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Quantitative Assays Based on the Use of Replicatable Hybridization Probes

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Amplifiable hybridization probes—molecules with a probe sequence embedded within the sequence of a replicatable RNA—will promote the development of sensitive clinical assays. To demonstrate their utility, we prepared a recombinant RNA that contained a 30-nucleotide-long probe complementary to a conserved region of the *pol* gene in human immunodeficiency virus type 1 (HIV-1) mRNA. Test samples were prepared, each containing a different number of HIV-1 transcripts that served as simulated HIV-1 mRNA targets. Hybridizations were carried out in a solution containing the chaotropic salt, guanidine thiocyanate. Probe–target hybrids were isolated by reversible target capture on paramagnetic particles. The probes were then released from their targets and amplified by incubation with the RNA-directed RNA polymerase, Qβ replicase (EC 2.7.7.48). The replicase copied the probes in an exponential manner: after each round of copying, the number of RNA molecules doubled. The amount of RNA synthesized in each reaction (~50 ng) was sufficient to measure without using radioisotopes. Kinetic analysis of the reactions demonstrated that the number of HIV-1 targets originally present in each sample could be determined by measuring the time it took to synthesize a particular amount of RNA (the longer the synthesis took, the fewer the number of targets originally present). The results suggest that clinical assays involving replicatable hybridization probes will be simple, accurate, sensitive, and automatable.

Additional Keyphrases: *recombinant RNA · reversible target capture · paramagnetic particles · exponential amplification · Qβ relicase · guanidine thiocyanate*

Infectious agents may be quite rare in asymptomatic individuals who are infectious to others. For example, an asymptomatic individual may have as few as one in 100 000 peripheral blood mononuclear cells infected with the pathogenic retrovirus, human immunodeficiency virus type 1 (HIV-1), yet donated blood from that person will readily infect others (*1*). It is thus imperative that very sensitive clinical assays be developed for detecting HIV-1, to screen donated blood and to identify asymptomatic carriers. Suitable assays would make use of a macromolecular probe having extremely high affinity for a particular component of the infectious agent and very low affinity for all the other components of the sample. The highest specificities and most stable interactions known occur when a single-stranded oligonucleotide probe hybridizes to a complementary oligonucleotide target (*2*). For example, oligonucleotide probes can seek out and bind to the integrated HIV-1 DNA, or the retroviral messenger RNA, present in a single infected cell.

However, the use of oligonucleotide probes is not sufficient to assure detection. An infected cell contains only about 6000 molecules of retroviral messenger RNA (*3*), so the problem becomes how to detect the probes once they are bound to such a small number of targets. The classic detection strategy is to attach reporter groups to the probes, such as fluorescent organic molecules or radioactive phosphate groups. More recently, biotin groups have been incorporated into probes (*4*). After the probes have bound to their targets, enzymes such as peroxidase or phosphatase are linked to the biotin, then incubated with a colorless substrate, leading to the accumulation of a large number of colored product molecules for each enzyme–probe adduct (*5*). However, the practical limit of detection of these schemes is about 10^6 target molecules. Clearly, they cannot be used to detect a single cell in a sample that contains only 6000 retroviral messenger RNAs.

A particularly attractive strategy for detecting rare targets is to link each probe to a replicatable reporter, which can be exponentially amplified after hybridization to reveal the presence of the probe (*6*). We recently described a novel version of this approach, in which a probe sequence was embedded within the sequence of a replicatable RNA (*7*). The resulting recombinant RNAs hybridize to their target sequences the same way as ordinary hybridization probes do and, as in a classical hybridization assay, nonhybridized probes are then washed away. The hybridized probes are then freed from their targets and released into solution. What makes these recombinant-RNA probes particularly useful is that they can then be exponentially amplified by incubation with the RNA-directed RNA polymerase, Qβ replicase (*8*). We have demonstrated that as many as 10^9 copies of each replicatable probe can be synthesized in a single 30-min incubation (*7*). Furthermore, the extreme specificity of Qβ replicase for its own template RNA (*9*) assures that only the replicatable probes will be amplified. The large number of copies synthesized can easily be quantified by incorporating radioactive nucleotides or by measuring the fluorescence of an intercalating dye such as ethidium bromide. Because as little as a single molecule of RNA can initiate exponential amplification (*10*), this approach offers the prospect of developing sensitive diagnostic assays.

Two developments led to our current work: the discovery that oligoribonucleotides can be inserted within the sequence of a small, naturally occurring template for Qβ replicase, MDV-1 RNA (*11*), without interfering with its replicatability (*12*); and the availability of a plasmid that serves as a template for the synthesis of MDV-1(+) RNA when the plasmid is incubated in vitro with bacteriophage T7 RNA polymerase. We modified this plasmid by inserting a polylinker into the MDV-1 cDNA sequence. The subsequent insertion of a probe sequence within the polylinker creates a plasmid that serves as a template for the transcription of recombinant-RNA probes (*7*). The site that we

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chose for inserting the polylinker and probe into MDV-1 RNA was known to be on the exterior of the molecule, where the presence of the inserted sequence was less likely to interfere with replication, and where the remainder of the molecule was less likely to interfere with the hybridization of the probe sequence to its target. In our previous report (*7*), we demonstrated that these recombinant RNAs are bifunctional, in that they hybridize specifically to complementary target sequences and retain the ability to be exponentially amplified by Qβ replicase.

Here, we demonstrate the use of replicatable hybridization probes in a model assay designed to detect very small amounts of HIV-1 mRNA. We had two main concerns in selecting the assay format: (*a*) because of the desirability of developing a method that can screen a large number of samples, the selected format had to be fast and simple, thus precluding the fractionation of cells or the isolation of nucleic acids; and (*b*) because nonhybridized probes are amplified by Qβ replicase along with hybridized probes, we needed an extremely efficient means of removing the nonhybridized probes. Our first problem was solved by the dual discoveries that hybridization is extremely efficient in solutions of the chaotropic salt, guanidine thiocyanate (*13*), and that concentrated solutions of guanidine thiocyanate will lyse cells, denature all proteins (including nucleases), liberate nucleic acids from cellular matrices, and unwind DNA molecules, permitting hybridization to occur without interference from cellular debris (*3*). Our second problem was solved by the development of the "reversible target capture" procedure (*14*). In this improved "sandwich hybridization" technique (*15, 16*), probe–target hybrids are bound to the surface of paramagnetic particles. After the particles are washed to remove nonhybridized probes, the hybrids are released from the particles and then bound to a new set of particles for another washing. Repeating this procedure several times dramatically reduces the number of nonhybridized probes (*14*).

We prepared recombinant RNAs containing a 30-nucleotide-long probe sequence complementary to a conserved region of the HIV-1 *pol* gene. We also prepared a set of serial dilutions of a stock solution of transcripts of the region of the HIV-1 genome that contains the *pol* gene, to simulate different amounts of HIV-1 mRNA that might be present in clinical samples. An excess of replicatable HIV-1 probes was added to each sample. After hybridization in the presence of guanidine thiocyanate, the probe–target hybrids were isolated by reversible target capture on paramagnetic particles. The probes were then released from their targets and exponentially amplified by incubation with Qβ replicase. Samples of each reaction were taken every minute during replication. The amount of RNA in each sample was then measured. The results demonstrate how kinetic data can be used for quantitative determination of the number of target molecules in a sample.

Materials and Methods

Enzymes and Oligodeoxyribonucleotides

Bacteriophage T7 RNA polymerase (EC 2.7.7.6) was purchased from New England Biolabs, Beverly, MA, and calf thymus terminal deoxyribonucleotidyltransferase (EC 2.7.7.31) was obtained from Supertechs, Bethesda, MD. Qβ replicase (EC 2.7.7.48) was isolated from bacteriophage Qβ-infected *Escherichia coli* Q13 by the procedure of Eoyang and August (*17*), with the hydroxylapatite step omitted. Qβ replicase is stable when stored in a glycerol solution at −20 °C: its activity remains unchanged after five years of storage. Single-stranded DNA fragments were prepared, by using β-cyanoethyl phosphoramidite chemistry, on a 380A synthesizer (Applied Biosystems, Foster City, CA).

Replicatable HIV-1 Probes

Recombinant MDV-1 RNA containing an inserted HIV-1 probe sequence was synthesized by transcription from a recombinant plasmid. The plasmid was constructed by inserting a synthetic probe sequence (prepared by annealing dGATCACCGTAGCACTGGTGAAATTGCTGCCATTGA to dGATCTCAATGGCAGCAATTTCACCAGTGCTACGGT) into the Bgl II site of a plasmid that is identical to plasmid pT7-MDV-poly (*7*), except that the polylinker sequence is in the opposite orientation. The nucleotide sequence in the recombinant region of the cloned plasmid was confirmed by the chain termination procedure (*18*). The synthesis of replicatable probes by transcription from linearized recombinant plasmids with T7 RNA polymerase is described in detail elsewhere (*7*). The resulting transcripts were recombinant MDV-1(+) RNAs containing a 30-nucleotide-long probe sequence that is complementary to nucleotides 4622–4651 in the *pol* gene of HIV-1 genomic RNA (*19*). Figure 1 shows the nucleotide sequence and predicted secondary structure of the transcribed RNA. MDV-hiv(+) RNA serves as an excellent template for exponential amplification by Qβ replicase.

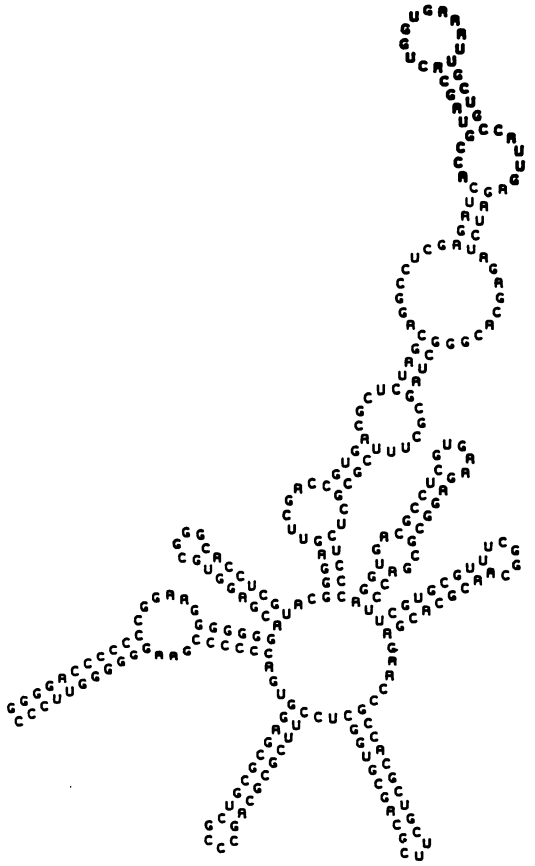

Fig. 1. Replicatable HIV-1 hybridization probe

The nucleotide sequence of MDV-hiv(+) RNA was folded into the secondary structures predicted to be most stable by a computer program (*20*). The probe sequence (*bold letters*) is located on the exterior of the molecule, where it is free to hybridize to its target, and where it is less likely to interfere with the sequences and structures required for replication (*21*)

Capture Probes

Single-stranded DNAs containing 3′-poly(dA) tails were synthesized for use in binding probe–target hybrids to oligo(dT) groups on the surface of paramagnetic particles. Four different oligodeoxyribonucleotides (of lengths 24, 40, 40, and 43 nucleotides) were prepared by automated synthesis. Each probe was complementary to a different region of the HIV-1 *pol* gene near to the target of the replicatable probe. A poly(dA) tail was added to the 3′ end of each probe by incubation with terminal deoxyribonucleotidyltransferase (*22*).

Hybridization

Simulated HIV-1 mRNA targets were purchased from Gene-Trak Systems, Framingham, MA. These transcripts included a complete copy of the HIV-1 *pol* gene. Seven reaction tubes were prepared. Each contained simulated HIV-1 mRNA targets, MDV-hiv(+) RNA (replicatable probes), and capture probes, dissolved in 70 μL of 2.5 mol/L guanidine thiocyanate (Fluka Chemical, Hauppage, NY), and placed in a polypropylene "titertube" (Bio-Rad, Richmond, CA). Each tube contained 2×10^9 molecules of MDV-hiv(+) RNA, 10^{11} molecules of each capture probe, and a different number of target molecules. The number of HIV-1 transcripts in each tube was: 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, and 10^3. The tubes were incubated at 37 °C for 18 h.

Reversible Target Capture

After the completion of hybridization, the probe–target hybrids were isolated from the reaction mixture by binding them to oligo(dT) groups on the surface of paramagnetic particles (*14*). These ferric oxide particles (<1 μm in diameter), purchased from Gene-Trak Systems, possess highly convoluted surfaces coated with silicon hydrides to which numerous oligo(dT) "hairs" have been covalently linked. We added 50 μL of a suspension of paramagnetic particles to each reaction tube, reducing the guanidine thiocyanate (GuSCN) concentration to 1.46 mol/L. The tubes were incubated at 37 °C for 10 min to allow the 3′-poly(dA) tails of the capture probes to hybridize to the oligo(dT) groups on the surface of the particles. The number of oligo(dT) groups available on the surface of the particles far exceeded the number of capture probes present in the reaction. Because the target molecules are hybridized to the 5′ end of the capture probes, the probe–target hybrids become linked to the surface of the particles (see Figure 2). If these had been actual clinical assays, and polyadenylated mRNAs had been present in the samples, the mRNAs would not have bound to the particles, because the poly(rA) on the end of an mRNA forms a much weaker bond with oligo(dT) in concentrated GuSCN solutions than does the poly(dA) on the end of a capture probe (*14*).

Because the particles are paramagnetic, they do not act as magnets, and they do not cling to each other. However, when placed in a magnetic field, they are drawn to the magnetic source. Accordingly, the paramagnetic particles, with the probe–target hybrids bound to their surface, were drawn to the walls of the titertubes by placing the tubes in the presence of the strong magnetic field provided by a magnetic separation device (purchased from Gene-Trak Systems). The supernates, which contained the nonhybridized probes, were then withdrawn from each tube by aspiration, and replaced by 200 μL of a wash solution containing GuSCN at 1.5 mol/L. The tubes were then withdrawn from the magnetic field, and the probe–target

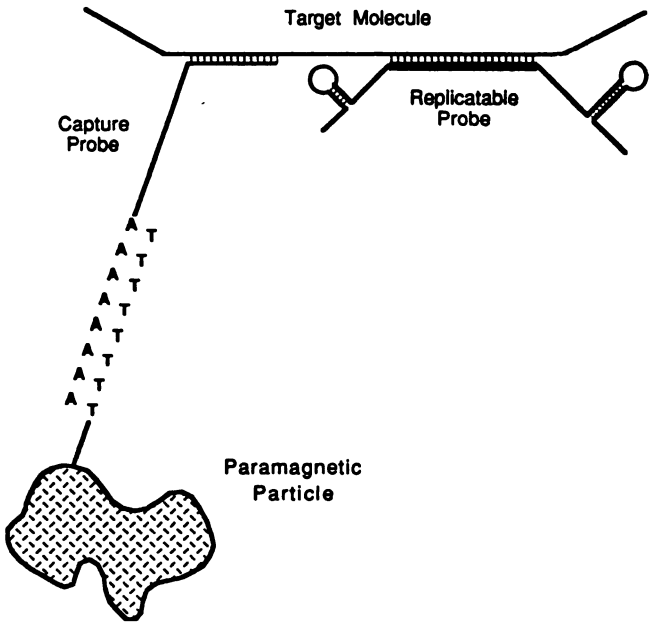

Fig. 2. Schematic representation of the complex formed when a capture probe links a probe–target hybrid to an oligo(dT) group on the surface of a paramagnetic particle

The replicatable probe is a single-stranded RNA containing a probe sequence (*thick line*) embedded within the sequence of a replicatable RNA (indicated by the *hairpin structures*). The replicatable probe is joined by hydrogen bonds (*cross-hatching*) to its complementary target sequence within the target molecule. The target molecule can be an RNA or a DNA. The capture probe is a single-stranded DNA that contains a 5′-probe sequence that is hybridized to a different complementary target sequence within the target molecule. The target sequence for the capture probe is located relatively close to the target sequence for the replicatable probe on the target molecule. The capture probe also contains a 3′-poly(dA) tail that is hybridized to an oligo(dT) group on the surface of the paramagnetic particle. The hybrid formed by the 3′-poly(dA) tail and the oligo(dT) group is relatively weak, permitting the more stable probe–target hybrid to be released from the particle at higher concentrations of guanidine thiocyanate (*14*)

hybrids attached to the resuspended particles were washed by vigorous agitation on a multi-tube vortex-type mixer (American Hospital Supply, McGaw Park, IL). The particles were again drawn to the walls of the tubes, the supernates were aspirated, another 200 μL of 1.5 mol/L GuSCN wash solution was added, and the particles were again agitated and drawn to the walls of the tubes. The supernates were again withdrawn by aspiration, but this time they were replaced with 50 μL of a release solution containing 3.25 mol of GuSCN per liter.

The tubes were again removed from the magnetic field, agitated to resuspend the particles, and then incubated at 37 °C for 5 min. In this more-concentrated GuSCN solution, the relatively weak hybrids formed between the 3′-poly(dA) tails of the capture probes and the oligo(dT) groups on the surface of the particles came apart, releasing the probe–target hybrids back into solution. However, the much stronger hybrids formed between the capture probes and the target (and between the replicatable probe and the target) remained intact (*14*). The stripped particles were then drawn to the sides of the tubes, and the supernates (containing the released probe–target hybrids) were transferred to new tubes. The stripped particles were discarded and 50 μL of a suspension of fresh particles was added to each new tube, reducing the GuSCN concentration to 1.62 mol/L. The tubes were incubated at 37 °C for 10 min to allow the probe–target hybrids to be recaptured by the hybridization of the 3′-poly(dA) tails of the capture probes

to the oligo(dT) groups on the surface of the new particles.

Transferring the probe–target hybrids from one set of solid surfaces to a new set of solid surfaces effectively removed any nonhybridized probes that adhere to surfaces. The entire process was repeated three times, which markedly reduced the number of nonhybridized probes (*14*). After the probe–target hybrids were captured onto the surface of particles for the third time, we washed them once in 200 μL of 1.5 mol/L GuSCN solution and then twice in 200 μL of a solution containing 100 mmol of KCl (a nonchaotropic salt that preserves the hybrids) per liter, to wash away the GuSCN. After removing the supernate from the last 100 mmol/L KCl wash by aspiration, we resuspended the particles in 50 μL of 10 mmol/L Tris-HCl buffer (pH 8.0) containing 1 mmol of EDTA per liter. The resuspended particles were then incubated in this salt-free buffer at 37 °C for 10 min, which completely dissociated the probe–target hybrids. The particles were then drawn to the walls of the tubes, leaving in each tube a supernate that contained an amount of MDV-hiv(+) RNA proportional to the number of target molecules originally present.

Exponential Amplification

We prepared seven replication reactions (*23*), each containing 35 μL of the final supernate from the corresponding hybridization reaction. These MDV-hiv(+) RNA molecules were then amplified by incubation with 5.76 μg of Qβ replicase at 37 °C in 105 μL of a solution containing, per liter, 400 μmol of ATP, 40 μmol of [α-^{32}P]CTP, 400 μmol of GTP, 400 μmol of UTP, 7.5 mmol of $MgCl_2$, and 45 mmol of Tris-HCl (pH 7.5). The reactions were incubated in parallel, and 4-μL aliquots were withdrawn from each of the seven reactions at 1-min intervals between 9 and 29 min of incubation and mixed with 36 μL of an ice-cold solution containing 120 mmol of NaCl and 20 mmol of EDTA–NaOH (pH 8) per liter. The EDTA in this solution chelated the magnesium in the sample, preventing further replication.

The size and homogeneity of the [^{32}P]RNA products in 5-μL aliquots of each of the 147 samples were determined by electrophoresis through 8% polyacrylamide slab gels in the presence of urea, 7 mol/L (*24*). Finally, the [^{32}P]RNA products in a 10-μL aliquot of each of the 147 samples were precipitated by the addition of 190 μL of an ice-cold solution containing 360 mmol of phosphoric acid, 20 mmol of sodium pyrophosphate, and 2 mmol of EDTA per liter. The precipitated RNA in each sample was then electrostatically bound to a "Zeta-Probe" quaternary-amine-derivatized nylon membrane (Bio-Rad) in a dot-blot vacuum filtering manifold (Bio-Rad). Each bound sample was washed 10 times with 200 μL of the ice-cold precipitation solution to remove unincorporated [^{32}P]CTP. The membrane was then air-dried, and the amount of [^{32}P]RNA present in each sample on the membrane was made visible by autoradiography. After the autoradiograph was prepared, the amount of RNA present in each dot-blot was measured in a scintillation counter.

Results

We carried out model assays in which replicatable HIV-1 probes were hybridized to serial dilutions of simulated HIV-1 mRNA target molecules in the presence of guanidine thiocyanate (*13*). After hybridization, the resulting probe–target hybrids were isolated by three cycles of reversible target capture (*14*) on paramagnetic particles. The hybridized probes were then released from their targets and exponentially amplified by incubation with Qβ replicase (*7*), with samples of each reaction being taken at 1-min intervals. Electrophoretic analysis of the size and homogeneity of the RNA in each sample indicated that only MDV-hiv RNA was synthesized.

An aliquot of each sample was bound to a nylon membrane in a dot-blot filtration apparatus, and the [^{32}P]MDV-hiv RNA in each aliquot was made visible by autoradiography. In the resulting autoradiogram (Figure 3), the density of each spot indicated the amount of RNA present at each time point. After the autoradiogram was prepared, the amount of RNA in each sample was measured with a scintillation counter. Although no RNA was apparent in the early time points, this was the period in which the RNA population increased exponentially (*8*). The RNA became visible in the autoradiogram at about the same time that the number of RNA molecules equaled the number of active replicase molecules (the "saturation point"). From that time on, the RNA population increased linearly (*23*). Thus, at those times when the RNA was visible in each reaction, the RNA population was increasing linearly.

The results demonstrate that the time at which the saturation point occurs in each amplification reaction is a function of the number of target molecules originally present in the corresponding hybridization reaction. The fewer target molecules that were originally present, the fewer the replicatable probes that were bound to targets, and thus the fewer replicatable probes that were available to initiate the amplification reaction. Because amplification reactions initiated with fewer replicatable probes must undergo more

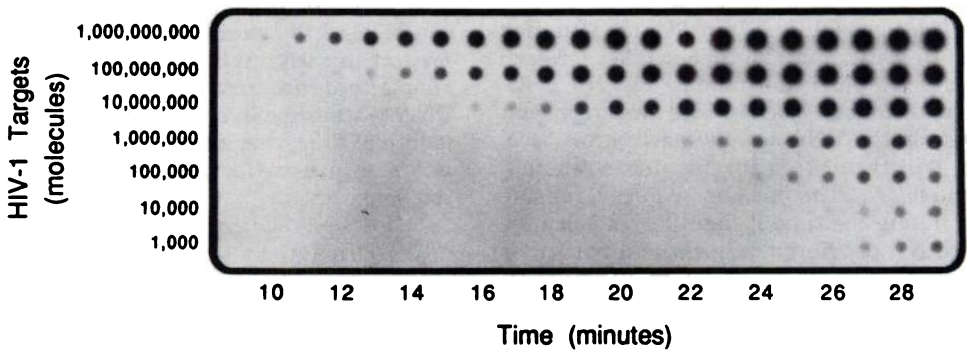

Fig. 3. Kinetic analysis of amplification reactions initiated with replicatable probes isolated from hybridization reactions

Approximately 50 ng of MDV-hiv RNA was synthesized in each amplification reaction. This amount of RNA is sufficient to have been accurately measured by the fluorescence of an intercalating dye, such as ethidium bromide

doublings of the RNA population before there are enough RNA molecules to achieve saturation, the kinetic data can be used to calculate the number of replicatable probes that were present at the beginning of the reaction. If known standards are included among the unknown samples to be tested, then these data can be used to determine the number of target molecules originally present in each unknown sample.

The results also indicate the limit of detection. The amplification reaction corresponding to the sample containing 10^5 targets achieved saturation at an earlier time than did the amplification reaction corresponding to the sample containing 10^4 targets. However, there was no significant difference in the amplification reactions corresponding to the samples containing 10^4 and 10^3 targets. Accordingly, the limit of detection was about 10 000 target molecules. Because electrophoretic analysis of the amplified RNA in each sample indicated that only recombinant RNA was synthesized, the limit of detection was determined by the level of persistence of nonhybridized replicatable probes. It is important to note that these were only preliminary assays, designed to demonstrate how replicatable probes might be used. Further experiments should lead to alterations in the assay format that will improve the sensitivity.

Discussion

During exponential synthesis, the time it takes for the RNA population to double is a constant for a given set of reaction conditions (*23*). If we know how many replicatable probes were initially present in a reaction, and if we know how long that reaction was incubated, then we can predict how many doublings have occurred and how many RNA molecules have been synthesized. Conversely, if we know how long it takes for a particular number of RNA molecules to be synthesized, then we can calculate how many molecules of replicatable probe were present initially. This relationship is summarized by the following equation:

$$N = N_0\, 2^{t/d}$$

where N_0 is the initial number of RNA molecules; t is the time of incubation; d is the characteristic time it takes for the RNA population to double; and N is the number of RNA molecules present at time t. Taking the logarithm of each side of the equation and rearranging algebraically:

$$\log N_0 = \left(\frac{-\log 2}{d}\right) t + \log N$$

where $(-\log 2)/d$ is a constant. If we consider the situation that occurs when we determine the time it takes for each reaction to synthesize a particular number of RNA molecules, then log N will also be a constant and t will represent the time it takes for the RNA population to grow to N molecules. There will then be an inverse linear relationship between t and log N_0. Therefore, if we have a reliable method for determining the time it takes for an exponentially replicating RNA population to grow to a particular (though arbitrary) number of molecules, then we can accurately determine the initial number of replicatable probes.

There is a good method for determining how long it takes for a particular number of RNA molecules to be synthesized. An intercalating fluorescent dye, such as ethidium bromide, could be included in the RNA amplification reaction mixture. Ethidium bromide becomes fluorescent when it interacts with the secondary structures present in replicatable probes. An ethidium bromide concentration of about 1 μmol/L would give a good signal, without significantly inhibiting replication (*23*). A simple instrument could periodically monitor the fluorescence of the ethidium bromide in an entire set of amplification reactions. Initially, the number of RNA molecules would be too low to produce an appreciable fluorescence. However, as exponential synthesis proceeds, the fluorescence would increase. The instrument would be programmed to store the kinetic data and to use these data to determine the time for each reaction at which the fluorescence corresponds to the presence of a particular number of RNA molecules (the "endpoint").

The inclusion of standards in the hybridization reaction, each containing a known number of target molecules, would permit the establishment of a "standard curve," in which the logarithm of the number of target molecules would be inversely proportional to the time at which the endpoint is reached (as described in the second equation). The number of target molecules in each of the unknown samples would then be determined by comparing their endpoints with those on the standard curve. This method is readily automatable; it would not require radioactive compounds; the magnitude of the fluorescent signal at the endpoint would be the same for all the reactions and would be well above the fluorescent background; the assay would be accurate; and the logarithmic nature of the standard curve would permit the determination of the number of targets in a sample over an extremely wide range of target concentrations.

There is an alternative method for analyzing the data. Once the saturation point is reached in an amplification reaction, the number of RNA molecules increases linearly with time. For example, in Figure 3, by the time the reactions had been incubated for 28 min, they had all passed the saturation point and were in the linear phase of synthesis. A comparison of the amounts of RNA present in each sample at 28 min shows that the most RNA is present in those samples that correspond to the hybridization reactions that contained the most targets. Because these reactions were initiated with the greatest number of replicatable probes, they reached the saturation point soonest and had the longest period of time to synthesize RNA in the linear phase. We can analyze the data by using the direct linear relationship between the amount of RNA present at a particular (though arbitrary) time in the linear phase and the logarithm of the number of replicatable probes that initiate a reaction (*7*). If known standards are included among the unknown samples to be tested, then these data can be used to determine the number of target molecules present in each unknown sample. Because it is relatively simple to devise an assay kit to measure the amount of RNA synthesized in a reaction that is incubated for a fixed length of time, this alternative analytical approach would be an inexpensive method for detecting infectious agents in the field.

The model assay we have described demonstrates that replicatable hybridization probes can be used in quantitative assays designed to detect rare targets. Further experiments will be needed to develop actual clinical assays for measuring the number of HIV-1 mRNA molecules in blood samples. However, it is clear that these assays will be simple, accurate, sensitive, and automatable.

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