Synthesis of Radioactive Single-Stranded DNA Probes Using Asymmetrical PCR and Oligonucleotide Random Priming

BioTechniques 27:674-678 (October 1999)

Single-stranded (ss)DNA labeled probes can be used in S1 nuclease analysis, Southern, northern blotting and in situ hybridization (1,4,7,8). They are especially useful in detecting the specific expression of genes in which both strands are transcribed. Several methods for synthesis of radioactive ssDNA probes have been described (3,6,9), for example, cloning the sequence into a suitable vector like the single-strand bacteriophage M13 for ssDNA production. Asymmetrical polymerase chain reaction (PCR), in which a large excess in the amount of one primer to the other, can also be used to produce an ssDNA for use as a probe. Other investigators using asymmetrical PCR have incorporated 32P into the PCR as 32P-labeled nucleotides (3,9) or as the 5′ 32P-labeled primers to directly radiolabel the probe during synthesis (8). Thus, all subsequent steps must be carried out under precautions for radioactive materials, and the probe must be used before decay of the 32P. In addition, if a 5′ 32P-labeled primer is used, the specific activity of the radioactive probe will be limited to that of the 5′ 32P-labeled primers. Unidirectional PCR has also been used to generate ssDNA probes (3,9). However, this approach requires a much larger amount of template. Here, we introduce a method that combines asymmetrical PCR and oligonucleotide random priming in separate steps to produce radioactive ssDNA probes for use in Northern blotting. We studied the expression of the UL34.5 gene of herpes simplex virus type-1 (HSV-1) grown in tissue culture cells. Both strands of the viral DNA are transcribed in the UL 34.5 gene locus (5).

A previously developed PCR protocol (2) was used to produce ssDNA probes. The template for the PCR was a pBR322 plasmid (2.0 µg) containing the EcoRI EK fragment of HSV-1 KOS strain and the UL34.5 gene. Viral DNA could also be used as template. Due to the high GC content of the gene and the failure of several other approaches, PCR was performed using the MasterAmp™ PCR Optimization Kit and T7 DNA Polymerase (both from Epicentre Technologies, Madison, WI, USA). Primers were chosen based on their unique sequences and to bracket the UL34.5 gene (2). The first step was to determine the appropriate ratio of primers (data not shown). The best ratio of primer pair was 50:1 (50 pmol vs. 1 pmol in 50-µL reaction volume) producing the largest amount of ssDNA (Figure 1A). The reaction mixture contained 50 mM Tris-HCl (pH 9.0, 25°C), 20 mM (NH4)2SO4 and 200 µM each dNTP, 2.5 mM MgCl2, 4× MasterAmp PCR Enhancer (Epicentre Technologies) and 1.0 U T7 DNA polymerase. The program consisted of 5 min at 96°C, 35 cycles of 1 min each at 95°C, 60°C and 68°C with an additional incubation of 10 min at 68°C. For comparison, the purified normal PCR product, with 800 bp double-stranded (ds)DNA, was denatured by heat or alkali to produce ssDNA and electrophoresed on a 1.2% agarose gel with the asymmetrical PCR products. The normal dsDNA product (ca. 800 bp long) and a (+) ssDNA encoding ICP34.5 were generated using equal amounts of primers: 50 pmol of 5′ primer vs. 50 pmol of 3′ primer (lanes 2–4). The Klenow polymerase-free H2O to 14 µL, it was boiled for 10 min and chilled on ice. The Klenow polymerase (1.0 µL), buffer (5.0 µL), 5× and 32P-CTP (5.0 µL) (10 mCi/mL; ICN Biomedicals, Costa Mesa, CA, USA) were added together and incubated for 1 h at room temperature. The radioactive probe was then purified from unincorporated nucleotides using a Sephadex® G-50 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The specific activity of the probe was approximately 2 × 109 counts per minute (cpm)/µg.

The specificity of the desired DNA strand was checked using a dot-blotting method. dsDNA from normal PCR of UL34.5 and ssDNA from either strand of the UL34.5 gene region, produced by asymmetrical PCR, were blotted as 100, 10 and 1 pg onto nitrocellulose membranes (NitroPure™; Osmonics, Minnetonka, MN, USA). After denaturation and neutralization, the membranes were hybridized with 32P-la-

Figure 1. Asymmetrical PCR amplification product of the HSV-1 (KOS strain) UL34.5 gene. (A) The normal dsDNA product (ca. 800 bp long) and a (+) ssDNA encoding ICP34.5 were generated using unequal amounts of primers: 50 pmol of 5′ primer vs. 1 pmol of 3′ primer (lanes 2–4). A (-) ssDNA was generated by using 1 pmol of 5′ primer vs. 50 pmol of 3′ primer (lanes 5–7). Note that the mobility of both ssDNAs are similar to 400 bp dsDNA. Lane 1 is the 800 bp dsDNA produced by normal PCR. (B) The mobilities of the 800-bp dsDNA from normal PCR (lane 1), the (+) ssDNA produced by asymmetrical PCR (lane 2), the NaOH (lane 3) and heat denatured normal dsDNA (lane 4) are compared. Only a weak band is present in lane 3 but with the same mobility as (+) ssDNA. Gel electrophoresis was performed on a 1.2% agarose gel containing etidium bromide.
beled ssDNA probe in Church Buffer [1% bovine serum albumin (BSA), 7% sodium dodecyl sulfate (SDS), 1.0 M sodium phosphate, pH 7.4, 1 mM EDTA], at 65°C overnight. As expected, both of the ssDNA probes bound to the dsDNA for UL34.5. However, the probe [corresponding to the (-) DNA strand] synthesized by random priming from (+) UL34.5 ssDNA, only recognized (+) ssDNA, on the membrane and did not bind to the (-) strand (Figure 2, inset). In agreement with this result, the probe synthesized from the (-) strand could only detect the (-) strand template (data not shown). These results indicated that the probes made by this technique were very specific for the ssDNA template from which they were derived.

For northern blotting, total RNA was purified from HSV-1 (SP7 strain) (2) infected Vero cells at different times post-infection using TRIzol® Reagent (Life Technologies, Gaithersburg, MD, USA) and then separated on a 1% agarose gel containing 2.2 M formaldehyde. The RNA was transferred to a nitrocellulose membrane, UV fixed (UV fixing the membrane was not required) and baked at 80°C for 2 h and prehybridized in Church Buffer at 65°C for 2 h. The concentrations of RNA samples were measured by spectrophotometry, and 8.0 µg of total RNA were loaded in each lane. The equal loading of samples was further checked by ethidium bromide staining of the membrane after transfer (data not shown). Probes (2 × 10⁶ cpm/mL, 15 ng) were added directly to the hybridization solution. After overnight incubation at 65°C, the membranes were washed twice with 2× standard saline citrate (SSC), 0.1% SDS, 5 min each at room temperature, and twice with 0.1× SSC, 0.1% SDS at 68°C for half-hour each. The radioactive signal on the membranes was detected by PhosphorImager® (Molecular Dynamics, Sunnyvale, CA, USA).

The genes for ICP34.5 and other transcripts, including latency associated transcripts (LATs) and open reading frames O and P (orfO and orfP) (5) are located on complementary strands of the same region of the long terminal repeats of the HSV-1 genome. Their transcripts overlap each other and can be detected using a dsDNA probe for the UL34.5 gene (data not shown). The ssDNA probe specific for ICP34.5 mRNA identified two main transcripts of 1.3 kb and 4.4 kb, but not other transcripts (Figure 2). However, the complementary ssDNA probe detected a series of fragments including primary LAT (8.3 kb) (data not shown). These transcripts were expressed at different times post-infection. RNA for ICP34.5 could be detected as early as 3 h post-infection, increasing to a peak at 9 h, and then they decreased in concentration (Figure 2).
In conclusion, the combination of asymmetrical PCR and oligonucleotide random priming is an alternative approach to synthesize a radioactive ssDNA probe. The approach can also be applied directly to genomic DNA as the template for PCR, bypassing the steps for constructing recombinant plasmids. Laboratories that lack established protocols for generating recombinant DNA may prefer this approach. Either strand of the DNA can be amplified by changing the amount of one of the primers in the asymmetrical PCR. This technique is also useful if an established PCR protocol has been developed or special PCR conditions are needed, similar to the UL34.5 gene that was studied. The ssDNA template can be stored and used to synthesize radioactive probes when desired. The ssDNA can also be used to generate nonradioactive probes. This approach is also safer because it limits the handling of radioisotope to the last step in the preparation, the random priming. Finally, the technique can synthesize a radioactive probe with a high specific activity up to $2-3 \times 10^9$ cpm/µg.

REFERENCES


Address correspondence to Dr. Ken S. Rosenthal, Northeastern Ohio Universities College of Medicine, Box 95, 4209 SR 44, Rootstown, OH 44272, USA. Internet: ksr@neoucom.edu

Received 30 April 1999; accepted 28 June 1999.

Hanwen Mao and Ken S. Rosenthal
Northeastern Ohio Universities College of Medicine
Rootstown, OH, USA