Simple and effective method for generating single-stranded DNA targets and probes

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A simple and efficient PCR method was developed for generating dye- or radiolabeled single-stranded DNA targets or probes used for hybridization studies. The method involved the use of a pair of long primers with high annealing temperatures and a short, labeled primer with a low annealing temperature in a PCR consisting of two cycles at different temperatures. We used this method to generate dye CyTM5-labeled and [32P]-radiolabeled single-stranded DNA targets and probes. These labeled probes were used successfully for the microarray identification of point mutations in Mycobacterium tuberculosis genes and for the Northern blot detection of expression changes of the GATA-2 gene in Pneumocystis carinii-infected rat lungs.

INTRODUCTION

The creation of amplification methods to generate single-stranded DNA (1,2) has represented a major advance in development of PCR technology. Single-stranded DNA has been shown to be very useful for DNA hybridization studies (3) with a highly efficient hybridization and no need to be denatured before hybridization. Single-stranded target DNAs have been efficiently used in the studies of microarray hybridization (4–7) and direct sequencing of DNA (1,8).

Single-stranded DNA can be generated by conventional asymmetric or real-time asymmetric PCR (9,10). Alternative methods of generating single-stranded DNA targets and probes include the utilization of biotin-streptavidin purification procedures (11,12). However, many of the methods of single-stranded DNA generation require extra steps to process the products and thus are time-consuming and costly. Conventional asymmetric PCR procedures using an unequal concentration of the forward and reverse amplification primers are theoretically simple (13), but they did not give us satisfactory results as other researchers have indicated (10).

The purpose of this study was to develop an improved asymmetric PCR procedure for generating single-stranded DNA. This novel method used a unique PCR nesting approach to generate single-stranded DNA probes. We demonstrated that these probes were effective for the DNA hybridizations associated with two different molecular biology procedures. The single-stranded DNA targets that we generated were used for the microarray detection of mutations in several drug-resistant strains of Mycobacterium tuberculosis. We also generated single-stranded DNA probes for use with a Northern blot procedure involving detection of the expression of a transcription factor, GATA-2, associated with Pneumocystis carinii infection. Overall, the method that we developed for asymmetric PCR generation of labeled, single-stranded probes or targets is relatively easy to perform and was effectively utilized to create DNA probes for two different biological assays.

MATERIALS AND METHODS

Oligonucleotide Primers and Probes

The design of PCR primers and probes used for microarray analysis was based on the published genome sequence of M. tuberculosis (14) and published data on the M. tuberculosis gene mutations associated with drug resistance (15). The DNA primers and amine-linked probes were synthesized by the Center for Biologics Evaluation and Research’s Facility for Biotechnology Resources (U.S. Food and Drug Administration, Rockville, MD, USA). They were prepared with a Model 394 automated DNA synthesizer (Applied Biosystems, Foster City, CA, USA) using cyanethyl phosphorodite nucleosides. The probes we designed and used for microarray studies included wild-type AOK463: 5′-TCCGATGCCGATCTTG-3′; mutant AOK463: 5′-TCCGATGCCAGATCTTG-3′; wild-type AOK587: 5′-GCTCCAAGCGCAGAAGG-3′; and mutant AOK587: 5′-GCTCCAAGCGCAGAAGG-3′.

Asymmetric PCR for Microarray Analyses

Double-stranded DNA templates containing point mutations in the M. tuberculosis gene katG were prepared by a recombinant PCR in vitro mutagenesis technique (16–18). Single-stranded DNA targets were then generated by our asymmetric PCR technique (Figure 1). This technique used three primers in a PCR. Two paired primers (forward and reverse), with a high annealing temperature (70°C) and a very low concentration (1 pmol/40 μL), were used for the generation of a small amount of primary double-stranded PCR product in the first round of PCR. A short CyTM5-labeled third primer, with a much lower annealing temperature (54°C) and a high concentration (15 pmol/40 μL), was designed to nest inside of the products generated in the first round of PCR to create single-stranded targets or probes in the second round of PCR. The forward and reverse primers, MTB-Kf2 (5′-CAAGCTGATCCACCAGACATG-3′) and MTB-Kr2 (5′-CTTGTCGAGCACGGCAAAGG-3′), were used to generate a 610-bp PCR fragment containing codon positions of 463 and 578 in the katG gene. The Cy5-labeled third primer, Cy5-Kf2s (5′-ATCCACCAGACATG-3′), nested inside of the PCR product generated by MTB-Kf2 and MTB-Kr2. For M. tuberculosis mutation detection, each 40 μL of PCR solution consisted of PCR buffer (2 mM Tris-HCl, pH 8.0, 1.5 mM MgCl₂, 10 mM KCl,
10 mM EDTA), 200 μM each dNTP, 1 pmol each forward and reverse primers, 15 pmol C5-labeled third primer, 50–60 pg recombinant PCR-generated template, and 2 U Takara Taq DNA polymerase (Takara Mirus Bio, Madison, WI, USA). The cycling program was 94°C for 3 min for DNA denaturation, followed by 20 cycles of 94°C for 20 s, and 70°C for 90 s. This was followed immediately by 20 cycles of 94°C for 20 s, 54°C for 20 s, and 72°C for 1 min. To test whether the PCR products consisted of single-stranded DNA, the PCR product was treated with S1 nuclease (20 μL PCR product/20 U S1 nuclease at 37°C for 30 min). Doubled-stranded PCR product generated with the first pair of primers was also treated at the same conditions as a comparison. Five microliters of each treated and untreated PCR product were electrophoresed side-by-side on a 1% regular agarose gel in TBE buffer (Roche, Indianapolis, IN, USA) at 150 V to compare the existence of single-stranded fragments. The gel was stained with ethidium bromide in TBE buffer for 30 min before photography under UV light. All the PCR-generated products to be used for hybridization (targets) were mixed together, purified by ethanol precipitation, and dissolved in 60 μL distilled water.

Asymmetric PCR for Northern Blot Analyses

For the GATA-2 expression experiments, the paired forward and reverse primers designed and used to generate a 390-bp PCR fragment were GATAratF (5′-GGCAAGCAGCTCTCTCTCGC-3′) and GATAratR (5′-GGACTGACCTCCCATCTATTC-3′), A-32P-labeled third primer, GATAratF2 (5′-TCTCTCTGCGCTCGTGC-3′), was used to generate a radiolabeled single-stranded DNA probe using the 390-bp fragment as a template generated in the first round of PCR. Each 40 μL of PCR solution consisted of PCR buffer, 200 μM each dNTP, 1.5 mM MgCl2, 1 pmol each forward and reverse primers, 15 pmol third primer, 50–60 pg cloned GATA-2 template, and 2 U Takara Taq DNA polymerase. The cycling program was 94°C for 3 min, followed by 20 cycles of 94°C for 20 s and 70°C for 90 s. This was followed immediately by 20 cycles of 94°C for 20 s, 54°C for 20 s, and 72°C for 1 min. The PCR-generated radioactive probes were purified by Sephadex® G-50 column and eluted into 60 μL distilled water and mixed with hybridization solution.

Microarray Spotting of DNA Probes

The M. tuberculosis wild-type and mutant probes were spotted onto CSS-silylated glass microscope slides (Fisher Scientific, Hampton, NH, USA) with an OmniGrid Accen™ robotic microarrayer (Genomic
Solutions, Ann Arbor, MI, USA). Each probe (100 μM) was mixed 1:1 with microarray spotting solution consisting of 100 mM NaOH and 0.02% sodium dodecyl sulfate (SDS). The average spot size was 200 μm. Each probe was spotted in triplicate, and the distance between spots was 500 μm. The spotted slides were left overnight at room temperature in a clean chamber for drying.

**Rate Lung Total RNA Isolation and Northern Blot Analysis**

The total RNA was isolated from normal and *P. carinii*-infected rat lungs by the acid guanidinium thiocyanate-phenol-chloroform extraction method (19). Fifteen micrograms of each isolated total RNA sample were electrophoresed on an RNA gel and then transferred to a Nitran membrane (Schleicher & Schuell, Keene, NH, USA). The transferred membrane was UV-linked and baked at 80°C for 1 h.

**Hybridization for Microarray Analysis**

Ten microliters purified Cy5-labeled targets were mixed with an equal volume of hybridization solution consisting of 50% formamide, 10× saline sodium citrate (SSC), 0.2% SDS, and 0.2 mg/mL sperm DNA and were added to the probe area on the slide. A coverslip was added, and the slides were kept in a humidified chamber at 40°C for 30 min for hybridization. The slides were subsequently washed with a series of solutions that descended from 6× SSC to 2× SSC for 2 min each at room temperature. The hybridization signals on the slides were visualized with a GenePix® 4000B Array Scanner (Axon Instruments, Union City, CA, USA) at 635 nm.

**Hybridization for Northern Blot Analysis**

The [³²P]-labeled GATA-2 single-stranded DNA probe was added directly into a hybridization bag containing a blotted membrane plus hybridization solution consisting of 0.25 M phosphate buffer, pH 7.0, 7% SDS, and 1 mM EDTA. The hybridization bag was kept in a shaking water incubator at 65°C for 16 h. After hybridization, the membrane was washed with a series of washing solutions that descended from 2× SSC, 0.5% SDS to 0.5× SSC, 0.1% SDS, each at 65°C for 20 min. The hybridization signal of GATA-2 was visualized by exposure of an X-ray film at -80°C for 72 h.

**RESULTS AND DISCUSSION**

Figure 2 shows the results of double-stranded and single-stranded PCR fragments treated with S1 nuclease. The single-stranded fragment (the product in the second round of PCR) showed a lower position on the gel due to its smaller molecule size as compared with that of the double-stranded fragment (the product in the first round of PCR). After treatment with S1 nuclease (as shown in Figure 2), the single-stranded fragment was degraded, while the double-stranded fragment remained almost intact.

Experiments were subsequently performed to show that this asymmetric PCR procedure generated single-stranded DNA targets and probes that were effective in hybridization studies. Three different Cy5-labeled, single-stranded target DNAs containing *katG* gene sequences were prepared by our method. One of these, *katG* WT, contained a wild-type *katG* sequence that included codons 463 and 587. The other two target DNAs each contained one point mutation at either position 463 (G to T) or 587 (C to A) and were also prepared by the same methods. The details of probe arrangement on the slide are given in the legend of Figure 3.

Each of the three single-stranded target DNAs was hybridized to a microarray containing the four immobilized probes. Figure 3 shows a typical example of the hybridization data obtained. In Figure 3A, *katG* WT target DNA was hybridized with the four probes. As expected, hybridization of this target DNA occurred with

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**Figure 3. Oligonucleotide microarray identification of *Mycobacterium tuberculosis katG* gene point mutations.** This figure shows a copy of the actual scanner hybridization results from the microarray experiment. Each microarray (A–C) consisted of 12 DNA oligomeric probe spots. Four different probes (two wild-type and two mutant) were each spotted in triplicate on a glass slide. The wild-type probes contained all wild-type *katG* codons, and the mutant probes contained wild-type *katG* codons plus one *katG* codon (463 or 587), with a point mutation. The target DNAs used in this experiment were all single-stranded, generated by asymmetric PCR. The label at the top of each subfigure indicates the particular target DNA used for hybridization with the spotted probes. It includes the *M. tuberculosis* gene plus, in the case of targets with a point mutation, the particular mutant codon selected. A simple representation of the target DNA is presented at the bottom of each subfigure and identifies the relevant codons used in the experiment and whether or not each codon is wild-type (W) or mutant (M). Target DNAs used were (A) *katG* WT Target, which consisted of all wild-type *katG* codons (including 463 and 587), (B) *katG* 463 MUT Target, which consisted of one wild-type *katG* codon 587 plus one *katG* codon 463 (a polymorphism) with a point mutation, and (C) *katG* 587 MUT Target, which consisted of the one wild-type *katG* codon 463 plus one *katG* codon 587 with a point mutation. The numbers in the column on the right side of each subfigure represent the two *katG* codons of the spotted probes that were designed to be complementary to the selected sequences of interest in the target DNAs. This figure shows that the asymmetric PCR-generated, single-stranded target DNAs successfully hybridized with the probes.
wild-type probes 463 and 587. Little or no hybridization was observed with mutant probes 463 and 587. Results for the remainder of the experiment were similar. As expected, Figure 3B shows that the katG 463 MUT target DNA hybridized with the mutant 463 probe, but not with the wild-type 463 probe. Since this target DNA contained wild-type codon 587, it hybridized with the wild-type probe 587, but not with the mutant probe 587. Figure 3C shows similar data: the katG 587 MUT target DNA hybridized best with the probe that contained the 587 point mutant anticodon and the probe that contained the wild-type 463 anticodon. Some minor nonspecific hybridization was observed with the 463 mutant probe and the 587 wild-type probe.

To further demonstrate the utility of our asymmetric PCR procedure, single-stranded probes were generated to detect expression of the gene of GATA transcription factor in the host infected by P. carinii (20–22) using Northern blot analysis. For Northern blotting, a high quality of labeled probes is critical for the successful detection of messenger RNAs (mRNAs) with low copy numbers such as transcription factors and kinases. After treatment, the membrane was hybridized with a single-stranded radiolabeled GATA-2 DNA probe that was generated by asymmetric PCR. The hybridization results are shown in Figure 4. A clear radioactive signal band was observed in the first lane (control), where the position of the GATA-2 mRNA (mRNA), as indicated by the hybridization band in the control lane, is about 3000 bp. This figure (control lane) shows that the asymmetric PCR-generated DNA probe successfully hybridized with the blotted rat RNA target.

**REFERENCES**


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