Research Report

Molecular Colony Diagnostics: Detection and Quantitation of Viral Nucleic Acids by In-Gel PCR

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INTRODUCTION

Currently, low amounts of DNA or RNA are commonly detected by quantitative PCR (13,23). Despite years of effort and thousands of published papers, quantitative PCR still suffers from technical complications and insufficient reliability. Since quantitative PCR is based on signal measurements and generates a logarithmic response to the target copy number, it relies on meticulous calibrations, careful control of reaction conditions, and is inherently error prone: an inevitable scatter of data results in exponentially large assay errors (12). The quantitative PCR results are further distorted by competing background DNA synthesis (e.g., caused by mispriming on non-target templates and the formation of primer dimers), in which the effect is most severe at low target amounts (28).

Many of these problems are overcome in the recently invented "digital" PCR format, in which a DNA sample is diluted to less than one template molecule per well of a multiwell PCR plate, and the number of target-positive wells is counted (26). Digital PCR linearly responds to the target titer, eliminates competition among templates, and needs no signal measurements, but it requires impractically large plates when, as is often true in diagnostics, target molecules constitute a minute fraction of the assayed nucleic acid sample.

An alternative approach is the molecular colony technique (MCT), which was invented in this laboratory (6). In contradiction to conventional-solution PCR methods such as digital PCR,

MCT amplifications are carried out in a solid medium, such as a gel, resulting in the formation of molecular colonies arranged in a 2-D pattern. Each colony comprises a molecular clone; that is, many copies of a single parental molecule.

The MCT can utilize any enzymatic reaction that allows the exponential amplification of nucleic acids. In the first-published version of MCT (8,10), RNA colonies were grown in agarose containing Φ replicase (the RNA-directed RNA polymerase of bacteriophage Φ) (9) and rNTPs. Φ -MCT does not require temperature cyclers and has proven to be a powerful tool for in vitro studies of very rare events of RNA recombination (7,11). In principle, it can also be used for diagnostics (e.g., by employing the protocol in which a replicable molecule carrying a target-specific insert is generated by ligating its non-replicable fragments that have been hybridized next to each other on a target RNA or DNA) (25). However, such an approach is of a limited utility because of the structural restraints Φ replicase imposes on its templates (2,9) and because RNA fragments are intrinsically prone to recombination and can thereby produce replicable RNAs in the absence of any target or ligase (11). These drawbacks are surmounted in the PCR version of MCT, in which DNA colonies are grown by carrying out PCR in heat-resistant polyacrylamide gel (6,16).

Here we describe a method for the accurate and direct quantification of viral nucleic acids using PCR-MCT. The technique was improved, and its perfor-

ABSTRACT

When PCR is carried out in a polyacrylamide gel, each target molecule forms a molecular colony that comprises many copies of the original template. By counting the number of colonies, one can directly determine the target titer, with 100% of the DNA molecules and approximately 15% of the RNA molecules being detected. Furthermore, because of the spatial separation of the products in the gel, no interference is observed from another simultaneously amplified target even if it is present at a 10^6 higher amount or from human nucleic acids that outweigh the target by up to a factor of 10^{12} , which is often true of clinical samples. All these features provide for an accurate and reliable assay of viruses even at very low amounts, that is, in cases most important to diagnostics.

mance was tested under conditions closely resembling real diagnostic assays. We found that, because of a spatial separation of products in the gel, one can reliably detect and count several viral nucleic acid molecules in a high excess of other target or non-target molecules. The results show that PCR-MCT possesses all the advantages of digital PCR but is technically simpler, more economical, and has a much greater resolving power.

MATERIALS AND METHODS

Targets

As an human hepatitis B virus (HBV) DNA target, we used a plasmid obtained by ligating the *Bam*HI fragment encoding core antigen HbcAg of the HBV subtype ayw genome (5), which had been excised from pUHbc (14), into pTZ19R. As an HIV-1 RNA target, we used an 879-nucleotide run-off transcript from a *Sma*I-digested plasmid carrying fragment 4230–5091 of the HIV-1 strain NL4-3 (25). The transcript was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) to eliminate the plasmid DNA. Approximately 10000 RNA molecules of the resulting preparation produced no specific PCR product if the reverse transcription step was omitted. Φ RNA was isolated by phenol extraction from the wild-type Φ phage that was purified as previously described (8). The concentration of the nucleic acids in the preparations was determined spectrophotometrically (20).

Primers

The following oligonucleotides were used as primers in this study: 5'-GCTTAATACGACTCACTATAGG GCTACTGTGGAGTTACTCTCGTT-TTTGC-3' and 5'-GTCTATAAGCTGGAGGAGTGCGAATC-3' (matching positions 1933–1960 and 2277–2302 of the HBV genome, respectively); 5'-CAAGTAGACTGTAGTCCAGGAAT-AT-3' and 5'-GCTTAATACGACTCACTATAGGGGATTGTAGGGAATGCA-3' (matching positions 4386–4410 and 4646–4665 of the HIV genome, respectively); and 5'-GCCGTCATCGT-

CATGGCATATCTT-3' and 5'-GCT-TAATACGACTCACTATAGGCCGC-CCATTTCGTTTAC-3' (matching positions 869–892 and 1145–1164 of Φ RNA, respectively). The longer primers introduced the T7 promoter (underlined) that provided for transcription of the DNA colonies in situ, resulting in up to 100-fold enhancement of the signal produced by the colonies (data not shown).

DNA Polymerases

The coding region of the *polA* gene (GenBank® accession no. D28878) was PCR-amplified using chromosomal *Thermus thermophilus* HB8 DNA as a template and primers that introduced an *Nde*I site and His₆-coding sequence upstream and an *Eco*RV site downstream, cloned in pET11c between an *Nde*I site and blunt-ended *Bam*HI site, and expressed in B834(DE3)/pLysS *E. coli* cells (22). A highly purified N-His₆-tagged *Tth* DNA polymerase was isolated from these cells by heating and (NH₄)₂SO₄ precipitation of the lysate (19), followed by chromatography on Zn²⁺-iminodiacetate-sepharose (15). N-His₆-tagged *Taq* DNA polymerase was prepared similarly.

Isolation of Nucleic Acids from Whole Blood

We used citrate-treated blood samples obtained from anonymous healthy donors and tested HBV-negative at a blood transfusion station; IRB required no informed consent in this case. A blood aliquot was treated for 3 min in a boiling water bath with 4 volumes of lysis solution (125 mM Tris-HCl, pH 7.5, 125 mM NaCl, 12.5 mM EDTA, 6.25% SDS, 2.5% 2-mercaptoethanol) and then for 1 h at 65°C with 0.2 mg/mL proteinase K (Amersham Biosciences, Vienna, Austria), extracted with phenol/chloroform, precipitated with ethanol (20), and extracted twice with 1 volume of a 45:55 (v/v) mixture of ethanol: 200 mM sodium-citrate/300 mM NaCl (this discolored the pellet and removed substances that are inhibitory to PCR). The residue was washed with 80% ethanol, dissolved in a minimal volume of 0.1 mM EDTA (pH 8.0), and used for the assays. The

yield of total nucleic acids was 30–60 µg/mL blood, as determined spectrophotometrically (20).

DNA Colony Growth and Detection

Amplification gels (8% acrylamide/0.12% N,N-methylene-bis-acrylamide) were cast into cylindrical wells (0.4 mm deep; 14 mm in diameter), produced on a 1.5-mm-thick microscopic glass slide, treated with PlusOne Bind-Silane™ (Amersham Biosciences), washed with deionized water, autoclaved, and dried. Before amplification, the gels were overlaid with 65 µL RT-PCR cocktail [40 mM bicine-potassium hydroxide, pH 8.3, 90 mM potassium-acetate, 7% glycerol, 1 mg/mL molecular biology-grade BSA (Roche Applied Science, Mannheim, Germany), 2.5 mM MnCl₂, 0.28 mM each dCTP, dGTP, and dTTP, 0.56 mM dATP, 0.4 µg *Tth* DNA polymerase, 0.08 µg *Taq* DNA polymerase, 0.45 µM each primer, and an assayed nucleic acid sample], and glass coverslips were applied. The slides were placed into an UNO-Thermoblock™ thermal cycler (Biotetra, Göttingen, Germany) on a flatbed thermoblock preheated to a reverse-transcription temperature (56°C for HIV-1 RNA, 64°C for Φ RNA, or either temperature for HBV DNA). After a 30-min incubation (during which time all liquid was entrapped by the gel), PCR cycles were performed as follows: the first three cycles (melting at 94°C for 15 s, annealing for 4 s, and extension at 72°C for 60 s) were followed by 40 cycles of 94°C for 6 s, annealing for 4 s, extension at 72°C, and then by incubation at 72°C for 5 min. The annealing temperature was 50°C for the HIV-1 target or 60°C for Φ target, either for the HBV target. After PCR, we transferred the gels to Hybond® N⁺ nylon membranes (Amersham Biosciences), fixed the membranes in 80% ethanol, dried them, incubated them at 80°C for 1.5 min on a filter paper moistened with denaturing solution (0.5 M NaOH, 10 mM EDTA), and then UV-cross-linked and hybridized them as previously described (10). For consecutive hybridizations, a previous probe was removed by washing the membranes in the denaturing solution and then in 2× SSC (20). RNA hybridization probes were prepared using T7 RNA poly-

Molecular Diagnostic Techniques

merase in the presence of [α - 32 P]ATP (7), the above plasmids carrying the HBV and HIV-1 sequences, and plasmid pQ β 7 carrying the Q β cDNA downstream from the T7 promoter (21). The membrane images of Figures 1 and 2 were obtained by autoradiography on an X-ray film, and those of Figures 3 and 4 were obtained by scanning with a CycloneTM phosphorimager (Packard Instrument, Meriden, CT, USA).

RESULTS AND DISCUSSION

Accuracy of DNA and RNA Quantification

Originally, we prepared amplification gels by polymerizing acrylamide in the presence of PCR components including DNA polymerase (6), and that protocol has been recently reproduced by others (16). Later, we found that more consistent results can be obtained

by first casting an “empty” gel and then washing it to remove any soluble substances, autoclaving, drying, and soaking the dehydrated gel in a complete PCR cocktail. Compared to the original protocol, this ensured the full preservation of the enzyme activity and the elimination of any DNA that might contaminate the gel during its preparation. Because our goal was to develop a universal assay for viruses whose genome can be either DNA or RNA, we used *Tth* DNA polymerase, which can function both as a DNA polymerase and a reverse transcriptase in the presence of Mn²⁺ ions (17,18). The gel was incubated at a temperature appropriate for reverse transcription, followed by PCR. DNA colonies were revealed by transferring the gel to a nylon membrane and hybridizing the membrane with target-specific labeled probes.

Performance of the assay was checked by quantifying targets in dilut-

Figure 1. Sensitivity of the assay. DNA colonies produced by the indicated number of target molecules that have been introduced into gels as aliquots of serially diluted preparations of viral DNA or RNA.

Molecular Diagnostic Techniques

Figure 2. Lack of competition between targets in a multiplex assay. Colonies produced by 300 molecules of HIV-1 RNA in the presence of the indicated number of concurrently amplifying molecules of HBV-DNA. Each of the nine blotting membranes was first hybridized with an HIV-specific probe (top row) and then with an HBV-specific probe (bottom row).

ed preparations of HBV DNA, HIV-1 RNA, and Φ RNA (Figure 1). The HBV DNA and HIV-1 RNA were cloned sequences from conservative regions of the viral genomes, and the Φ RNA was the entire Φ genome extracted from phage particles. The amplified fragments were 392, 300, and 315 bp, respectively. With the DNA target, the number of colonies was equal to the number of DNA molecules added to the gel ($98 \pm 21\%$ for HBV DNA; $n = 23$). The standard deviation seems to reflect the error of serial dilutions and the stochastic nature of the distribution of molecules, rather than errors in the assay itself. As expected, the yield of colonies produced by RNAs was lower, reflecting the efficiency of reverse transcription, but was similar for the two targets tested ($12.9 \pm 3.2\%$ for HIV-1 RNA, $n = 13$; $16.5 \pm 4.3\%$ for Φ RNA; $n = 16$) and proportional to the number of RNA molecules added. Therefore, the assay provides for a direct determination of the target titer, although at a lower sensitivity for RNA than for DNA. By comparing the signals produced by the colonies with those by known amounts of PCR products spotted on the blotting membrane (data not shown), we determined that a DNA colony contained 10^7 – 10^8 copies of the progenitor template.

Mixed Targets

In practice, it can be useful to assay simultaneously several viruses, in which the amounts may differ by orders of magnitude. Therefore, we tested to see if the amplification of two targets in the same gel would result in their mutu-

al competition. Surprisingly, we observed no interference to the amplification of one species (HIV-1 RNA) from the concomitant amplification of another (HBV DNA), even if the latter was 3 million-fold more abundant (Figure 2). This result demonstrates an extremely high intrinsic resolving power of the PCR-MCT gel. The gel was only 14 mm in diameter and had a 65- μ L volume (which is close to the standard PCR volume, 50 μ L), yet it accommodated 10^9 HBV colonies, the largest number tested, without affecting the growth of HIV colonies.

Effects of Background Synthesis

In real assays, the goal is to detect a low quantity of viral molecules in samples containing a huge amount of human

Figure 3. Lack of interference from non-target nucleic acids. Patterns generated by 50 molecules of HBV DNA in the presence of total nucleic acids isolated from the indicated aliquots of whole human blood (top row) and control samples, without added HBV DNA (bottom row). In this experiment, the yield of the nucleic acids was 5.5 μ g/100 μ L blood. This amount corresponds to 1.7×10^6 human genome equivalents and exceeds the amount of the target by a factor of 2.7×10^{11} .

(or other non-target) DNA and RNA. Given the complexity of the human genome and the limited specificity of nucleic acid hybridization, it is not surprising that virus-specific oligonucleotides sometimes anneal and prime on human sequences, resulting in a background that competes with the amplification of the proper target (1). At low virus amounts, the background may completely hide the signal generated by the target. If that happens, then assays produce false-negative results (3,27). Of course, the MCT format cannot eliminate background synthesis; however, because of the high resolving power of the MCT gel, it could negate that effect by spatially separating background colonies from those produced by the target. Indeed, the number of target-specific colonies remained unchanged even in the presence of total nucleic acids isolated from 0.1 mL whole human blood, which outweighed the target by a factor of almost 1 trillion-fold (Figure 3).

Assay of Blood Samples

Finally, we tested the method by assaying human blood samples containing a known number of HBV DNA molecules (Figure 4). These assays closely resembled a real diagnostic test and accounted for any target degradation or loss during sample preparation, as well as for the effects of any PCR inhibitors present in the whole blood. However, the number of DNA colonies did not deviate from the expected number of target molecules compared to experiments with purified HBV DNA (see Figure 1).

Figure 4. Detection of HBV DNA molecules in human blood. Total nucleic acids were isolated from 100- μ L aliquots of whole human blood, to which diluted samples expected to contain 0, 50, 100, or 200 molecules of HBV DNA had been added. A portion (60%) of the preparations was used for assays.

Molecular Diagnostic Techniques

CONCLUSION

Here we explored the potentials of PCR-MCT by using the assay of viral nucleic acids as an example. Methods for the reliable detection of pathogenic viruses at very low amounts are urgently needed for early diagnostics, testing a blood sample to be transfused or an organ to be transplanted, environmental monitoring, etc. Quantitative assay for a virus is important for observing the disease progression and the efficacy of the medicine being taken, for optimizing the treatment, and for studying the disease mechanism (4). Our results show that the MCT format provides for the detection of a single specific DNA molecule and approximately seven RNA molecules (which may potentially be improved by increasing the yield of reverse transcription), allows the target titer to be determined accurately and directly (without a reference to a standard), eliminates the competition between targets in multi-target assays, and substantially reduces the interference from background DNA synthesis. It is technically as simple as the standard one-tube PCR protocol (one just needs to introduce the tube contents into a gel) and is almost as inexpensive. In this study, we used radioactive probes, but there is enough material in a DNA colony to be detected in situ by fluorescence (16). In particular, it would be tempting to employ real-time detection techniques such as molecular beacons (24) to monitor target-specific DNA colonies as they grow. Of course, the same technology can be used for detecting other infectious agents, such as bacteria, protozoa, or fungi, as well as for diagnosing cancer or genetic diseases.

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