Agarose Gel Analysis of 15–40-kb PCR Amplimers

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ABSTRACT

Accurate resolution of PCR products in the range of 15–40 kb may be obtained in agarose gels without pulsed-field electrophoresis. A gel of 0.3% SeaKem[®] Gold agarose cast on GelBond[®] support film provides good resolution and sufficient gel strength to reliably allow staining and photography. This paper describes a test system for Long PCR and demonstrates analysis of the PCR products on a gel run under standard low-voltage electrophoresis conditions.

INTRODUCTION

Amplification of target fragments in the kilobase range by Long polymerase chain reaction (Long PCR) has become possible by using a combination of thermostable polymerases with and without the proofreading activity of 3' exonuclease (1–3). The addition of a proofreading enzyme in very small amounts allows removal of mismatched nucleotides without degradation of oligonucleotide primers (1), generating longer extension products at high fidelity (1,4). For analysis of PCR products, standard agarose gel procedures resolve fragments well up to about 20 kb, but it has been necessary to use pulsed-field electrophoresis for accurate analysis of fragments between 20 and 40 kb. To simplify this process, we screened for a high gel strength agarose, which could be used under standard electrophoresis conditions (without pulsed-field) to obtain good resolution of these large DNA fragments. SeaKem[®] Gold (FMC BioProducts, Rockland, ME, USA), with the highest gel strength of all the available agaroses, met these requirements at a concentration of 0.3%. Although other agaroses theoretically have the resolving power to separate fragments in this range (e.g., SeaKem LE and FastLane[®] agaroses, both from FMC BioProducts), they are not practical alternatives, since at the low concentrations required, their gel strengths are too low to allow the routine handling of staining and photography.

DISCUSSION

The LA PCR kits Versions 1 and 2 from Takara (PanVera, Madison, WI, USA) were used to generate a series of amplified fragments from 15–40 kb with very little modification of the suggested protocols. The template used was purified phage lambda DNA. A set of primers was prepared consisting of one forward primer and a series of reverse primers 31–32 bases long, whose target sites in the template were spaced at 5-kb intervals to give amplimers from 15–40 kb. The primer sequences were from the product insert in the Expand[™] Long template PCR system (Boehringer Mannheim, Indianapolis, IN, USA). They were synthesized at Genosys (The Woodlands, TX, USA) with cartridge purification.

Reaction mixtures were set up as specified in the Takara product literature and using AmpliWax[™] PCR Gems (Perkin-Elmer, Norwalk, CT, USA) to facilitate the hot-start procedure. The Version 1 PCR kit amplified the 15–30-kb fragments well. The Version 2 kit readily produced good yields of 35- and 40-kb targets when the magnesium concentration was increased to 5.0 and 5.4 mM, respectively, with appropriate thermal cycling. Reactions were performed in a PTC-100[™] Model 60 Thermal Cycler (MJ Research, Watertown, MA, USA). For all reactions, the block was preheated to 98°C before putting in the tubes. The 15–30-kb targets were amplified successfully by 30 cycles of a simple two-step cycle: 98°C for 20 s, then 68°C for 15 min. For the two largest fragments, an extra step was included at the start (94°C for 1 min), and a longer extension time (20 min) was used for the first 14 cycles. Then for the remaining 16 cycles, the extension time was further increased by 15 s more in each successive cycle.

The PCR products were analyzed on 0.3% SeaKem Gold agarose gels in 1× TAE buffer (40 mM Tris-acetate, 1.0 mM EDTA, pH 8.3), which gave the optimum combination of resolution and gel strength at low concentration. The results are shown in Figure 1. The gel must be at least 15-cm-long for good separation of DNA bands in the 15–40-kb range. For ease of handling, gels of 2.5-mm-thickness were cast either in

Figure 1. Long PCR products analyzed on 0.3% SeaKem Gold agarose cast and photographed on GelBond support film. Lane 1, 2-kb ladder (100 ng); lane 8, 5-kb ladder (100 ng). Lanes 2–7, amplified fragments loaded with approximately equal amounts of DNA: 15 kb (0.25 μ L of the unpurified PCR), 20 kb (0.35 μ L), 25 kb (2.0 μ L), 30 kb (4.0 μ L), 35 kb (2.0 μ L) and 40 kb (4.0 μ L). The length of the gel portion in the photograph is 10.5 cm, and the 40-kb band has migrated approximately 5.5 cm. Photography with Polaroid Type 55 film. Lane 7 also contains an artifact band at about 14 kb.

Figure 2. A plastic frame was used to cast 0.3% agarose gels on GelBond. (A) Strips 1.5-cm-wide were cut from 3-mm-thick plastic sheet and cemented together to form a rectangle of internal dimensions of 17 $\times$ 9.5 cm. To cast the gel, the frame was placed on GelBond film and weighted. The edge was sealed by pipetting gel solution at about 45°C around the inside perimeter. About 1 min later, the bulk of the gel solution was poured in and the comb was placed, supported by spring binder clips. (B) After the gel solidified, the comb and frame were removed, leaving a border of film for handling.

a frame of 1% agarose or on GelBond® support film (FMC BioProducts). Unsupported 0.3% gels are surprisingly resilient and flexible but do take some practice to handle. Gels of 0.2% SeaKem Gold agarose, while giving excellent separation of the largest fragments, are fragile and difficult to manipulate even with support film.

The gel shown in Figure 1 was cast on GelBond support film using a workshop-made acetal plastic frame to contain the gel and leave a border of film around its edge for handling (Figure 2). To preserve the wells, after the gel was completely set, buffer was layered with a transfer pipet onto the gel surface in the well area as the comb was removed, so that the wells filled immediately. The supported gel was then peeled away from the frame, leaving a border of film for handling. PCR products were loaded along with 2-kb and 5-kb ladders (Superladder High; GenSura Laboratories, Del Mar, CA, USA) as size markers. Electrophoresis was carried out at 1 V/cm for 16 h at room temperature. Shortening the electrophoresis time below 10 h or increasing the voltage twofold both markedly decreased resolution of fragments above 25 kb. Overloading the DNA also decreased resolution; between 15 and 40 ng should be loaded. The run time is comparable with pulsed-field electrophoresis since a field inversion gel electrophoresis (FIGE) or contour-clamped homogeneous electric field (CHEF) gel for this range of fragments sizes requires 9–12 h, an overnight gel run for most laboratories.

The gel was stained after electrophoresis with ethidium bromide and photographed gel-side down on the transilluminator. GelBond appears fluorescent under UV light since the

UV wavelength is blocked. However, the emission wavelength of ethidium bromide is not blocked; therefore, even though the DNA bands were not easily visible by eye, clear pictures were readily obtained by using Polaroid Type 57 or 55 film (Polaroid, Cambridge, MA, USA) with only small amounts of DNA loaded, at settings of *f* stop 4.5, exposure time 1 s for Type 57 and 60 s for Type 55, using both orange and UV filters for both film types.

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