynucleotides to the total number of templates in any of the mixtures was minimal. Thus, all of the amplification reactions behaved

as though they contained roughly 10^6 templates even though the number of analyte polynucleotides varied widely. This effectively standardized the amplification reactions to 10^6 template polynucleotides regardless of actual analyte level.

II. Controlling Amplicon Production

It has been further discovered that pseudo targets can be employed to overcome obstacles associated with the "over production" of analyte amplicons that can lead to inaccurate quantitation of high levels of input target polynucleotide. If excessive amounts of amplicon are produced in an amplification reaction, and if those amplicons are to be quantitated by hybridizing a detection probe to saturation, then large quantities of detection probe necessarily will be consumed in the detection step of the assay. Conversely, if reduced levels of amplicon are produced, then less detection probe will be required to carry out the detection step. Still another advantage of reducing the amount of amplicon produced in an amplification reaction relates to the another aspect of the detection apparatus used for detecting the amplicon. Since detection means such as luminometers often will have linear response ranges that can be saturated at high signal levels, it is an advantage to be able to conduct amplification reactions such that the signal produced in a detection step falls within the linear response range for the detection apparatus. Thus, the ability to control amplicon synthesis clearly is beneficial with respect to subsequent detection steps.

The amount of analyte amplicon generated in the amplification reaction can be controlled by including a pseudo target in the amplification reaction to compete with the target polynucleotide for amplicon synthesis. When the pseudo target is present, reactants in the amplification reaction mixture will be used to synthesize both analyte amplicons and pseudo target amplicons. If the amplification reaction proceeds to reagent exhaustion, and if higher numbers of pseudo target amplicons are produced at the expense of analyte amplicons, then the relative proportion of analyte amplicons can be reduced by increasing the starting amount of pseudo target in the amplification reaction. An appropriate level of pseudo target to be included in the amplification reaction can be determined using no more than routine experimentation.

An alternative approach to reducing analyte amplicon production in a polynucleotide amplification reaction such as the TMA reaction would be to perform the reaction under sub-optimal conditions, such as those described in U.S. Patent Nos. 5,705,365 and 5,710,029. This alternative may at times be less desirable than the above-described approach because different

conditions may be required to "de-optimize" the reaction for different analytes to be detected. In contrast, reducing the amount of analyte amplicon produced in a reaction by including a pseudo target allows all reactions to be performed under optimal conditions.

Thus, including a pseudo target in an amplification reaction provides a means for "tuning" a quantitative amplification reaction by competing high target levels with even higher levels of pseudo target.

III. Specimen Processing in the Presence of Added Pseudo Targets

Uniform recovery of target polynucleotides from different biological samples is very important for many quantitative assays. For example, assays for determining plasma levels of HIV virions easily could lead to an inaccurate estimation of virion levels in the plasma if the precision of target polynucleotide recovery is low. Herein there is described an alternative approach that is relatively tolerant to variability in the level of input analyte polynucleotides.

Rather that striving for quantitative recovery of polynucleotides that will serve as templates in an amplification reaction, one aspect of the invention is directed to a method of normalizing variability of target polynucleotide recovery at the specimen processing step. According to this approach, the final amount of a target polynucleotide amplification product is easily controlled when the level of a pseudo target is sufficiently high that pseudo target amplification competes with true target amplification and when the pseudo target and the analyte polynucleotide are co-amplifiable with similar amplification efficiencies. In this range, the amount of analyte amplicon is inversely proportional to the input level of the pseudo target. For example, if the input level of pseudo target is increased by X fold, then the analyte amplicon will be reduced to 1/X. When pseudo target is present in specimen processing steps, the pseudo target and the true target will be recovered with similar efficiencies. Therefore, if the recovery efficiency for the analyte polynucleotide is K%, then the pseudo target also will be recovered at an efficiency of K%.

Under otherwise identical conditions, reactions will produce a relatively constant amount of amplicon. Thus, addition of pseudo target diverts the reaction components from making nonspecific amplicon and ensures that effectively all of the amplicon produced in the reaction is either pseudo target amplicon or analyte amplicon representing the amplification product of an analyte polynucleotide. The pseudo target amplicon and the analyte amplicon will

be produced in the same ratio as the ratio of pseudo target and analyte polynucleotide at the time the amplification reaction was initiated.

When analyte polynucleotides and pseudo targets are co-isolated and then added to an amplification reaction that proceeds to reactant exhaustion, the relative proportion of resulting analyte and pseudo target amplicons will be the same as the relative proportion of analyte and pseudo target polynucleotides in the sample isolated from the biological specimen. This means that, regardless of the efficiency of the polynucleotide recovery in specimen processing, the amount of analyte amplicon synthesized in an amplification reaction will be the same as the amount that would have been synthesized if 100% of the analyte polynucleotide and pseudo target had been recovered in the specimen processing step. Thus, by adding a pseudo target to a biological specimen at the time of processing to isolate polynucleotides for subsequent amplification, the target recovery efficiency will be normalized in the subsequent amplification step.

Amplification Reactions that Include Pseudo Targets

Two convenient formats can be used for performing the TMA reactions that were employed in Examples described below. In the first format, all materials are in a liquid state at all times. For example, solutions of reagents, templates and enzymes are combined in a reaction vessel and the amplification is allowed to proceed. This is most convenient when the target polynucleotide is available in a purified or semi-purified state. In the second format, the polynucleotide template to be amplified in the TMA reaction is first collected on a solid phase (such as a bead), and the complex that includes the solid phase and the template combined with other reagents in the amplification reaction. Useful solid phase supports include but are not limited to nitrocellulose, nylon, glass, coated magnetic particles, polyacrylamide, polystyrene and derivatized polymers such as epoxies. This second format is especially convenient when the template polynucleotide is available in limiting amounts. Those having an ordinary level of skill in the art will appreciate that manipulating small samples of polynucleotides easily can be replaced by manipulating suspensions of the larger and more manageable beads. Moreover, the beads may represent one component in a scheme for purifying the template. For example, beads having an oligo(dT) polynucleotide disposed thereon can be mixed with a cell lysate such that poly(A)$^+$ mRNA becomes immobilized on the beads. Thus, the complex that includes the beads and immobilized mRNA can be combined with reagents and enzymes so that a TMA reaction

can be performed using the RNA joined to the beads as templates for the amplification. Under this circumstance the beads may be added directly to the reaction vessel. If instead of an oligo(dT) polynucleotide a polynucleotide having a different sequence is immobilized onto beads, that different sequence can be used to capture a complementary analyte polynucleotide or pseudo target from a collection of polynucleotides. This method of immobilizing a particular polynucleotide to a solid support can provide a means for isolating particular polynucleotides from a complex mixture of polynucleotides. Other methods of isolating polynucleotides can involve standard procedures such as extraction with organic reagents such as mixtures of phenol and chloroform, optionally including alcohol precipitation steps.

The following Examples demonstrated that the presence of pseudo targets advantageously reduced variability of amplicon production in TMA reactions that included magnetic beads derivatized with oligo(dT). This decreased variability alternatively can be expressed as an increase in the "precision" of amplification. More particularly, the results presented below demonstrated that different reactions performed using substantially identical amounts of starting template polynucleotides advantageously gave more reproducible results where variability from sample to sample was reduced.

Although many different methods of detecting amplified polynucleotides can be used in connection with the present invention, the "Hybridization Protection Assay" (HPA) disclosed in U.S. Patent No. 5,639,604, the disclosure of which is incorporated herein by reference, represents a particularly useful method. In one embodiment, the HPA detection method involves hybridizing amplified polynucleotides with a complementary polynucleotide probe that is labeled with a chemiluminescent acridinium ester. When hybridized in a duplex structure, the acridinium ester is protected from degradation under mild hydrolysis conditions. Acridinium ester in unhybridized probe molecules is susceptible to such degradation and is selectively destroyed by appropriate chemical treatment. Determining the amount of undegraded acridinium ester indicates the amount of probe that was hybridized to complementary polynucleotides. This determining step involves adding hydrogen peroxide to the mixture and measuring the amount of light emitted during a subsequent base-catalyzed chemiluminescence reaction. The HPA method of quantitating amplicon synthesis is preferred because there is no requirement for tedious and time consuming steps to remove excess unhybridized probe which otherwise would result in high levels of background hybridization. However, other methods for

detecting and quantifying amplicons, such as procedures that employ radioactive, flourescent or enzyme-labeled probes or other detection methods which use separation methods including but not limited to solid-phase support formats, HPLC and electrophoresis, can be used in the practice of the invented method with equally good results. Indeed, the method used to detect amplicons is not expected to influence the quality of the results that would be obtained in the following procedures.

Preferred Analyte Polynucleotides

As described herein, quantitative methods employing pseudo targets can be used for conducting amplification reactions regardless of the origin of the analyte polynucleotide. Preferred analyte polynucleotides include nucleic acids from disease-causing organisms, including viruses, bacteria, fungi and protozoa. Examples of highly preferred analyte polynucleotides from viruses are nucleic acids from the human immunodeficiency viruses (HIV-1 and HIV-2), the hepatitis B virus (HBV), and the hepatitis C virus (HCV). Preferred analyte polynucleotides from bacteria, fungi and protozoa that can be quantitated according to the methods disclosed herein include the ribosomal RNAs (rRNA). Examples of bacteria that are highly preferred as sources of analyte polynucleotides include *Chlamydia trachomatis* (Gram-negative cells that are obligate intracellular organisms), members of the genus Campylobacter (*C. jejuni, C. coli, C. laridis*), members of the genus Enterococcus (*E. avium, E. casseliflavus, E. durans, E. faecalis, E. faecium, E. gallinarum, E. hirae, E. mundtii, E. pseudoavium, E. malodoratus, and E. raffinosus*), *Haemophilus influenzae, Listeria momocytogenes, Neisseria gonorrhoeae, Staphylococcus aureus, Group B Streptococci, Streptococcus pneumoniae, Mycobacterium tuberculosis, Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium gordonae, Mycobacterium kansasii*. Examples of fungi that are highly preferred as sources of analyte polynucleotides include: *Blastomyces dermatitidis*, members of the genus Candida (*C. albicans, C. glabrata, C. parapsilosis, C. diversus, C. tropicalis, C. guilliermondii, C. dubliniensis*), *Histoplasma capsulatum, Coccidioides immitis*. Examples of protozoa that are highly preferred as sources of analyte polynucleotides include blood and tissue protozoa, such as members of the genus Plasmodium (*P. malariae, P. falciparum, P. vivax*), as well as protozoa which infect the gastrointestinal tract such as *Giardia lamblia* and *Cryptosporidium parvum*.

The invented method also can be used for quantifying nucleic acids that are of human origin, such as mRNAs that are over-expressed or under-expressed in disease states, including cancers. One example of gene that is present at an increased copy number in breast and ovarian adenocarcinomas is the HER-2/neu oncogene which encodes a tyrosine kinase having certain features in common with the epidermal growth factor receptor (EGFR). U.S. Patent No. 4,968,603 describes the value of measuring the increased copy number of the HER-2/neu gene, or the HER-2/neu mRNA as a tool for determining neoplastic disease status. Thus, for example, the method described herein can be employed in quantitative nucleic acid amplification protocols whereby the cellular content of HER-2/neu polynucleotides is determined.

Indeed, the polynucleotide amplification method described herein is broadly applicable to numerous nucleic acid targets and is easily extended to procedures for quantifying any given analyte polynucleotide in a test sample.

Although other materials and methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. General references for methods that can be used to perform the various nucleic acid manipulations and procedures described herein can be found in *Molecular Cloning: A Laboratory Manual* (Sambrook, et al. eds. Cold Spring Harbor Lab Publ. 1989) and *Current Protocols in Molecular Biology* (Ausubel, et al. eds., Greene Publishing Associates and Wiley-Interscience 1987). Methods of performing the TMA reaction are disclosed in U.S. Patent No. 5,399,491. Improvements to the TMA reaction protocol, such as that disclosed in U.S. Patent No. 5,786,183, are embraced within the scope of Transcription Mediated Aamplification for the purpose of the present disclosure. Methods of preparing and using acridinium ester labeled probes are given by Arnold et al., in U.S. Patent No. 5,639,604. The disclosures of these three patents are hereby incorporated by reference. A description of the experiments and results that led to the creation of the present invention now follows.

Example 1 describes methods which demonstrated that polynucleotide amplification reactions which included a pseudo target showed reduced variability of amplicon production.

Example 1

Pseudo Target Amplification Reduces Variability in the Quantity of Amplicon Produced in TMA

A series of TMA reactions was prepared using primers specific for a segment of the HIV *pol* transcript. All reactions were performed in replicates of eight. Each reaction received: 50 µl containing 60 copies of RNA transcripts of the complete HIV genome diluted in specimen buffer consisting of 10 mM HEPES (pH 7.5) and 1 mM EDTA. The RNA transcript had been synthesized using the plasmid BH10 as a template. In this procedure, the BH10 RNA was used as an exemplary analyte polynucleotide. The polynucleotide sequence of the BH10 RNA is given by the sequence of SEQ ID NO:3. Reactions also included either 0, 10^5, 10^6 or 10^7 copies of the IAC-Ascr pseudo target RNA having the sequence of SEQ ID NO:4. There were added 25 µl of amplification reagent containing 10 pmols of a T7A(-)4190 primer having the sequence AATTTAATACGACTCACTATAGGGAGAGTTTGTATGTCTGTTGCTATTATGTCTA (SEQ ID NO:1); 10 pmols of the (+)4108 primer having the sequence ACAGCAGTACAAATGGCAG (SEQ ID NO:2); 160 mM Tris buffer (pH 7.5); 16 mM each of ATP, CTP, GTP and UTP; 4 mM each of dATP, dCTP, dGTP and dTTP; 100 mM $MgCl_2$; 70 mM KCl; 20% glycerol; 0.6 mM zinc acetate and 20% polyvinylpyrrolidone. Samples were then overlaid with 200 µl of mineral oil, incubated first at 65°C for 10 minutes to allow primer-target annealing, and then incubated at 42°C for 5 minutes. Thereafter, each reaction received a 25 µl aliquot of enzyme mixture that contained 2,000 GP units of MMLV reverse transcriptase; 2,000 GP units of T7 RNA polymerase; 140 mM Tris buffer (pH 8.0); 100 mM N-acetyl-cysteine as a reducing agent; 20% glycerol; 70 mM KCl; 80 mM trehalose; 8 mM HEPES; 1.04 mM EDTA; 10% TRITON X-102 and 0.01% phenol red. One GP unit of reverse transcriptase corresponds to the amount of enzyme that synthesizes 5.75 fmols of cDNA from an RNA template in 15 minutes at 37°C. One GP unit of RNA polymerase is defined as the amount of enzyme that synthesizes 5 fmols of RNA transcript from a double stranded DNA template containing a promoter sequence in a period of 20 minutes at 37°C. Reactions were incubated at 42°C for an additional 60 minutes. Thereafter, 100 µl samples of the reaction mixtures were combined with an equal volume of the HIV-specific AE(+)4134 probe bearing an acridinium ester moiety as the label and having the sequence CCACAATTTTAAAAGAAAAGGGGGGATTGG (SEQ ID NO:5). This labeled

polynucleotide probe was prepared and used essentially according to the method disclosed in U.S. Patent No. 5,639,604 and dispersed in a solution that included 100 mM lithium succinate buffer (pH 4.7), 2% (w/v) lithium lauryl sulfate, 1.2 M lithium chloride, 15 mM ardrithiol-2, 20 mM EDTA, 20 mM EGTA and 3% ethanol. Substantially all of the ionic strength for promoting the hybridization reaction was provided by the 600 mM lithium chloride and 1% lithium lauryl sulfate components of the final hybridization solution. Importantly, the sequence of the AE(+)4134 probe permitted hybridization through complementary base pairing only with the analyte amplicon and not with the pseudo target amplicon. After hybridizing the mixture at 60°C for 15 minutes, 300 µl of a selection reagent containing 600 mM sodium borate (pH 8.5) and 1% TRITON X-100 was added and the mixture incubated at 60°C to inactivate unhybridized probe. Finally, the mixtures were cooled to room temperature, placed into a luminometer and the amount of analyte amplicon quantitated by measuring the light emitted from a chemiluminescent reaction (in RLUs). Detection reagent I included hydrogen peroxide solution in 0.001 N nitric acid. Detection reagent II included 1 N NaOH solution. Each reaction tube was injected first with detection reagent I, then with detection reagent II in order to stimulate light emission. Notably, probes used to detect and quantify the analyte amplicon were as follows: reactions not receiving the pseudo target were probed with 100 fmols of labeled AE(+)4134 probe (SEQ ID NO:5) and 3.9 pmols of unlabeled 2'methoxyribonucleotide (2'OMe) (+)4134; reactions that included 10^5 copies of the pseudo target were probed using 100 fmols of labeled probe and 3.9 pmols of unlabeled 2'OMe (+)4134; reactions that included 10^6 copies of the pseudo target were probed using 100 fmols of labeled probe and 0.2 pmols of unlabeled 2'OMe (+)4134; reactions that included 10^7 copies of the pseudo target were probed using 100 fmols of labeled probe alone. Notably, 2'OMe has substituted a methoxy moiety for the hydroxyl moiety at the 2' position of the ribose in RNA. It should be noted that the (+)4134 polynucleotide had the same base sequence as the AE(+)4134 polynucleotide, but did not include the N-acridinium ester label. The different probe specific activities were employed to facilitate luminometry readings in a linear detection range.

The results presented in Table 1 indicated that amplification reactions which included a pseudo target advantageously gave more uniform results with less variability among the collection of samples. Table 1 shows the number of copies of IAC-Ascr pseudo target and BH10 RNA analyte polynucleotide included in each reaction. Also shown are the raw data

representing the light emission from the reactions (in RLUs) and the net emission which has been corrected to subtract out the background emission measured in the negative control reactions. The column marked "Corrected to Uniform Sp. Act." indicates the value of the net RLUs that would be obtained if all HPA assays had been performed using the same high specific activity probe. This value was included in the analysis so that the different reactions could be compared directly. The net averages of all determinations for a given reaction condition are also presented along with the calculated values for standard deviation (SD) and coefficient of variability (CV%). The last column in the Table shows that the CV% values decreased as the number of copies of pseudo target in the reactions increased. This result quantitatively indicated that variability in the amount of amplicon produced in different reactions decreased as the amount of pseudo target was increased.

TABLE 1

Pseudo Target Amplification and Analyte Amplicon Synthesis
Using Soluble Polynucleotides (Pure System)

IAC-Ascr (copies)	BH10 RNA	RLUs	net RLUs	Corrected to Uniform Sp. Act.	net Avg.	Standard Deviation (SD)	Coefficient of Variability (CV%)
None	60	60758	55366	2214640	2340305	1465936	62.6
		100316	94924	3796960			
		116898	111506	4460240			
		80682	73500	3012000			
		78084	72692	2907680			
		31443	26051	1042040			
		29419	24027	961080			
		13587	8195	327800			
	None	5392	0	0	0		
1.0×10^5	60	85700	78257	3130280	3029790	1891104	62.4
		64487	57044	2281760			
		54852	47409	1896360			
		41357	33914	1356560			
		51353	43910	1756400			
		93974	86531	3461240			
		190926	183483	7339320			
		82853	75410	3016400			
	None	7443	0	0	0		
1.0×10^6	60	580816	572789	1718367	1036444	323123	31.2
		401651	393624	1180872			
		302690	294663	883989			
		222165	214138	642414			
		275810	267783	803349			
		322421	314394	943182			
		349792	341765	1025295			
		372722	364695	1094085			
	None	8027	0	0	0		
1.0×10^7	60	155174	146863	146863	134795	30825	22.9
		138647	130336	130336			
		102893	94582	94582			
		157291	148980	148980			
		166824	158513	158513			
		149727	141416	141416			
		181812	173501	173501			
		92482	84171	84171			
	None	8311	0	0	0		

Example 2 describes the methods used to demonstrate that enhanced precision of amplicon production was a general feature of reactions that included a pseudo target. More particularly, the following procedures showed that a second exemplary pseudo target, called IAC-Bscr, also reduced the variability of amplicon production in an exemplary TMA reaction.

Example 2

Reduced Variability in the Production of Analyte Amplicons by TMA is a General Feature of Reactions that Included a Pseudo Target

30 µl containing 60 molecules of BH10 RNA diluted in specimen buffer (1 mM EDTA, 10 mM HEPES) was added to a series of reaction tubes. 20 µl containing 0, 10^5, 10^6, 10^7 or 10^8 IAC-Bscr pseudo target RNA molecules (SEQ ID NO:9) in specimen buffer was added to appropriate tubes. After vortexing, each tube received a 25 µl aliquot of amplification reagent described in Example 1, with the exception that the concentrations of the T7A(-)4190 and (+)4108 primers were used at 5 pmols each instead of 10 pmols. The liquid contents of each tube were then overlaid with 200 µl of oil to prevent evaporation. Tubes were incubated at 65°C for 10 minutes and then at 42°C for 5 minutes. A 25 µl aliquot of enzyme reagent as described in Example 1 was subsequently added to each tube. The contents of all tubes were mixed and the reactions incubated at 42°C for 1 hour. At the end of the reaction period, all samples were subjected to analysis by standard HPA. Accordingly, 100 µl of a solution of acridinium labeled 2'methoxy AE(+)4134 probe was added to each tube. Different specific activities of the probe were used to detect analyte amplicons in samples produced using different amounts of the IAC-Bscr pseudo target. This ensured that light emission readings in the detection procedure would fall within the linear range of the luminometer that was used for quantifying analyte amplicons. Probes used to detect and quantify the analyte amplicon were as follows: reactions not receiving the pseudo target were probed with 100 fmols of labeled 2'OMe(+)4134 probe and 20.0 pmols of unlabeled probe; reactions that included 10^5 copies of the pseudo target were probed using 100 fmols of labeled probe and 3.0 pmols of unlabeled probe; reactions that included 10^6 copies of the pseudo target were probed using 100 fmols of labeled probe and 0.4 pmols of unlabeled probe; reactions that included 10^7 or 10^8 copies of the pseudo target were probed using 100 fmols of labeled probe alone. Mixtures of the amplification products and the probe were incubated at 60°C for 15 minutes, mixed with 300 µl

of selection reagent and then incubated at 60°C for an additional 10 minutes. The mixtures were cooled to room temperature and chemiluminescence was read after adding detection reagents I and II.

The results presented in Table 2 confirmed that amplification reactions that included a pseudo target advantageously produced more uniform amounts of analyte amplicon with less variability among the collection of sample readings. Table 2 shows the number of copies of IAC-Bscr pseudo target and BH10 RNA analyte polynucleotide that were included in each of 8 replicate amplification reactions that were conducted for each level of input pseudo target. Also shown are the results representing the average net light emission readings from all HPA reactions. Background emission values measured for reactions that included the pseudo target without including the model analyte polynucleotide were subtracted to obtain the net results. The average amounts of analyte amplicon produced for each reaction condition ("Amplicon") are presented so that the products of different HPA reactions hybridized with probes having different specific activities could be compared directly. Also presented are values for standard deviation (SD) and coefficient of variability (CV%) that were calculated for all luminometry determinations for a given reaction condition. The results confirmed that the amounts of analyte amplicon synthesized in reactions that included a pseudo target were produced with greater precision than in reactions that did not include a pseudo target. More particularly, reactions that were conducted using greater than 1×10^5 pseudo target molecules yielded CV% values that were lower than the CV% value obtained for the data set produced from reactions conducted in the absence of a pseudo target. Indeed, the statistical "p" value was less than 0.05 for reactions in our data set that were performed using at least 10^6 molecules of pseudo target. This quantitatively confirmed that variability in the amount of amplicon produced in different reactions decreased when the pseudo target was present.

TABLE 2

Pseudo Target Amplification and Analyte Amplicon Synthesis

IAC-Bscr (copies)	BH10 RNA (copies)	Avg. net (RLU)	Amplicon (pmols)	Standard Deviation (SD)	Coefficient of Variability (CV%)
None	60	165085	1.84	103009	62.4
1×10^5	60	193642	0.29	134671	69.5
1×10^6	60	164908	0.039	46096	28.0
1×10^7	60	96749	0.0029	30116	31.1
1×10^8	60	10199	0.0003	2475	24.3

Example 3 describes methods that were used to investigate whether amplification precision would also be enhanced for reactions performed in the presence of derivitized magnetic beads. In this procedure the beads were processed according to a standard specimen processing procedure which included a synthetic "target capture polynucleotide."

Example 3

Precision of Amplicon Synthesis Improved for Reactions Performed in the Presence of Processed Magnetic Beads

100 µl of a target capture reagent was combined with an equal volume of HIV seronegative plasma. The target capture reagent included 17% lithium lauryl sulfate; 190 mM succinic acid; 250 mM lithium hydroxide; 3 mM EDTA; 3 mM EGTA; 3.5 nM deoxy (-)3737 capture probe having the sequence CCCTGTTTCTGCTGGAATAACTTCTGCTTCTATATTTAAAAAAAAAAAAAAAAAAA AAAAAAAAAAA (SEQ ID NO:6) and 3.5 nM 2'methoxy (-)4258 A30 capture probe having the sequence TCTGCTGTCCCTGTAATAAACCCGTTTAAAAAAAAAAAAAAAAAAAAAAAAAAA AA (SEQ ID NO:7). The mixture was incubated at 60°C for 20 minutes then combined with 20 µl of bead suspension including 120 µg of magnetic beads derivitized with oligo(dT) (Novagen; Madison WI). Reactions were then cooled to room temperature for 15 minutes to allow the hybridization of the capture probe and the immobilized oligo(dT). The beads were collected against the side of the vessel wall for 15 minutes upon positioning in a magnetic holder, and the supernatant aspirated. The beads were washed three times using 1 ml aliquots of wash reagent and used in a TMA reaction as described in Example 1, except that 100 copies of the BH10 RNA, and 0, 10^3, 10^4 or 10^5 copies of the IAC-Ascr pseudo target were used. HPA was carried

out using 100 fmols of labeled AE(+)4134 probe and 200 fmols of unlabeled (+)4134 according to the procedure described above.

The results presented in Table 3 confirmed that amplification reactions that included a pseudo target advantageously yielded more uniform production of the analyte amplicon. More particularly, these results, which are based on replicates of 8 amplification reactions conducted for each level of input pseudo target, again indicated that the CV% values decreased for trials that had been performed in the presence of increasing amounts of pseudo target. Notably, levels greater than 10^4 copies of pseudo target per reaction gave the most statistically significant improvement in the precision of analyte amplicon synthesis.

TABLE 3

Pseudo Target Amplification and Analyte Amplicon Synthesis in the Presence of Derivitized Magnetic Beads

IAC-Ascr (copies)	BH10 RNA (copies)	net Avg. (RLU)	Standard Deviation (SD)	Coefficient of Variability (CV%)
None	100	97105	37798	39.0
1.0×10^3	100	152313	38630	25.4
1.0×10^4	100	155062	67377	43.5
1.0×10^5	100	12385	19655	15.9

The results presented in the following Example confirmed that TMA reactions that included a pseudo target, and that employed analyte polynucleotides captured on an immobilized support, exhibited enhanced precision with respect to the amount of analyte amplicon synthesized in the amplification reaction. Whereas the procedures described under Example 3 proved that the presence of a solid phase capture substrate did not adversely affect the TMA reaction, the procedures set forth below more closely parallel the manner in which diagnostic testing procedures are carried out in accordance with the invention. More particularly, the procedures used in the following Example employed captured HIV RNA as amplification templates. Variability arising from inconsistent target recovery in these procedures was normalized to permit precision of amplification to be examined independently. More specifically, the HIV RNA was first collected on magnetic beads according to a standard specimen processing protocol and then pooled and redistributed into individual tubes so that all amplification reactions could be initiated with equal amounts of HIV RNA, but with different amounts of pseudo target.

Example 4 describes the methods used to demonstrate that TMA reactions conducted using pseudo targets and analyte polynucleotide templates captured on solid substrates gave enhanced precision of amplicon production in amplification reactions.

Example 4

Pseudo Targets Enhance the Precision of Amplification Reactions that Employ Captured Analyte Polynucleotides as Templates

100 µl aliquots of target capture reagent and HIV virion suspension diluted in seronegative plasma containing either 0 or 200 copy equivalents of the HIV RNA/100 µl of plasma were combined in individual reaction tubes. Target capture reagent included the following reagents at the specified concentrations: 3 mM disodium EDTA; 3 mM EGTA; 17% lithium lauryl sulfate; 190 mM succinic acid (adjusted to a final pH 5.1); 250 mM lithium hydroxide; 3.5 nM deoxy HIV(-)3837 A30 (SEQ ID NO:6); and 3.5 nM 2'methoxy HIV(-)4258 A30 (SEQ ID NO:7). Samples were incubated at 60°C for 20 minutes to liberate the HIV RNA from virions, to denature all polynucleotides and to allow the hybridization of capture probes to the target *pol* sequence. 20 µl aliquots of oligo(dT) bead suspension containing 120 µg of oligo(dT) derivatized beads were then added to each reaction tube. After mixing thoroughly, the samples were cooled to room temperature for 15 minutes to permit hybridization of the oligo(dA) tail of the capture probe and the bead-immobilized oligo(dT), thereby linking the analyte polynucleotide to the magnetic bead through a bridging polynucleotide. Beads and the polynucleotides immobilized thereon were isolated from plasma and free polynucleotides by placing the tubes on a magnetic rack for a period of 5 minutes, during which time the beads were collected against an inner surface of each tube. Supernatants were aspirated and the isolated beads washed three times using 1 ml aliquots of wash reagent (0.1% SDS, 10 mM HEPES (pH 7.5), 150 mM NaCl) with magnetic isolation of the beads between each step. The beads were next combined with 40 µl of specimen buffer (1 mM EDTA, 10 mM HEPES), mixed and pooled. 40 µl aliquots of the pooled bead suspension were then distributed to fresh reaction tubes so that all samples contained substantially identical amounts of bead-captured analyte polynucleotides. 10 µl aliquots of pseudo target diluted in specimen buffer (1 mM EDTA, 10 mM HEPES) were distributed to appropriate tubes. Each aliquot contained either 0, 2×10^6, 2×10^7 or 2×10^8 molecules of the IAC-Ascr or IAC Bscr pseudo target RNA. TMA amplification reactions were performed as described above in Example 2. Analyte amplicons were detected using a modified version of the HPA procedure called, "Adduct Promoted

Hydrolysis" (APH). Following the amplification reactions, each tube received a 100 µl aliquot of acridinium labeled 2'OMe (+)4134 probe. Probes having different specific activities were used in this procedure so that amplification reactions performed using different amounts of pseudo target, whether IAC-Ascr or IAC-Bscr, would give light emission readings that fell in a linear response range for luminometry. These different specific activities were achieved by mixing labeled and unlabeled probes. Probes used to detect and quantify the analyte amplicon were as follows: reactions not receiving the pseudo target were probed with 1.0 pmol of labeled 2'OMe (+)4134 probe and 100.0 pmols of unlabeled probe; reactions that included 10^6, 10^7 or 10^8 copies of the pseudo target were probed using 1.0 pmol of labeled probe and 1.0 pmol of unlabeled probe. Reactions were incubated at 60°C for 15 minutes, mixed with 300 µl of sodium metaarsenite-containing selection reagent, and incubated at 60°C for 20 minutes. The mixtures were cooled to room temperature and chemiluminescence was read after adding detection reagents I and II.

The results in Tables 4 and 5 confirmed that amplification reactions that included a pseudo target advantageously produced analyte amplicons in more uniform amounts and with less variability among the collection of sample readings. The Tables show that each reaction was primed using either 0 or 200 RNA equivalents of the HIV virion (strain HIV IIIb) as an analyte polynucleotide, and one of seven pseudo target conditions. The first condition was a negative control where the reactions were conducted in the absence of the pseudo target. The remaining conditions used either the IAC-Ascr (Table 4) or the IAC-Bscr (Table 5) pseudo target in one of three amounts. The summarized data in both Tables represents the results of 8 replicate trials conducted for each level of input pseudo target. Background emission values produced in reactions that included the pseudo target without including the model analyte polynucleotide template were subtracted to obtain the net results. The results clearly indicated that the amount of amplicon produced in the reactions decreased as the number of pseudo target molecules in the reaction increased, as expected. All amplification reactions that included a pseudo target resulted in the production of more uniform amounts of analyte amplicon. More particularly, the CV% values were lower among all data sets derived from reactions that included pseudo targets when compared to the negative control that was conducted in the absence of a pseudo target. These results supported the conclusion that precision in the amount of analyte amplicon produced in an amplification reaction can be improved by including a pseudo target in the reaction. The fact that two different pseudo targets gave similarly good

results showed that the improved precision did not depend of the particular sequence of the pseudo target. These results further showed how precision in the amount of analyte amplicon produced in an amplification reaction can be improved by including pseudo targets in reactions that employed analyte polynucleotides captured by a solid support, such as a magnetic bead, as templates for the amplification reaction.

TABLE 4

Different Pseudo Targets Improve the Precision of Analyte Amplicon Production

IAC-Ascr Pseudo Target (copies)	HIV Virion (copies)	Avg. net (RLU)	Amplicon (pmol)	Standard Deviation (SD)	Coefficient of Variability (CV%)
None	200	69858	0.95	57195	81.9
2×10^6	200	166824	0.03	62799	37.6
2×10^7	200	26733	0.0041	13616	50.9
2×10^8	200	3043	0.0005	585	19.2

TABLE 5

Improved Precision of Analyte Amplicon Production Using Different Pseudo Targets

IAC-Bscr Pseudo Target (copies)	HIV Virion (copies)	Avg. net (RLU)	Amplicon (pmol)	Standard Deviation (SD)	Coefficient of Variability (CV%)
None	200	69858	0.95	57195	81.9
2×10^6	200	109125	0.0166	37519	34.4
2×10^7	200	14904	0.0023	10669	71.6
2×10^8	200	1492	0.0002	697	46.8

Yet another advantage of conducting amplification reactions in the presence of a pseudo target relates to normalizing the amount of amplicon produced when the input analyte polynucleotide is recovered from a biological sample at less than quantitative yield. The basis of this advantage, which is illustrated in Figure 5, has been addressed above. The following Example was used to model situations wherein the recovery of analyte polynucleotides from a biological sample differed substantially. More particularly, conditions examined ranged from the equivalent of from 100% to 25% recovery. Such differences in the efficiency of analyte polynucleotide recovery could arise for reasons including variable recovery from phenol extraction procedures, ethanol precipitation procedures, difficult specimen collection or

extraction conditions or even a laboratory spill leading to sample loss. In each case, the amount of analyte polynucleotide recovered would be less than a quantitative recovery.

As described below, the variable efficiency of analyte polynucleotide recovery was modeled by performing amplification reactions under three different conditions. Under the first condition, reactions were performed using three different amounts of input analyte polynucleotide without pseudo target. The second condition involved reactions performed using the same three different amounts of input analyte polynucleotide and a constant amount of pseudo target. Finally, the third reaction condition employed the same three different amounts of input analyte polynucleotide, where the ratio of the amounts of analyte polynucleotide and pseudo target were constant. It will be apparent that this third condition represents a case which would result when the pseudo target was added to a biological sample containing analyte polynucleotide at a time before nucleic acids were isolated from the sample. Under this circumstance, loss of a portion of the sample during processing steps would result in identical percentage losses of both analyte polynucleotide and pseudo target, yet the ratio of the two species would remain fixed. As will be apparent from the results that follow, amplification reactions that included a constant ratio of pseudo target and analyte polynucleotide advantageously gave improved synthesis of analyte amplicons. Thus, even reactions having a limited number of input analyte polynucleotides behaved as though the starting number of templates was larger.

The results obtained in the Example which follows provided the basis for the improved method of biological specimen processing that includes adding pseudo targets to the specimen before nucleic acids are isolated. One method of normalizing the level of analyte amplicon produced in an amplification reaction involves first adding the pseudo target to a biological specimen, then isolating polynucleotides from the specimen and thereafter using the polynucleotides isolated in this fashion to conduct the amplification reactions.

Example 5 describes the methods that were used to represent amplification reactions that were initiated using variable amounts of analyte polynucleotide. More particularly, the reactions were performed so that the amounts of analyte polynucleotide represented "100%," "50%" and "25%" values.

Example 5

Normalizing Amplicon Synthesis in Amplification Reactions Primed with Variable Amounts of Analyte Polynucleotide

Amplification reactions were prepared according to the method of Example 1 with the following changes. First, replicates of 10 reactions for each condition were prepared instead of replicates of eight. Second, primer amounts used in the reactions were reduced to 5 pmols each, instead of 10 pmols each. Third, 20% polyvinylpyrrolidone was substituted by 10% trehalose. Fourth, the amounts of analyte polynucleotide and pseudo target were as presented in Table 6. In our procedures, the polynucleotide mixtures presented in this Table were first combined, then mixed with other reagents in the reaction mixture, and finally mixed with the two polymerase enzymes to initiate the TMA reaction.

TABLE 6

Mixtures of Analyte Polynucleotide and Pseudo Target

Condition	BH10 RNA (copies)	IAC-Ascr (copies)
No Pseudo Target	500	0
	1000	0
	2000	0
Constant Pseudo Target	500	6×10^6
	1000	6×10^6
	2000	6×10^6
Constant Ratio of Pseudo Target and Analyte Polynucleotide	500	1.5×10^6
	1000	3.0×10^6
	2000	6.0×10^6

At the conclusion of the amplification reactions, all reaction mixtures were probed according to the APH protocol described above in Example 4 to detect and quantitate analyte amplicons. AE labeled HIV (+)4134b probe having the sequence CCACAATTTTAAAAGAAAAGGG (SEQ ID NO:8) of different specific activities was used in this procedure so that the amplification reactions performed using different amounts of pseudo target would give light emission readings that fell within a linear response range for luminometry. Again, these different specific activities were achieved by mixing different amounts of labeled and unlabeled probes. Probes used to detect and quantify the analyte amplicon were as follows: reactions not receiving the

pseudo target were probed using 1.3 pmols of labeled probe and 400 pmols of unlabeled probe; reactions that included 1.5×10^6, 3×10^6 or 6×10^6 copies of pseudo target were probed using 1.3 pmols of labeled and 8.7 pmols of unlabeled probe.

The quantitative results presented in Tables 7 - 9 and in Figure 6 clearly indicated that reactions in which the ratio of pseudo target to analyte polynucleotide was held constant yielded substantially smaller differences in the amounts of analyte amplicon synthesized from variable amounts of input analyte polynucleotide. All results were based on replicates of 10 trials conducted for each level of input analyte polynucleotide template. In Figure 6, 100% of input analyte polynucleotide was represented by 2,000 copies of BH10 RNA. In the absence of pseudo target the slope of the line representing the amount of analyte amplicon produced at decreasing levels of input analyte polynucleotide, declined sharply as the number of these template decreased from 2,000 to 500. A similar result was obtained in the trials containing a constant level of pseudo target. Thus, procedures that involved merely adding pseudo target to a sample having a low level of input analyte polynucleotides had substantially no effect on increasing the amount of analyte amplicon that was synthesized. However, the amplification reactions that were carried out using a constant molar ratio of pseudo target to analyte polynucleotide yielded smaller differences in the amounts of analyte amplicon synthesized from variable amounts of input analyte polynucleotide. For example, the results shown in the Figure indicate that at 500 copies of input BH10 RNA, the yield of analyte amplicons (measured in RLU) was about 68% of the value obtained using 2000 copies of the template, while the corresponding result obtained in the absence of pseudo target or when pseudo target was held constant only was about 22%. Conducting amplification reactions using a constant ratio of pseudo target and analyte polynucleotide tended to normalize the amount of analyte amplicon synthesized in the amplification reaction. Also, the ratio may be varied somewhat depending on input level and desired accuracy of quantitation. Significantly, substantially similar results were obtained when the reactions were conducted in the presence of magnetic beads and a capture reagent, as described under Examples 3 and 4. Moreover, the data presented in Tables 7 - 9 show that precision of amplification reactions was improved by including a pseudo target in the amplification reaction.

TABLE 7

TMA Reactions Conducted in the Absence of a Pseudo Target

BH10 RNA (copies)	net Avg (RLU)	% of 2000	Standard Deviation (SD)	Coefficient of Variability (CV%)
None	0	0	N/A	N/A
500	35279	22.4	10033	28.4
1000	70202	44.7	36070	51.4
2000	157176	100	26792	17.0

TABLE 8

TMA Reactions Having Constant Pseudo Target Levels

BH10 RNA (copies)	net Avg (RLU)	% of 2000	Standard Deviation (SD)	Coefficient of Variability (CV%)
None	0	0	N/A	N/A
500	96434	22.2	23442	24.3
1000	224493	51.6	49903	22.2
2000	434899	100	30382	7.0

TABLE 9

TMA Reactions Having a Constant Ratio of Analyte Polynucleotide and Pseudo Target

BH10 RNA (copies)	net Avg (RLU)	% of 2000	Standard Deviation (SD)	Coefficient of Variability (CV%)
None	0	0	N/A	N/A
500	294660	67.8	43197	14.7
1000	340594	78.3	72128	21.2
2000	434899	100	30382	7.0

The following Example describes experiments that were carried out to show how incorporating a pseudo target into an amplification reaction can be used to control the amount of amplicon produced in the reaction. As indicated above, reducing the amount of amplicon produced in a reaction advantageously: (1) reduces the likelihood of positive carry-over contamination; (2) allows for more efficient use of labeled probes; and (3) may be used to "tune" signal strength to fall within a linear range for detection apparatus such as a luminometer.

With respect to this second point, with reduced numbers of product amplicon produced in a reaction it becomes possible to employ very high specific activity probes in quantities sufficient to provide probe excess. Those having an ordinary level of skill in the art will appreciate that the specific activity of a hybridization probe refers to the amount of detectable label per probe molecule. High specific activity probes are useful for detecting minute quantities of complementary polynucleotides. However, if the probe is expensive to prepare, or is labeled with a radioactive label that requires special handling and disposal precautions, it may not be desirable to use high specific activity probes in large quantities that would be needed to carry out quantitative hybridizations using probe excess conditions. Thus, reducing the amount of analyte amplicon produced in an amplification reaction advantageously can facilitate efficient use of probes that are employed for detecting the amplicons.

Example 6 describes methods that were used to demonstrate how pseudo targets can be used to control the amount of analyte amplicon produced in an amplification reaction.

Example 6

Employing Pseudo Targets to Control the Production of Analyte Amplicons

100 µl of target capture reagent (17% lithium lauryl sulfate; 190 mM succinic acid; 250 mM lithium hydroxide; 3 mM EDTA; 3 mM EGTA; 3.5 nM 2' methoxy (-)3837 A30 capture probe (SEQ ID NO:6) and 3.5 nM 2' methoxy (-)4258 A30 capture probe (SEQ ID NO:7) was combined with 100 µl of HIV virion diluted in HIV seronegative plasma. Samples contained either no HIV RNA; 200; 2,000; 20,000; 200,000 or 2,000,000 RNA equivalents/ml of plasma. Mixtures were incubated at 60°C for 20 minutes to allow hybridization of the capture probe with *pol* gene sequences present in target polynucleotides, and then combined with 20 µl of oligo(dT) bead suspension (120 µg of oligo(dT) beads/20 µl). After mixing thoroughly, samples were cooled to room temperature over a period of 15 minutes to allow hybridization of the oligo(dA) of the capture probe and the bead-immobilized oligo(dT), thereby linking the *pol* gene sequence and the magnetic bead. Beads were collected against the sides of the tubes using a magnetic rack and the supernatants aspirated. Beads were washed three times using 1 ml volumes of wash reagent (0.1% SDS; 10 mM HEPES (pH 7.5); 150 mM NaCl). 50 µl aliquots of specimen buffer (10 mM HEPES; 1 mM EDTA) were added to tubes that did not receive any pseudo target. 50 µl aliquots of pseudo target diluted in specimen buffer were added to tubes that did receive the pseudo target. After mixing, each sample received a 25 µl aliquot of

amplification reagent containing: 5 pmols of a T7A(-)4190 primer; 5 pmols of the (+)4108 primer; 160 mM Tris buffer (pH 7.5); 16 mM each of ATP, CTP, GTP and UTP; 4 mM each of dATP, dCTP, dGTP and dTTP; 100 mM $MgCl_2$; 70 mM KCl; 20% glycerol; 0.6 mM zinc acetate and 10% trehalose. Samples were overlaid with 200 µl of mineral oil and then incubated at 42°C for 10 minutes. Amplification reactions were initiated by adding 25 µl aliquots of enzyme reagent containing 2000 GP units of MMLV reverse transcriptase; 2000 GP units of T7 RNA polymerase; 140 mM Tris buffer (pH 8.0); 100 mM N-acetyl-cysteine as a reducing agent; 20% glycerol; 70 mM KCl; 80 mM trehalose; 8 mM HEPES; 1.04 mM EDTA; 10% TRITON X-102 and 0.01% phenol red. All reactants were mixed and allowed to incubate at 42°C for 1 hour.

At the conclusion of the reaction period, analyte amplicons were quantified using the above-described APH procedure. A 100 µl aliquot of a solution of acridinium labeled probe AE(+)4134b was added to each sample. Samples corresponding to reactions that included the pseudo target received 1.3 pmols of labeled probe and 38.7 pmols of unlabeled probe, while samples corresponding to reactions that did not include the pseudo target received 1.3 pmols of labeled probe and 400 pmols of unlabeled probe. Mixtures were incubated at 60°C for 15 minutes and then combined and mixed with 300 µl of APH selection reagent containing sodium metaarsenite. Reaction mixtures were incubated at 60°C for 20 minutes and then cooled to room temperature. Chemiluminescence was read following addition of detection reagents I and II.

Notably, preliminary experiments were carried out in which routine APH procedures were conducted using a range of specific activities to identify conditions that would give results falling within the linear detection range of the luminometer used in our experiments. Those having ordinary skill in the art readily will appreciate that many sorts of detection apparatus, whether a luminometer or an X-ray film, have a range within which the intensity of a signal and the amount of material that produced the signal are linearly or exponentially related. Above that range, the correspondence does not hold. Thus, determining such linear ranges is a matter of routine experimentation for those having ordinary skill in the art.

The probe mixtures employed for detecting analyte amplicons in our procedure were: 401.3 pmols of probe consisting of 1.3 pmols labeled probe and 400 pmols of unlabeled probe for the reaction conducted in the absence of a pseudo target; and 40 pmols of probe consisting of 1.3 pmols labeled probe and 38.7 pmols of unlabeled probe for the reaction that included the

pseudo target. In order to normalize the results of the assay, light intensity readings (measured in RLUs) were converted into pmols of amplicon in the hybridization step by multiplying the average net RLU values by a conversion factor. This conversion factor was established by hybridizing, in parallel reactions, known amounts of target polynucleotide and excess amounts of labeled probe and then determining the light output generated by the known amount of target. This allowed correlation of light output and the amount of amplicon hybridized to the probe.

The results presented in Tables 10 and 11 and in Figure 7 indicated that the presence of a pseudo target in an amplification reaction did not compromise the correlation between the amount of input analyte polynucleotide and the amount of analyte amplicon produced in amplification reactions. All results were based on replicates of 5 trials conducted for each level of input HIV IIIb RNA used in the procedure. The log plot shown in Figure 7 clearly indicates a strong relationship between the amount of HIV IIIb RNA equivalents input into a reaction and the amount of analyte amplicon produced. Clearly, this same strong linear relationship prevailed when the amplification reactions additionally included the pseudo target. The downward shift observed for the line representing analyte amplicons produced in reactions that included pseudo targets indicates that fewer molecules were produced when compared with reactions that did not include pseudo targets. For example, the results shown in Table 10 indicate that approximately 520 pmols of analyte amplicon were produced in the reaction that included 200,000 HIV RNA equivalents, and that this number was reduced by about ten fold when the pseudo target was included in the reaction. Thus, the number of analyte amplicons produced in the amplification reaction was reduced by including a pseudo target in the reaction.

TABLE 10

Controlling Analyte Amplicon Production Using Pseudo Targets

HIV IIIb RNA equivalents/reaction	No Pseudo Target		
	Avg net (RLU)	Amplicon (pmols)	Standard Deviation (SD)
None	0	0	N/A
20	47878	2.5	55529
200	137756	7.2	143360
2000	794621	41.7	174616
20,000	4762815	250	609171
200,000	9908427	520	639895

TABLE 11
Controlling Analyte Amplicon Production Using Pseudo Targets

HIV IIIb RNA equivalents/reaction	2 x 10^6 Pseudo Target Molecules (IAC-Ascr)		
	Avg net (RLU)	Amplicon (pmols)	Standard Deviation (SD)
None	0	0	N/A
20	1623	0.01	2224
200	10473	0.067	8000
2000	84435	0.54	8449
20,000	802975	5.1	189079
200,000	8083585	51.5	1567615

Qualitative Format Assays

Although the foregoing description relates to quantitative assays, other useful procedures that employ pseudo targets in amplification reactions relate to qualitative assays that provide information about the presence or absence of an analyte polynucleotide in a test sample. Qualitative tests can also be used for indicating whether or not an analyte polynucleotide in a test sample is present at a level falling within a specified range. These assays could, for instance, be used to monitor a patient's response to drug therapy. For example, a patient infected with a blood borne virus may experience a change in the plasma titer following therapeutic drug treatment. A physician can monitor whether the patient's virus titer increases or decreases with respect to a particular threshold value using a qualitative assay that incorporates pseudo target amplification. It is to be understood that a qualitative testing format involves only detection of a signal and so would not necessarily require quantitative measurement of the signal or the production or use of a standard curve by an end-user of a diagnostic assay.

In certain preferred embodiments of the invention qualitative assays are performed to indicate whether a biological sample contains an analyte polynucleotide. In other preferred embodiments of the invention assays that produce only qualitative results (i.e., a result is either positive or negative) but can provide semi-quantitative information about an analyte polynucleotide in a sample.

Qualitative assays that co-amplify analyte polynucleotides and pseudo targets are especially versatile when combined with detection protocols having specified thresholds of

detection. These thresholds can be manipulated by adjusting the specific activity of a hybridization probe, or by calibrating the detection device to specify a negative result below a certain numerical value or a positive result above a certain value. For example, a luminometer can be set to indicate a positive result for RLU values greater than a certain threshold level. Alternatively, the amount of pseudo target included in the amplification reaction can be increased or decreased so that certain levels of analyte amplicon produce detectable signals that are either above or below the limit of detection for a particular device. Thus, the amount of pseudo target input into an amplification reaction for a diagnostic assay can be adjusted or "tuned" through routine experimentation so that a detection signal falling within a desired range is produced.

When an analyte polynucleotide and a pseudo target are co-amplified according to the above-described procedures, the amount of analyte amplicon synthesized in the reaction will naturally be related to the amount of analyte polynucleotide that was input into the reaction. Since the magnitude of a hybridization signal can be tuned by one of the procedures described above, since amplification reactions that incorporate pseudo targets advantageously are characterized by enhanced precision, and since it is possible to tune a diagnostic reaction so that a given level of input analyte polynucleotide produces a hybridization signal that is above or below a detection threshold for a testing instrument it is possible to produce qualitative assays that also provide quantitative information.

The following Example illustrates how semi-quantitative information about the amount of analyte polynucleotide in a test sample can be obtained using a qualitative assay that provides only positive or negative results. For illustrative purposes, the HIV polynucleotide serves as the analyte polynucleotide and the indicated titer range is based on results presented in the previous Example. Of course, other analyte polynucleotides and different threshold ranges also can be employed in this qualitative testing format. Also, detection by luminometry can be substituted by fluorescence or other optical or electro-chemical detection methods. Pseudo target can be combined with a biological sample and nucleic acids isolated thereafter, or simply combined with pre-isolated analyte polynucleotide prior to the co-amplification step. In this Example the detection system includes a detection device (luminometer), a labeled hybridization probe that can be detected by the detection device. Based on the preceding description it should be clear that the specific activity of the labeled probe and the amount of pseudo target included in the co-

amplifying step are both variables that can be manipulated to contro the threshold of detection in the detection system.

Example 7 describes how amplification reactions that include pseudo targets can be used in a qualitative assay format to derive semi-quantitative information about pre-amplification amounts of analyte polynucleotide.

Example 7

Qualitative Assay Formats

A physician treating a patient infected with HIV desires to monitor the effectiveness of a drug treatment protocol. The physician specifically desires to know when the patient's plasma titer is reduced from a high starting level to a lower level that corresponds to below about 200 RNA equivalents in 100 µl of plasma.

First and second plasma samples are obtained from the patient at times before and after commencing drug therapy. Samples are prepared and used for amplification reactions essentially as described under Example 6. Individual 100 µl aliquots of the plasma samples are mixed with 100 µl aliquots of target capture reagent and the mixtures incubated, combined with oligo(dT) bead suspension, mixed again and then cooled to room temperature. Beads are collected, washed and then combined with 50 µl aliquots containing 2×10^6 copies of pseudo target diluted in specimen buffer. After mixing, each sample receives a 25 µl aliquot of amplification reagent containing primers and nucleotide reactants. Samples are overlaid with 200 µl of mineral oil and then incubated at 42°C for 10 minutes. Amplification reactions are initiated by adding 25 µl aliquots of enzyme reagent containing 2000 GP units of MMLV reverse transcriptase and 2000 GP units of T7 RNA polymerase in a buffered solution. All reactants are mixed and allowed to incubate at 42°C for 1 hour. Amplified samples are then subjected to an APH detection procedure. A solution of acridinium labeled probe AE(+)4134b is added to each sample. Each sample receives 1.3 pmols of labeled probe and 38.7 pmols of unlabeled probe, where each probe is specific for authentic HIV amplicons but not pseudo target amplicons. These amounts of probe represent saturating hybridization amounts so that analyte amplicons will be quantitatively detected. Mixtures are incubated at 60°C for 15 minutes and then combined and mixed with 300 µl of APH selection reagent containing sodium metaarsenite. Reaction mixtures are incubated at 60°C for 20 minutes and then cooled to room temperature. Chemiluminescence is read following addition of detection reagents I and II using a luminometer programmed to indicate a positive result for RLU values of 10,000 or greater and

a negative result for RLU values less than 10,000. The pre-treatment plasma sample gave a positive result, thereby indicating a level of at least about 200 RNA equivalents. Conversely, the post-treatment plasma sample gave a negative result, thereby indicating a level of less than 200 RNA equivalents in the 100 µl sample. The physician judges that the drug treatment is effective at reducing viral load.

This invention has been described with reference to a number of specific examples and embodiments thereof. Of course, a number of different embodiments of the present invention will suggest themselves to those having ordinary skill in the art upon review of the foregoing detailed description. Thus, the true scope of the present invention is to be determined upon reference to the appended claims.

EDITORIAL NOTE

APPLICATION NUMBER - 62322/00

The following sequence listing pages 1-9 are part of the description.

The claims' pages follow on pages 55-60

SEQUENCE LISTING

<110> GEN-PROBE INCORPORATED

<120> POLYNUCLEOTIDE AMPLIFICATION METHOD

<130> GP104-PCT

<160> 9

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<212> RNA
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<220>
<223> Sequence of (-)3837 A30 capture probe.

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WHAT IS CLAIMED IS:

1. A method of quantifying analyte polynucleotides present in a test sample, comprising the steps of:

obtaining a test sample that contains an unknown amount of an analyte polynucleotide;

combining a predetermined amount of said test sample with a predetermined amount of a pseudo target;

co-amplifying in a polynucleotide amplification reaction the pseudo target and any of the analyte polynucleotide contained in said test sample to produce a collection of amplification products, said collection including both an analyte amplicon if said test sample contained the analyte polynucleotide and a pseudo target amplicon; and

quantifying the analyte amplicon without reference to the amount of pseudo target amplicon, whereby the quantity of analyte amplicon is related in a manner that is dose-dependent on the unknown amount of the analyte polynucleotide contained in said test sample.

2. The method of Claim 1, further comprising a step for detecting the pseudo target amplicon.

3. The method of Claim 1, wherein the step for quantifying comprises hybridizing said collection of amplification products from the co-amplifying step with a labeled probe specific for the analyte amplicon but not the pseudo target amplicon and then detecting any labeled probe that specifically hybridized the analyte amplicon.

4. The method of Claim 3, wherein said polynucleotide amplification reaction in the co-amplifying step is selected from the group consisting of a Transcription Mediated Amplification reaction, a NASBA reaction and a Polymerase Chain Reaction.

5. The method of Claim 4, wherein said polynucleotide amplification reaction is the Transcription Mediated Amplification reaction.

6. The method of either Claim 4 or Claim 5, wherein the obtaining step comprises first collecting a biological specimen and then releasing nucleic acids contained therein to result in said test sample that contains the unknown amount of said analyte polynucleotide.

7. The method of Claim 6, further comprising a step for capturing said analyte polynucleotide onto a solid support prior to said co-amplifying step.

8. The method of Claim 7, wherein the solid support is a bead derivatized with a synthetic polynucleotide.

9. The method of Claim 7, wherein the predetermined amount of said pseudo target in the combining step ranges from between 1.0×10^3 and 2×10^8 molecules.

10. The method of Claim 9, wherein the predetermined amount of said pseudo target in the combining step ranges from between 1.0×10^4 and 2×10^8 molecules.

11. The method of Claim 10, wherein the predetermined amount of said pseudo target in the combining step ranges from between 1.0×10^5 and 2×10^8 molecules.

12. The method of Claim 6, wherein the biological specimen is a blood sample or a plasma sample and wherein said nucleic acids comprise viral nucleic acids.

13. The method of Claim 12, wherein the analyte polynucleotide is released from HIV virions.

14. The method of Claim 6, wherein the predetermined amount of the pseudo target in the combining step is between 1×10^3 and 2×10^8 molecules.

15. The method of Claim 14, wherein the predetermined amount of the pseudo target in the combining step is between 1×10^4 and 2×10^8 molecules.

16. The method of Claim 15, wherein the predetermined amount of the pseudo target in the combining step is between 1×10^5 and 2×10^8 molecules.

17. The method of Claim 5, further comprising a step for isolating said analyte polynucleotide and said pseudo target after the combining step and before the co-amplifying step.

18. The method of Claim 5, wherein the predetermined amount of the pseudo target is between 1×10^3 and 2×10^8 molecules.

19. The method of Claim 18, wherein the predetermined amount of the pseudo target is between 1×10^4 and 2×10^8 molecules.

20. The method of Claim 19, wherein the predetermined amount of the pseudo target is between 1×10^5 and 2×10^8 molecules.

21. The method of Claim 3, wherein said labeled probe is labeled with acridinium ester.

22. The method of Claim 21, wherein the quantifying step comprises measuring by luminometry the probe labeled with acridinium ester that specifically hybridized the analyte amplicon.

23. The method of Claim 6, wherein the analyte polynucleotide is a viral polynucleotide.

24. The method of Claim 1, further comprising a step for consulting a standard curve that relates pre-amplification amounts of analyte polynucleotide and post-amplification amounts of analyte amplicon.

25. The method of Claim 5, further comprising a step for consulting a standard curve that relates pre-amplification amounts of analyte polynucleotide and post-amplification amounts of analyte amplicon.

26. The method of Claim 22, further comprising a step for consulting a standard curve that relates pre-amplification amounts of analyte polynucleotide and post-amplification amounts of analyte amplicon.

27. The method of Claim 5, wherein the Transcription Mediated Amplification reaction employs a paired set of oligonucleotide primers have the sequences of SEQ ID NO:1 and SEQ ID NO:2.

28. The method of Claim 27, wherein the pseudo target has a polynucleotide sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:9.

29. A method for relating pre-amplification amounts of analyte polynucleotide and post-amplification amounts of analyte amplicon, said method comprising the steps of:

obtaining a plurality of control samples, wherein each of the control samples has a different predetermined amount of an analyte polynucleotide;

combining each of said plurality of control samples with a constant predetermined amount of a pseudo target to result in a plurality of mixed control samples;

co-amplifying in a plurality of amplification reactions both the pseudo target and the analyte polynucleotide present in each of said plurality of mixed control samples to produce a collection of amplification products that include a pseudo target amplicon and an analyte amplicon;

quantifying the analyte amplicon for each of said plurality of amplification reactions without reference to the amount of pseudo target amplicon present in said collection of amplification products; and

preparing a standard curve having the different predetermined amounts of said analyte polynucleotide plotted against the quantified amounts of said analyte amplicon

produced in each of said plurality of amplification reactions, thereby relating the pre-amplification amounts of said analyte polynucleotide in said plurality of control samples and the post-amplification amounts of analyte amplicon synthesized in the plurality of amplification reactions.

30. The method of Claim 29, further comprising a step for detecting the pseudo target amplicon.

31. The method of Claim 29, wherein said analyte polynucleotide is a viral polynucleotide.

32. The method of Claim 31, wherein the viral polynucleotide is an HIV polynucleotide.

33. The method of Claim 29, wherein said constant predetermined amount of said pseudo target is between 1×10^3 and 2×10^8 molecules.

34. The method of Claim 33, wherein said constant predetermined amount of said pseudo target is between 1×10^4 and 2×10^8 molecules.

35. The method of Claim 34, wherein said constant predetermined amount of said pseudo target is between 1×10^5 and 2×10^8 molecules.

36. The method of Claim 29, wherein said plurality of amplification reactions in the co-amplifying step is selected from the group consisting of a plurality of Transcription Mediated Amplification reactions, a plurality of NASBA reactions and a plurality of PCR reactions.

37. The method of Claim 36, wherein said plurality of amplification reactions in the co-amplifying step are a plurality of Transcription Mediated Amplification reactions.

38. The method of Claim 36 or Claim 37, wherein the quantifying step comprises hybridizing said collection of amplification products from the co-amplifying step with a labeled probe specific for the analyte amplicon but not the pseudo target amplicon and then quantitatively detecting any labeled probe that specifically hybridized the analyte amplicon.

39 The method of Claim 38, wherein the labeled probe is labeled with acridinium ester.

40. The method of Claim 38, further comprising a step for capturing said analyte polynucleotide onto a solid support prior to said co-amplifying step.

41. A method of determining whether a biological sample contains an analyte polynucleotide, comprising the steps of:

obtaining a biological sample to be tested for the presence of the analyte polynucleotide;

combining the biological sample with a pseudo target to result in a mixed sample;

isolating nucleic acids from the mixed sample, whereby there is obtained a collection of molecules comprising the pseudo target and any of said analyte polynucleotide present in the biological sample;

conducting a polynucleotide amplification reaction to co-amplify the pseudo target and any of said analyte polynucleotide contained in said collection of molecules to produce amplification products, whereby pseudo target amplicons are formed, and whereby analyte amplicons are formed if said collection of molecules included said analyte polynucleotide;

detecting in said amplification products any of said analyte amplicons without detecting said pseudo target amplicons; and

determining that the biological sample contains said analyte polynucleotide if said analyte amplicons are detected in the amplification products.

42. The method of Claim 41, wherein the amplification reaction is selected from the group consisting of a Transcription Mediated Amplification reaction, a NASBA reaction and a PCR reaction.

43. The method of Claim 42, wherein the amplification reaction is a Transcription Mediated Amplification reaction.

44. The method of Claim 43, wherein the obtaining step comprises drawing blood.

45. The method of either Claim 42 or Claim 43, wherein the detecting step comprises first hybridizing a labeled polynucleotide probe having binding specificity for the analyte amplicons and then measuring the extent of specific binding of the labeled polynucleotide probe and the analyte amplicons.

46. The method of Claim 45, wherein the isolating step comprises immobilizing said pseudo target and said analyte polynucleotide to a solid support.

47. The method of Claim 45, wherein the detecting step comprises detecting by luminometry.

48. The method of Claim 47, wherein the analyte polynucleotide is from HIV virions.

49. The method of claim 48, wherein the pseudo target has a sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:9.

50. A method of determining whether an analyte polynucleotide is present in a test sample in an amount greater or less than a pre-determined value, comprising the steps of:

obtaining a test sample to be analysed for the presence of said analyte polynucleotide;

combining said test sample with an amount of a pseudo target;

co-amplifying in a polynucleotide amplification reaction the pseudo target and any analyte polynucleotide contained in said test sample to produce amplification products that include a pseudo target amplicon and an analyte amplicon, wherein said analyte amplicon is present in an amount that is dose-dependent on the amount of said analyte polynucleotide present in said test sample; and

quantitatively detecting said analyte amplicon using a detection system calibrated to have a detection threshold corresponding to a signal strength arising from co-amplification of said amount of said pseudo target and an amount of analyte polynucleotide corresponding to said pre-determined value, wherein detection of a signal above or below said threshold of detection indicates that said analyte polynucleotide is present in said test sample in an amount that is respectively greater or less than said pre-determined value.

Dated 8 April, 2005
Gen-Probe Incorporated

Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

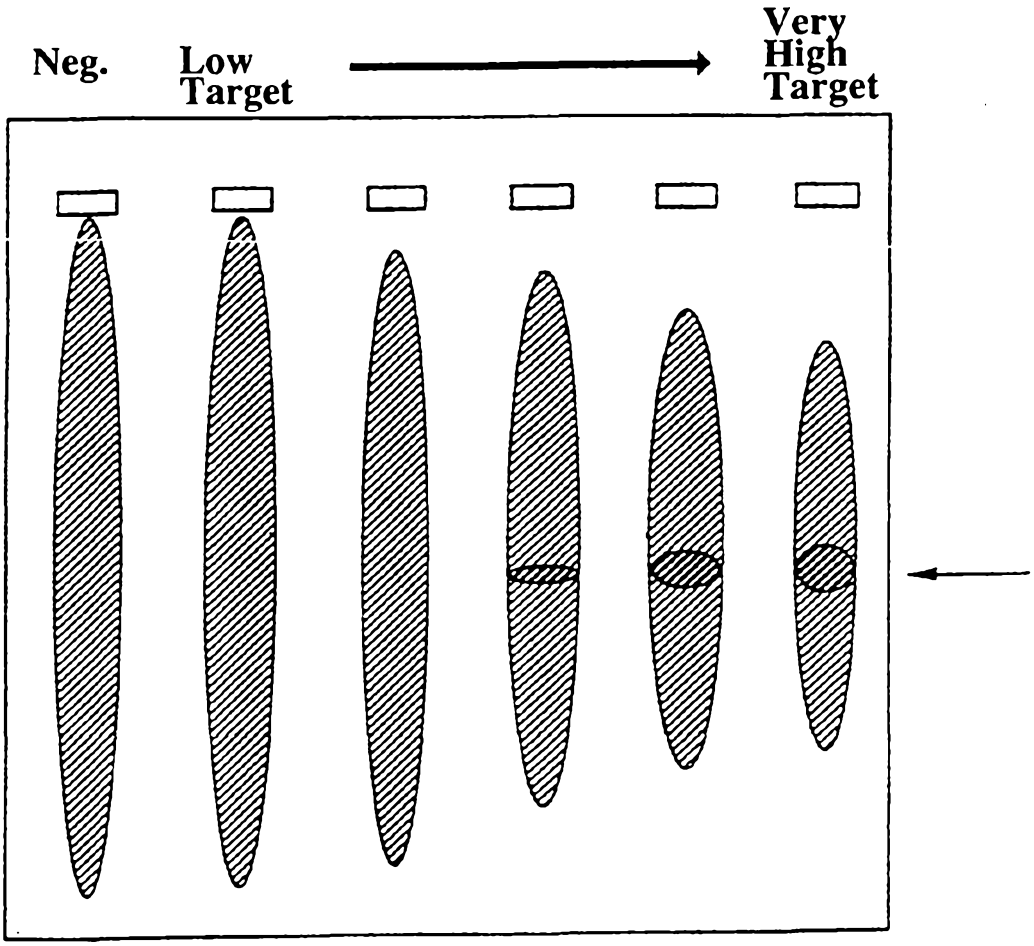

Fig. 1

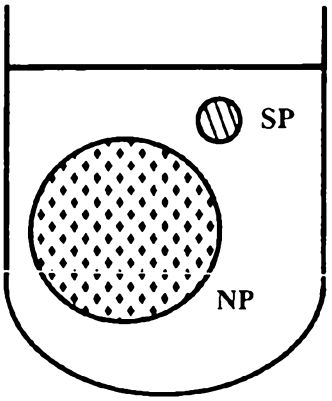

Fig. 2a

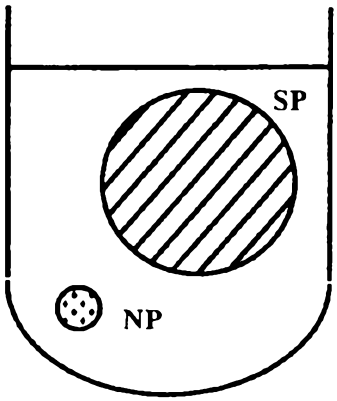

Fig. 2b

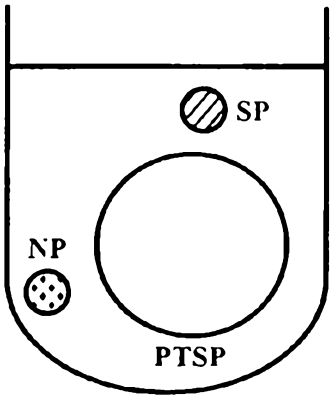

Fig. 2c

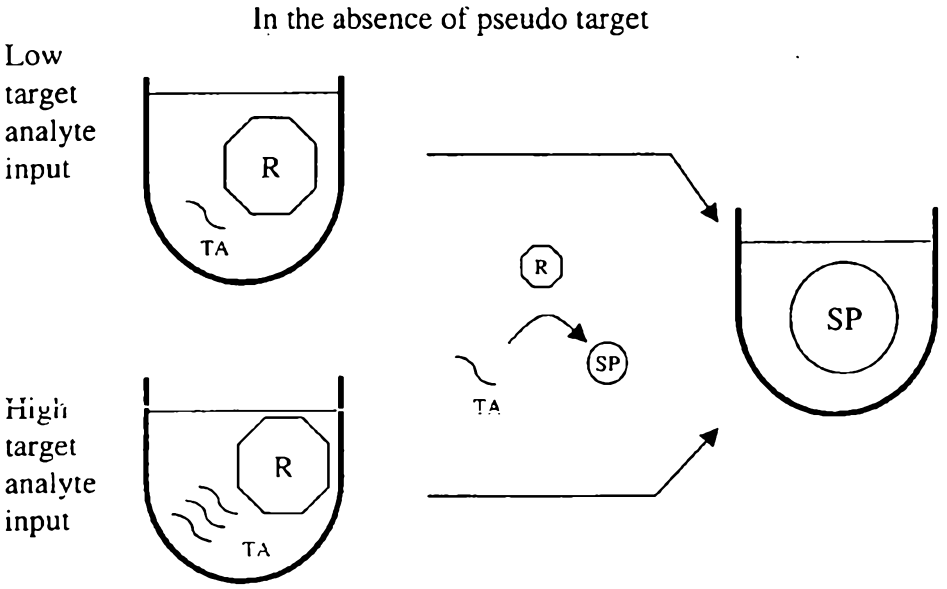
In the absence of pseudo target
Low target analyte input
R
TA
R
SP
TA
SP
High target analyte input
R
TA

Fig. 3a

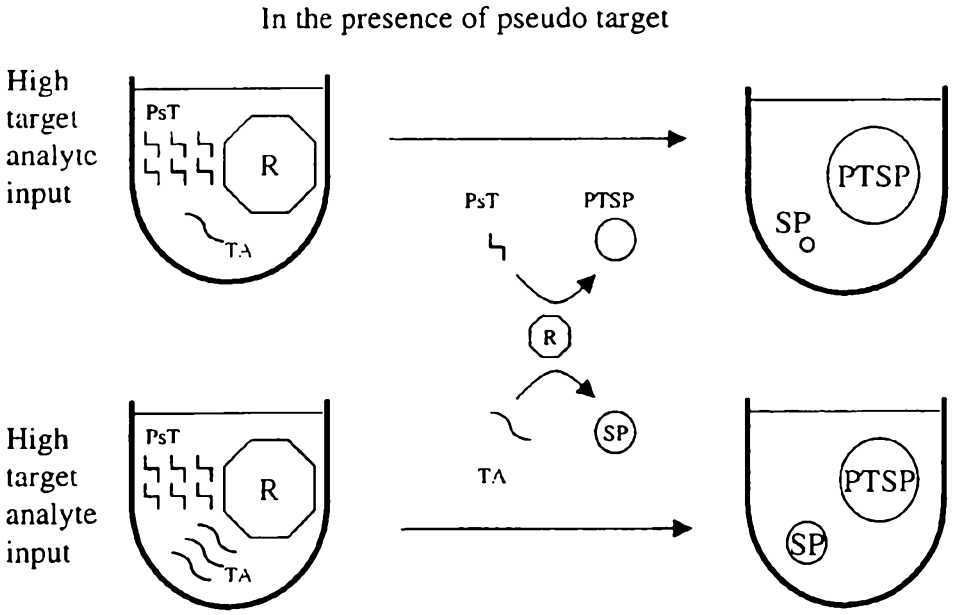
In the presence of pseudo target
High target analyte input
PsT
R
TA
PsT
PTSP
PTSP
SP
R
SP
TA
High target analyte input
PsT
R
TA
PTSP
SP

Fig. 3b

Fig. 4a

Fig. 4b

Fig. 4c

Fig. 4d

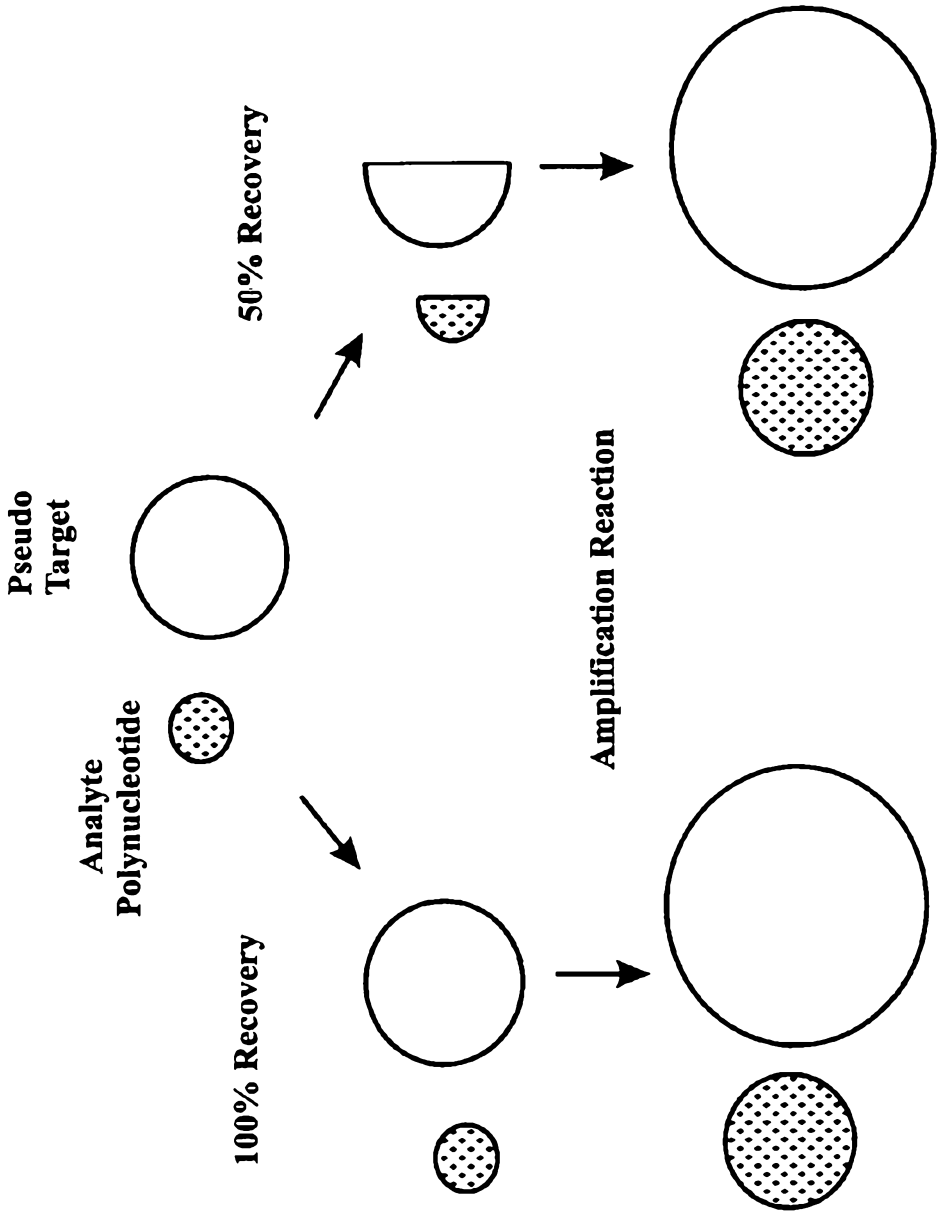
Pseudo Target
Analyte Polynucleotide
50% Recovery
100% Recovery
Amplification Reaction

Fig. 5

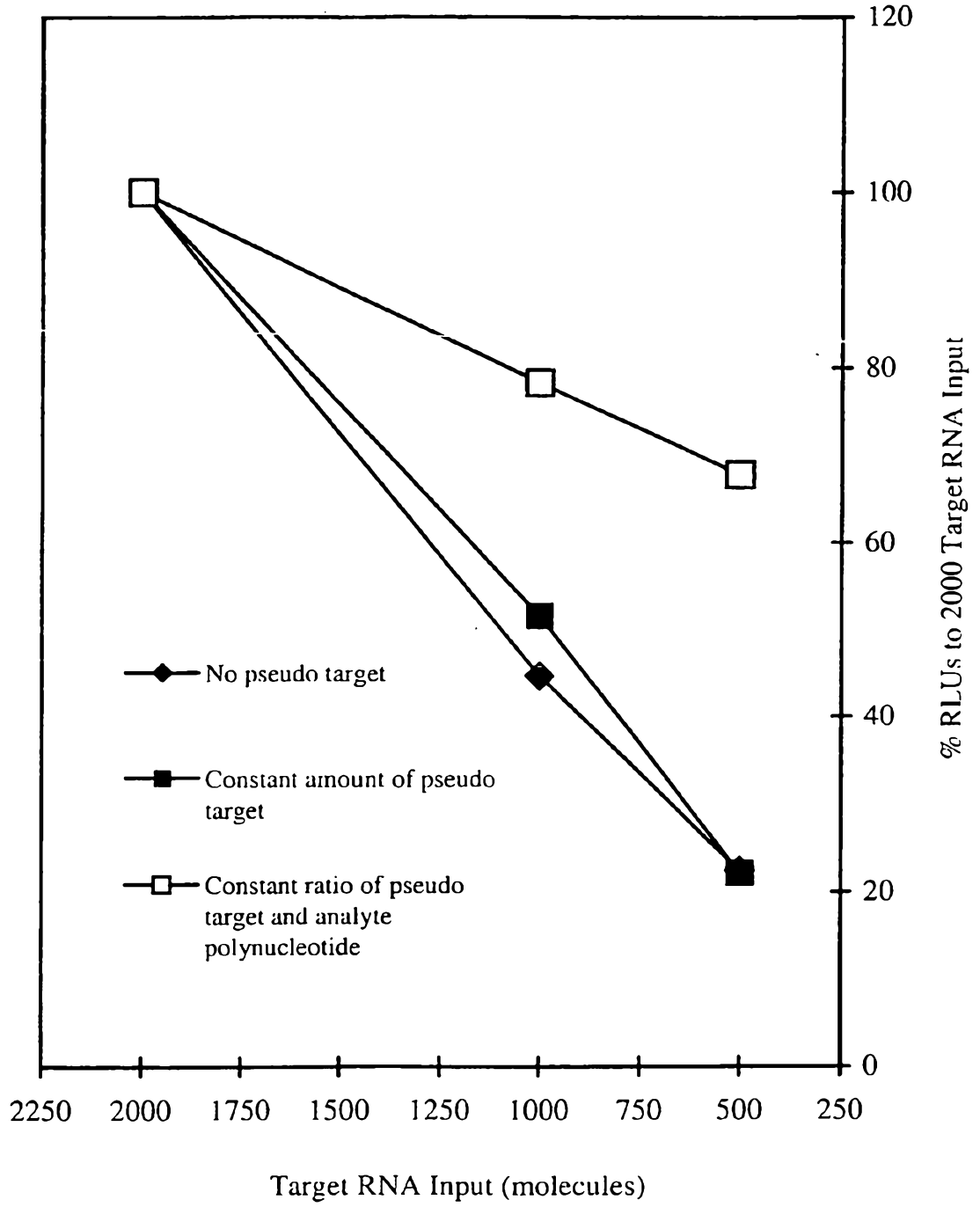
120
100
80
60
40
20
0
% RLUs to 2000 Target RNA Input
No pseudo target
Constant amount of pseudo target
Constant ratio of pseudo target and analyte polynucleotide
2250
2000
1750
1500
1250
1000
750
500
250
Target RNA Input (molecules)

Fig. 6

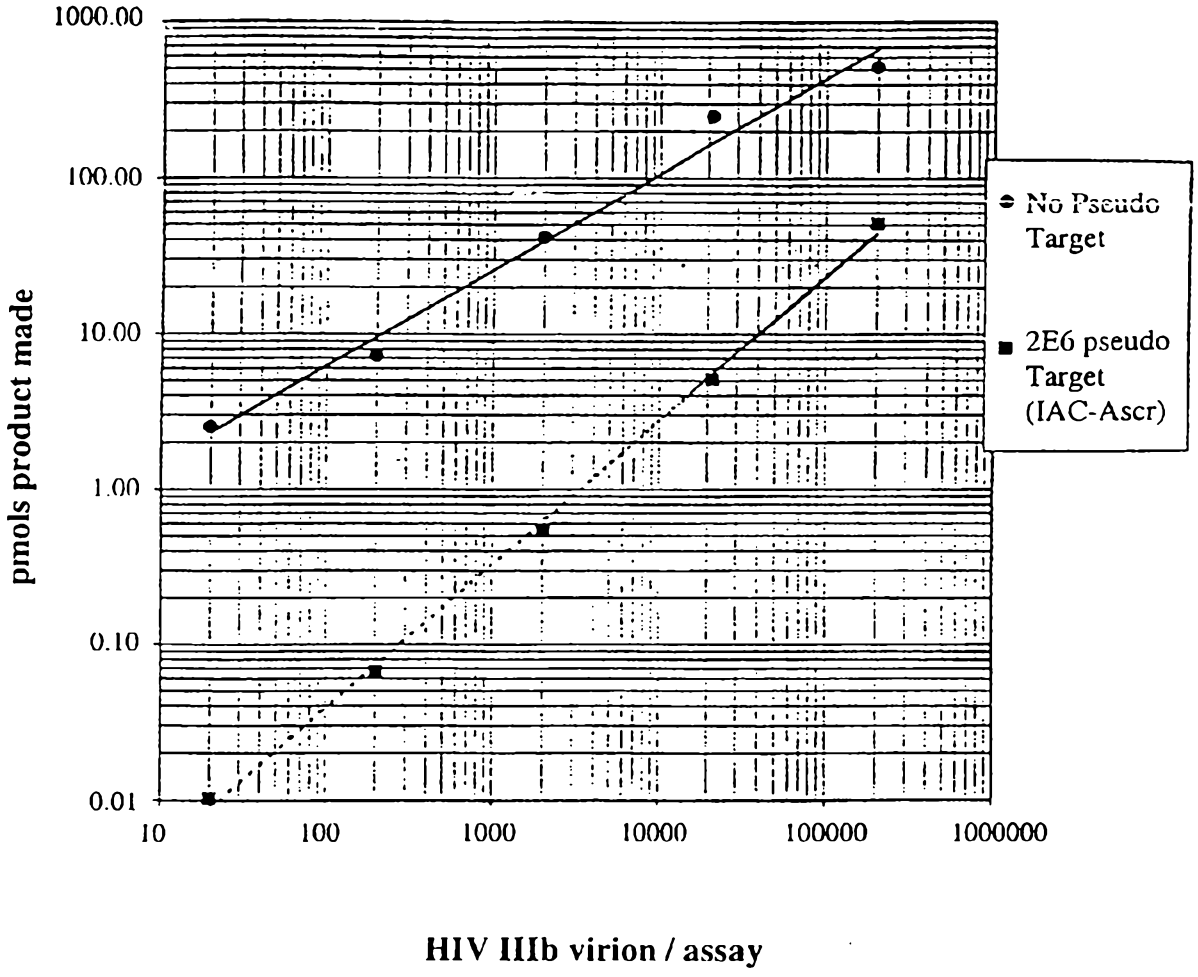
1000.00
100.00
10.00
1.00
0.10
0.01
pmols product made
10
100
1000
10000
100000
1000000
HIV IIIb virion / assay
No Pseudo Target
2E6 pseudo Target (IAC-Ascr)

Fig. 7