

Extremely sensitive, background-free gene detection using binary probes and Q β replicase

(diagnostic clinical assays/isolation of probe-target hybrids/T4 DNA ligase/target-dependent signal generation/exponential amplification of reporter RNAs)

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ABSTRACT We have developed a specific and sensitive nucleic acid amplification assay that is suitable for routine gene detection. The assay is based on a novel molecular genetic strategy in which two different RNA probes are hybridized to adjacent positions on a target nucleic acid and then ligated to form an amplifiable reporter RNA. The reporter RNA is then replicated up to a hundred billion-fold in a 30-min isothermal reaction that signals the presence of the target. The assay can detect fewer than 100 nucleic acid molecules; it provides quantitative results over a wide range of target concentrations and it employs a universal format that can detect any infectious agent.

Extremely specific and sensitive assays are needed for the routine detection of rare pathogenic agents in clinical samples. The most specific assays employ single-stranded oligonucleotide probes to seek out and bind to unique regions of a pathogen's nucleic acid. The resulting probe–target hybrids are the most specific and stable intermolecular complexes known. When a high concentration of probes is incubated with a sample, virtually every target molecule forms a hybrid. One need only remove the probes that are not hybridized and then count the remaining hybridized probes to determine the degree of infection. However, the number of target molecules in a sample is often so low that classical detection schemes, such as labeling the probes with radioactive atoms or fluorescent moieties, are not sufficiently sensitive. Thus it is necessary to design assays in which a large number of reporter molecules are generated for every target that is present.

Although a very large number of copies are synthesized in target–sequence amplification assays (1–5), such as those that use the polymerase chain reaction, their design creates practical problems that restrict their use to specialized laboratories. These assays are usually carried out in crude cellular extracts, where they can be inhibited by cellular components and where the presence of unrelated nucleic acids can lead to false-positive signals; different sample preparation protocols are needed for different tissues and for different infectious agents; relatively expensive equipment is often required to alternately raise and lower the temperature; and additional steps are needed to detect the amplified nucleic acid, increasing the risk of contaminating other samples. Although there are a variety of solutions for each of these problems, the resulting assays are complex and difficult to quantitate.

We have been working on an alternative amplification scheme that avoids these problems. In our approach, the probes, rather than the targets, are amplified exponentially (6–8). Unlike target amplification schemes, where repeated cycles of hybridization and polymerization are carried out in

the same solution, our assays employ a single hybridization step that is carried out under universal and highly stringent conditions. The probe–target hybrids are then isolated and the bound probes are amplified exponentially in a brief isothermal reaction. Our probes are recombinant RNAs (9), in which a probe sequence is embedded at an appropriate site within the sequence of MDV-1 RNA (10, 11), which is a naturally occurring template for the RNA replicase of bacteriophage Q β . These recombinant RNAs hybridize to their targets as do ordinary probes, but unlike ordinary probes, more than a billion copies of each probe can be synthesized in a 30-min incubation with Q β replicase (12). This amplification does not require primers, and strand separation occurs naturally at 37°C (13, 14). The large number of RNA molecules that are synthesized signals the presence of the target nucleic acid. Because a single probe molecule can initiate exponential amplification (15), these assays can be extremely sensitive. In practice, however, their sensitivity has been limited by how well the probe–target hybrids can be separated from the large number of nonhybridized probes that are present to ensure that hybridization occurs rapidly. Despite the use of reversible target capture (16), which is an extremely efficient hybrid isolation procedure, we found that 10,000 nonhybridized probes could not be removed, generating a background signal that obscured the presence of rare targets (8).

In this report, we describe a new strategy that solves the problem posed by the persistence of nonhybridized amplifiable probes. The probe molecules were redesigned so that they cannot be amplified unless they hybridize to their target. We divided the recombinant-RNA probes into two separate molecules, neither of which can be amplified by itself, because neither contains all the elements of sequence and structure that are required for replication. The division site is located in the middle of the embedded probe sequence. When these “binary probes” are hybridized to adjacent positions on their target, they can be joined to each other by incubation with an appropriate ligase, generating an amplifiable reporter RNA. Nonhybridized probes, on the other hand, because they are not aligned on a target, have a very low probability of being ligated. By combining this target-dependent ligation step with a new and simpler hybrid-isolation step, signal generation is strictly dependent on the presence of target molecules, no background signals are generated, and the resulting assays are extraordinarily sensitive.

MATERIALS AND METHODS

Synthetic Target Molecules. A cDNA encoding a portion of the polymerase gene of human immunodeficiency virus type 1 (HIV-1) strain NL4-3 (17) was subcloned between the *Hind*III and *Xma*I restriction sites in the polylinker of plasmid pGEM-4Z (Promega). The resulting plasmid was linearized by digestion with endonuclease *Sma*I and transcribed by incuba-

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tion with bacteriophage T7 RNA polymerase. The 875-nt transcripts contained nucleotides 4229–5091 of the HIV-1 (NL4-3) genome (listed in the GenBank data base as HIVNL43).

Binary Probes. Both the left and the right probe were prepared by transcription from DNA templates. The first 63 nt at the 5' end of the left probe (shown in Fig. 1) were identical to the 5'-terminal sequence of MDV-1 (+) RNA; the next 6 nt served as a spacer (which is not essential); and the last 23 nt were complementary to nucleotides 4596–4618 of HIV-1 (NL4-3) RNA. The 3' ends of the left probe transcripts naturally terminate in the hydroxyl group required for ligation. The first 19 nt at the 5' end of the right probe (also shown in Fig. 1) were complementary to nucleotides 4577–4595 of HIV-1 (NL4-3) RNA; the next 10 nt served as a spacer (also nonessential); and the last 156 nt were identical to the 3'-terminal sequence of MDV-1 (+) RNA. Normally, the 5' ends of the right probe transcripts would have contained a triphosphate group, which cannot participate in ligation; however, in addition to providing guanosine triphosphate as a precursor for transcription, we also provided a 20-fold excess of guanosine monophosphate, which is incorporated into the 5'-terminal position, assuring that almost all of the right probe transcripts contained the monophosphate group required for ligation. Many of the left probe transcripts possessed additional (nontemplated) nucleotides at their 3' end. Electrophoretic isolation of correct-length left probe transcripts, though not done for the experiments reported here, improves ligation efficiency from 8% to 40%.

The DNAs used as templates for the synthesis of these probes were prepared in polymerase chain reactions initiated with plasmid, pT7MDV (18), which contains a cDNA copy of the MDV-1 sequence. The primers used in these reactions possessed sequences at their 5' ends that added a T7 RNA polymerase promoter and a probe sequence to the amplified DNA. The first primer for the left probe template contained the promoter sequence and the other primer contained a portion of the target sequence, while the first primer for the right probe template contained the promoter sequence directed toward the complement of the remainder of the target

sequence and the other primer was complementary to the 3' end of the MDV-1 (+) sequence.

Capture Probes. These single-stranded oligodeoxynucleotides are complementary to the target (19) and possess a biotin moiety at their 5' end that enables them to bind strongly to streptavidin (20), which is covalently linked to the surface of paramagnetic particles (21). The use of two different capture probes improves the efficiency of target capture by 60%. These oligonucleotides were prepared commercially (Operon Technologies, Alameda, CA) by chemical synthesis and contained 4 nt at their 5' end that served as a spacer between the probe sequence and the 5'-terminal biotin moiety. The sequence of one capture probe was 5'-biotin-TACGATGTCTGTTGC-TATTATGTCTACTATTCTTTCCCTGCACTGTAC-3', which is complementary to nucleotides 4808–4852 of HIV-1 (NL4-3) RNA; and the sequence of the other capture probe was 5'-biotin-TACGACTGCTACCAAGATAACTTTTC-CCTTCTAAATGTGTACAATCTAGC-3', which is complementary to nucleotides 4415–4459 of HIV-1 (NL4-3) RNA.

HIV-1 Infected Lymphocytes. A suspension of H9 cells (22) was infected with HIV-1 isolate HTLV-III_{MN} (23). Histological staining with serum from an HIV-positive individual, 24 hr after infection, indicated that more than 99% of the cells were infected. Stock samples were prepared by serially diluting the HIV-1-infected H9 cells with uninfected H9 cells. Each stock sample contained 600,000 cells. The cells were then washed and concentrated by centrifugation, dissolved in 240 μ l of 5 M guanidine thiocyanate, and incubated at 37°C for 60 min. Concentrated solutions of this chaotropic salt lyse cells, inactivate enzymes, liberate nucleic acids from cellular matrices, unwind DNA molecules, and relax RNA secondary structures (24). Each lysate was mixed thoroughly and one sixth of its volume was assayed.

Assay Protocol. Some 10^{10} molecules of each binary probe and 10^{11} molecules of each capture probe were added to each sample. Hybrids were formed by incubation at 37°C for 60 min in 100 μ l of buffer A [2 M guanidine thiocyanate (Fluka)/400 mM Tris-HCl, pH 7.5/5 mg of sodium N-lauroylsarcosine per ml/5 mg of bovine serum albumin fraction V per ml (Boehringer Mannheim)/and 80 mM EDTA] in polypropylene titer-

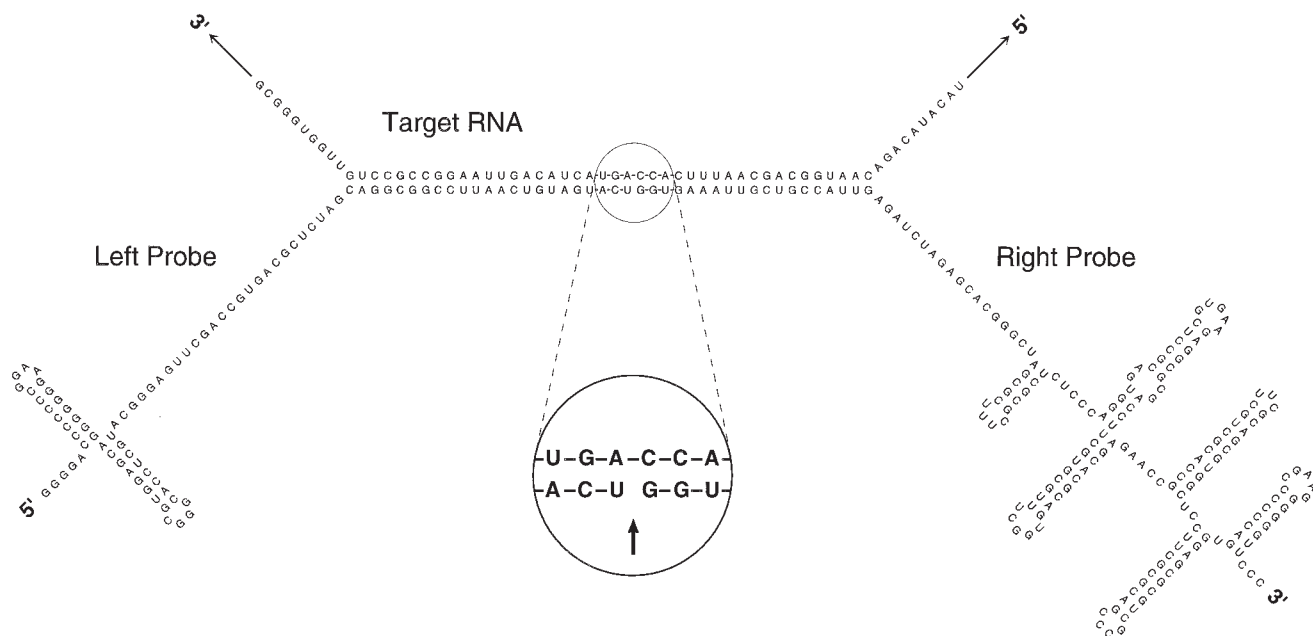


FIG. 1. Binary probes bound to a complementary HIV-1 target molecule. Neither the left probe nor the right probe can be amplified by incubation with Q β replicase. However, if the binary probes are hybridized to adjacent positions on a target RNA (as shown), they can be ligated to each other to form a reporter RNA that can be amplified exponentially by Q β replicase. The magnified view shows the phosphodiester bonds (short lines). An arrow points to the location where a phosphodiester bond will be formed when the hybrid is incubated with T4 DNA ligase. Ligated probes form a naturally occurring Q β replicase template, MDV-1 RNA, containing an embedded HIV-1 probe sequence.

tubes (Bio-Rad). The presence of 2 M guanidine thiocyanate in the hybridization mixture promotes the formation of hybrids without interference from denatured cellular debris (25). The hybrids were then captured by adding 20 μ l of a suspension of streptavidin-coated paramagnetic particles, as supplied by the manufacturer (Promega), and incubating at 37°C for 10 min. We discovered that the presence of 2 M guanidine thiocyanate does not prevent the biotinylated capture probes from binding to streptavidin. The particles were then washed four times with 200 μ l of buffer A at 37°C to remove excess probes and cellular material, and washed an additional four times at 37°C with 200 μ l of buffer B [5 mM MgCl₂/66 mM Tris·HCl, pH 7.5/1 mM ATP/0.5 mg of Nonidet P-40 per ml (Sigma)/1 mM dithiothreitol] to remove the guanidine thiocyanate. During each wash cycle, the suspended particles were agitated vigorously on a multitube vortex-type mixer (American Hospital Supply, McGaw Park, IL); they were then drawn to the walls of the titertube with the aid of a magnetic separation device (Vysis, Downers Grove, IL); and the wash solution was withdrawn by aspiration and replaced with a new solution. After the last wash, the particles were suspended in 50 μ l of buffer B containing 1 unit of *Escherichia coli* ribonuclease H (Pharmacia) and incubated at 37°C for 10 min to release the hybrids. The particles were then magnetically drawn to the sides of the titertube and the supernatant containing the hybrids (\approx 45 μ l) was transferred to a new titertube. Ligation was carried out by adding 5 μ l of buffer B containing 25 units of bacteriophage T4 DNA ligase (Boehringer Mannheim) and incubating at 37°C for 60 min. The resulting reporter RNAs were exponentially amplified by adding 100 μ l of buffer C (15 mM MgCl₂/45 mM Tris·HCl, pH 8/100 μ M ATP/600 μ M [α -³²P]CTP/600 μ M GTP/and 600 μ M UTP) containing 6 μ g of Q β replicase (Vysis) and incubating at 37°C for 31 min. Samples (5 μ l) of each amplification reaction were withdrawn every minute (beginning at 8 min) and were added to 100 μ l of a stop solution containing 20 mM EDTA (pH 8) and 120 mM NaCl. The RNA in each sample was precipitated by adding 400 μ l of a solution containing 360 mM phosphoric acid, 20 mM sodium pyrophosphate, and 2 mM EDTA. The precipitated RNA was bound to a Zeta-Probe nylon membrane (Bio-Rad) on a dot-blot vacuum filtration manifold (Bio-Rad). The membrane was then washed with 500 ml of the precipitation solution to remove unincorporated [³²P]CTP. Finally, the membrane was air-dried and the [³²P]RNA present in each sample was visualized by autoradiography.

RESULTS

Design of the Assay. Our previous probe-amplification assays used recombinant RNAs that consisted of a probe sequence embedded within a template for Q β replicase (8). To obtain hybridization probes that cannot be exponentially amplified, we divided the recombinant RNA probes into two separate molecules. The division site was located approximately in the middle of the embedded probe sequence (Fig. 1). To be replicated exponentially, an RNA must possess an internal replicase binding site (26), a particular 3'-terminal sequence for the initiation of replication (27), and a particular 5'-terminal sequence that encodes the 3'-terminal initiation sequence needed for the replication of the complementary strand (28). Neither fragment of the recombinant RNA probe possessed all of these sequences, and preliminary experiments confirmed that neither fragment could be replicated exponentially. However, each fragment retained the ability to hybridize to the target. When these binary probes are hybridized to a target strand, the partial probe sequence at the 3' end of one molecule is brought immediately adjacent to the partial probe sequence at the 5' end of the other molecule. Incubation of the hybrid with a target-dependent ligase covalently links the two probes, generating an amplifiable reporter RNA. Although

other researchers obtained contrary results (29–31), we found that RNA fragments hybridized to an RNA target can be ligated efficiently by incubation with T4 DNA ligase. Consequently, we were able to design an assay in which nonamplifiable RNA fragments are used as probes and the generation of exponentially amplifiable reporter RNAs is strictly dependent on the presence of target strands.

The assay is shown schematically in Fig. 2. The sample is first dissolved in a guanidine thiocyanate solution. Binary probes and biotinylated DNA capture probes are added, and the

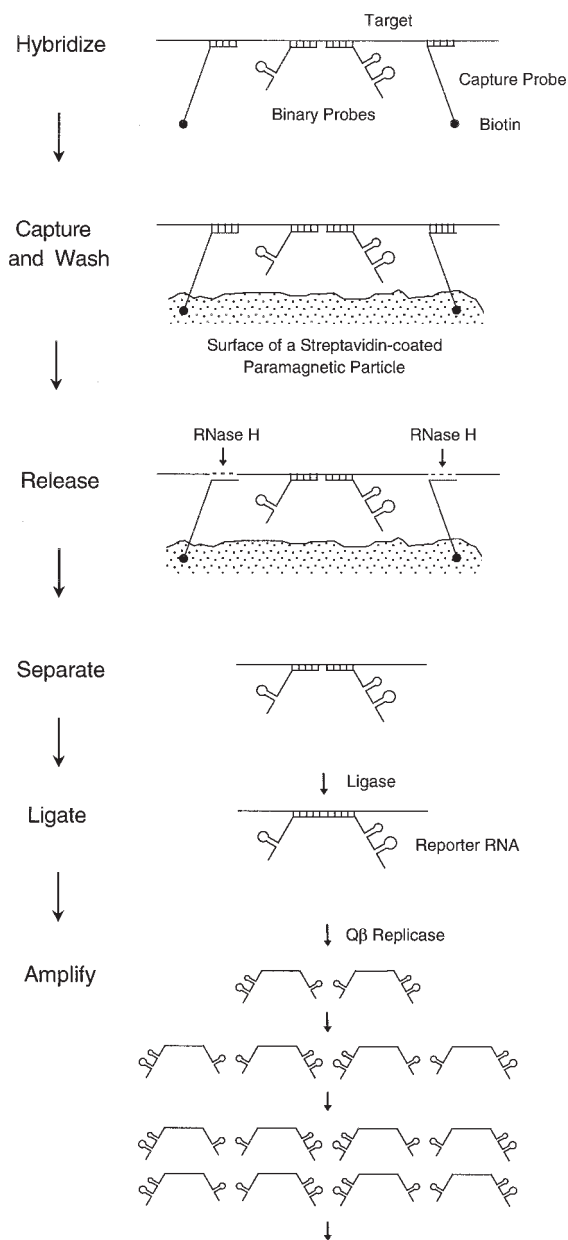


FIG. 2. Schematic representation of the key physical and enzymatic steps used in the assay. Both the target and the binary probes are RNA molecules. The capture probes are DNA molecules. Two different capture probes are used to increase the efficiency of capture. They hybridize to the target RNA on opposite sides of the sequence to which the binary probes are bound. Incubation with ribonuclease H digests the target RNA where it is bound to each capture probe, selectively releasing the binary probe–target hybrid from the surface of the paramagnetic particle. After removal of the particle with a magnet, the isolated hybrid is incubated with T4 DNA ligase (which serves here as an RNA-dependent RNA ligase), resulting in the formation of a reporter RNA that is then amplified exponentially by incubation with Q β replicase.

mixture is incubated to form hybrids, which are then collected on the surface of streptavidin-coated paramagnetic particles. The particles are washed to remove excess probes and cellular material. Despite vigorous washing, some probes remain bound in a nonspecific manner to the walls of the assay tube, to the surface of the particles, and to the capture probes (32). Since nonhybridized binary probes can be ligated through chance alignment, it is necessary to further reduce their concentration. Therefore, the hybrids are separated from the nonspecifically bound probes by incubating the particles with ribonuclease H, which cleaves the target RNA where it is bound to the DNA capture probes, selectively releasing the probe–target hybrids into solution. The particles are then magnetically drawn aside and the supernatant containing the hybrids is transferred to a new tube. The isolated hybrids are then incubated with T4 DNA ligase to covalently link the binary probes, forming amplifiable reporter RNAs. When Q β replicase is added, the only RNA that is synthesized is derived from binary probes that were hybridized to target strands.

In this assay format, hybridization precedes amplification. This is fundamentally different from the format used in target–sequence amplification assays, where repeated cycles of hybridization and amplification are carried out in the same solution. Because the nonenzymatic steps in our assay (sample preparation, hybridization, capture, and washing) are carried out in the presence of guanidine thiocyanate, which is an extremely effective denaturant, the same protocol can be used for all samples, irrespective of the type of tissue being tested or the nature of the suspected infectious agent. Furthermore, the enzymatic steps (hybrid release, ligation, and amplification) are deferred until the hybrids have been isolated and placed in a defined environment; thus they cannot be inhibited by cellular components, and false-positive signals cannot arise from the presence of irrelevant nucleic acids.

Assay with Simulated HIV-1 mRNA Targets. To determine the sensitivity of the assay, eight samples were prepared, each containing a different number of transcripts of the HIV-1 integrase gene. We used binary probes (shown in Fig. 1) that were complementary to a conserved sequence within the integrase gene and capture probes that were complementary to sequences on either side of the target sequence. Probe–target hybrids were formed, bound to the surface of paramagnetic particles, washed vigorously, and released into solution by digestion with ribonuclease H. The isolated hybrids were incubated with T4 DNA ligase and then incubated with radioactive nucleotides and Q β replicase. Aliquots of each amplification reaction were taken at 1-min intervals, and the RNA in each aliquot was bound to a nylon membrane and visualized by autoradiography. The results are shown in Fig. 3. Reporter RNA was synthesized in the amplification reactions from samples that contained 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , and 10^2 HIV-1 target molecules. However, no reporter RNA was

synthesized in the amplification reaction from the sample that contained 10 target molecules, nor was any RNA synthesized in the amplification reaction from the sample that contained no target molecules, even after 31 min of incubation. These results demonstrate that the sensitivity of the assay lies between 10 and 100 target molecules.

Because there is no background reaction, the sensitivity of the assay is not limited by the occurrence of obscuring signals. Instead, it is determined by the efficiency of the individual steps required to generate a reporter RNA. In experiments that followed the fate of labeled target strands and labeled probes through the various steps of the assay, we measured the number of hybrids that survived each step and found the following efficiencies: hybridization and capture, 95%; washing, 56%; hybrid release, 60%; and ligation, 8%—resulting in an overall efficiency of 2.6%. Thus, for every 100 target molecules in a sample, two or three reporter RNAs were formed, and they were exponentially amplified by Q β replicase to generate a detectable signal. Samples containing less than 40 target molecules were unlikely to generate even a single molecule of amplifiable RNA.

Assay with HIV-1 Infected Human Lymphocytes. To demonstrate the specificity of the assay and to confirm that the presence of cellular material in the sample does not compromise sensitivity, seven samples were prepared by mixing HIV-1-infected lymphocytes with uninfected lymphocytes. Although each sample contained a different number of infected cells, the total number of cells in each was 100,000. A mock sample was also prepared by adding 10^6 HIV-1 transcripts to a lysate from 100,000 uninfected cells. The same protocol and probes were used as in the assays with HIV-1 transcripts. The results are shown in Fig. 4. Every sample that contained infected cells gave a clear signal, including the sample that contained only a single infected cell in the presence of 100,000 uninfected cells; yet the sample that did not contain any infected cells gave no signal, despite the presence of cellular components and nucleic acids from 100,000 uninfected cells. These results demonstrate that the assay is highly specific for the presence of target nucleic acid.

The results also illustrate how kinetic data can be used to determine the number of target molecules in an unknown sample. Because the amount of reporter RNA doubles at regular intervals, it takes longer for a given (arbitrary) amount of RNA to be synthesized in a reaction initiated with less RNA. We measured the amount of time it took for 100 ng of reporter RNA (6.4×10^{11} molecules) to be synthesized in each of the reactions shown in Fig. 4. This amount of RNA was just enough to be visible in the autoradiogram. We then plotted these “response times” against the number of infected cells in each sample. The results (Fig. 5) demonstrate that response time is inversely proportional to the logarithm of the number of targets. For every 10-fold decrease in the number of infected

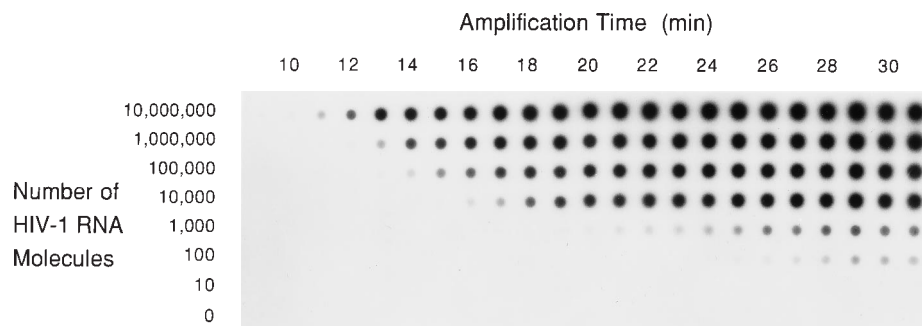


FIG. 3. Demonstration that binary probe assays are background-free and sufficiently sensitive to detect 100 target molecules. Each amplification reaction was sampled at 1-min intervals. The fewer the number of target molecules in the original sample, the longer it took before sufficient reporter RNA was synthesized for it to be visible in the autoradiogram. Measurements of the amount of radioactive reporter RNA synthesized in the reaction from the sample containing 100 molecules indicated that it was amplified a hundred billion-fold.

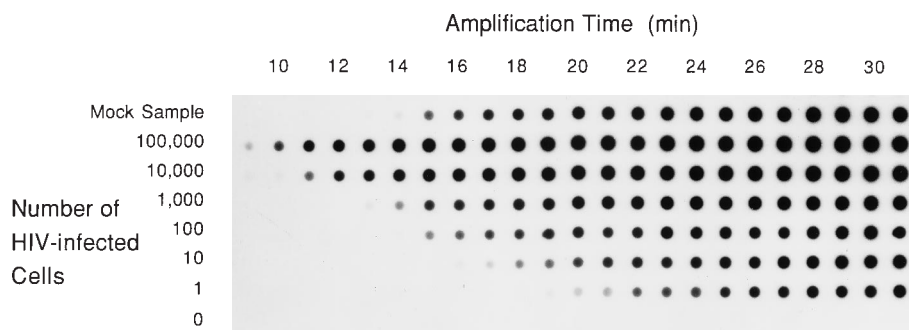


FIG. 4. Demonstration that binary probe assays are highly specific. Every sample that contained HIV-1-infected lymphocytes gave a positive signal, including the sample that contained only one infected cell in 100,000 uninfected cells; yet the sample that contained only uninfected cells gave no signal at all.

cells, it took about 2.1 min longer to synthesize 100 ng of reporter RNA. This relationship holds over an extremely wide range of target concentrations, extending from 1 cell to at least 100,000 cells. Thus, the number of infected cells in an unknown sample can be determined by comparing its response time to the results obtained from a set of reference standards.

DISCUSSION

Both of the enzymatic steps that occur before amplification are necessary. When the ligation step is omitted, the number of reporter RNAs generated is five orders of magnitude lower. The only reason that any reporter RNAs occur in the absence of ligation is that Q β replicase can occasionally continue polymerization across the gap in the ligation junction. When the enzymatic hybrid isolation step is omitted, the assays are

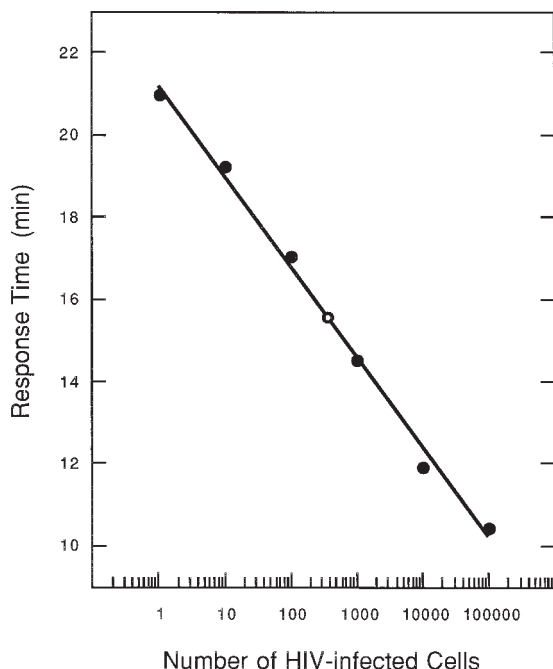


FIG. 5. Linear relationship between response time and the logarithm of the number of targets in a sample. The response time was measured for each amplification reaction shown in Fig. 4 and plotted against the number of HIV-1-infected lymphocytes in the corresponding sample. For every 10-fold decrease in the number of infected cells, it took about 2.1 min longer to synthesize 100 ng of reporter RNA. The response time of the mock sample (indicated by an open circle) corresponded to the response time that would have been obtained from a sample containing 360 infected cells. Because the mock sample contained 10^6 HIV-1 transcripts, we infer that each infected cell contained ≈ 2800 HIV-1 target molecules.

no longer background-free because persistent nonhybridized binary probes on the surface of the particles are occasionally ligated to each other. However, target-independent ligation is second order and depends on the concentration of the probes. When the hybrids are isolated, the concentration of these probes is reduced to such a low level that not even a single reporter RNA is generated.

Several modifications will expand the utility of the assay. Virtually the only nucleic acid that is present in the amplification reactions is the reporter RNA. Therefore, the kinetic course of the reactions can be followed in real-time by including an intercalating fluorescent dye, such as propidium iodide, in the reaction mixture and measuring the increase in its fluorescence as it binds to the RNA being synthesized (33). Because there is no need to isolate the amplified RNA to detect it, the reaction tubes can be permanently sealed, eliminating the risk of contaminating other samples.

Binary probes that are specific for different target RNAs can be combined in a single assay tube, thus enabling the simultaneous detection of entire panels of pathogens. When these assays give a positive signal, the responsible pathogen can be identified because the amplified reporter RNA contains a unique embedded probe sequence. We recently devised novel nucleic acid detector probes, called “molecular beacons,” that only fluoresce when they hybridize to their target (34). A series of molecular beacons, each specific for a different embedded probe sequence and each labeled with a fluorophore of a different color, can be included in an amplification reaction, enabling homogeneous, real-time detection in a multiplex format.

Binary probe assays are particularly amenable to distinguishing genetic alleles. Efficient ligation only occurs when the terminal nucleotides on either side of the ligation junction are correctly base-paired to the target strand (35). The occurrence of a terminal mismatch (due to an allelic difference) will result in a marked reduction in the number of amplifiable reporter RNAs. In these assays, the targets will be DNA, the ligation reaction will utilize an RNA–DNA heteroduplex (36), and selective hybrid release will be achieved by incubation with an appropriate restriction endonuclease.

Finally, binary probe assays will require little in the way of instrumentation. They can be carried out in a hermetically sealed device containing two reaction chambers, where hybridization, capture, washing, and hybrid release occur in one chamber, and ligation, amplification and signal detection occur in the other. Because these assays are simple in design and practice, they can routinely be used for gene detection.

We dedicate this paper to the memory of Sol Spiegelman, who first conceived quantitative nucleic acid hybridization assays (37, 38) and who introduced an entire generation of molecular biologists to the exponential amplification of nucleic acids (39). We thank Claire Grigaux for her expert technical assistance; David Ho, William

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