Bandstab: A PCR-Based Alternative to Cloning PCR Products

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In many DNA amplification reactions, extra fragments arising from nonspecific mispriming, a mixture of target templates or allelic variation may be generated in addition to the desired product. Some nonspecific bands can be reduced by more stringent annealing conditions, but in cases of allelic variation or other mixed targets, the amplification products may be of the same length but differ by only a single base in the DNA sequence. In these situations, it is often desirable to purify the expected product(s) from contaminating fragments or alleles in order to undertake further analysis such as direct DNA sequencing. The conventional cloning-based approach for preparing a pure template for DNA sequencing involves ligation of the polymerase chain reaction (PCR) products (usually gel-purified) into an appropriate vector, followed by transformation and then propagation in a suitable host (4). Several steps are required to generate the recombinants, after which it is necessary to sequence several independent clones to ensure that any observed base changes reflect genuine allelic variation and not mistakes introduced during the PCR, especially if *Taq* DNA polymerase is used in the amplification (3). Problems with *Taq* DNA polymerase-induced errors can be minimized by using high-fidelity amplification systems such as the GeneAmp® XL PCR Kit (Perkin-Elmer, Norwalk, CT, USA) and the Expand™ High Fidelity PCR System (Boehringer Mannheim, Indianapolis, IN, USA).

It is possible to isolate pure species of PCR products by electrophoretically separating the mixture on the basis of length or shape (single-strand conformation polymorphism [SSCP]) and then eluting the bands of interest from the gel slice. A simpler and more efficient approach is to stab the band(s) of interest with a pipet tip(s) and use this to inoculate a second round of DNA amplification. This procedure, called "bandstab", can readily generate sufficient material for direct DNA sequencing, for use as a hybridization probe or for other detailed analysis. This bandstab approach requires much less effort and is quicker and easier than even band slicing or excision techniques, where the desired band(s) is cut out of a gel with a scalpel blade, and the gel slices are transferred to another tube before elution or other manipulations. The limitation on how many fragments can be bandstabbed from a gel is often determined by the number of individual PCRs that can be set up conveniently. This bandstab approach is easier, faster and more convenient than cloning, and the ability to analyze a product directly from a gel can help to overcome any problems encountered with errors that may have arisen during the amplification reaction.

This report provides two examples where the bandstab method was used to select and re-amplify individual bands from a mixture of amplification products after electrophoretic separation based on SSCP analysis (5) and length. DNA fragments may be detected and bandstabbed after staining in ethidium bromide (EtdBr; 0.5 μg/mL) and visualization on a transilluminator or after silver staining. Since the bandstab procedure can be carried out very quickly, there is minimal exposure of the PCR products to damage from the ultraviolet light. Cross-contamination with other PCR products was not a problem if fresh EtdBr was used each time and an excessive number of thermal cycles was not carried out in the re-amplification. In situations where EtdBr staining was not sufficiently sensitive (short DNA fragments or single-stranded material such as that found after SSCP analysis), a simple silver staining method was used where the polyacrylamide gel was soaked in approximately 20 vol of 10% ethanol for 3 min, 1% nitric acid for 3 min and 0.1% silver nitrate for 8 min. The gel was kept in developer (6% sodium carbonate, 0.12% formaldehyde) until a satisfactory signal-to-background ratio was achieved (usually 4–5 min), at which time the reaction was stopped by placing the gel in 10% acetic acid for 10 min. (The gel was rinsed twice with distilled water between each solution). Although it is preferable to carry out the bandstab re-amplification soon after the gel has been stained/developed, we have re-amplified fragments from silver-stained gels that had been developed 48 h earlier. In cases where the silver-stained gel could not be bandstabbed immediately, the gel was rinsed in water and then kept in a 1% glycerol solution in subdued light at room temperature.

Bandstab Amplification. After gel fractionation and staining (either EtdBr or silver), the gel was rinsed briefly with distilled water before a sterile micropipet tip (yellow P200 tip; Quality Scientific Plastics, USA) was used to stab the band of interest. A single stab contained sufficient material for re-amplification from either polyacrylamide or agarose gels. The pipet tip was left in

a 50-μL amplification reaction for 15–30 s before being withdrawn (a micropipet was used to expel any PCR mixture that had entered the tip because of capillary action). At this time, an oil overlay can be added (if necessary), and the amplification can be carried out (under conditions previously used to generate those particular products) for 20–25 cycles. The quality and quantity of the re-amplification can be quickly checked on an agarose gel, and if there are insufficient amounts of the re-amplified material, an additional 5 rounds of amplification could be carried out.

There was little difference in the amount of material synthesized if single or multiple stabs or even portions of a polyacrylamide gel slice were added to the re-amplification mixture. We have used two different PCR systems: conventional amplification using *Taq* DNA Polymerase (Biotech International, Perth, Western Australia) and long-range PCR using the GeneAmp XL PCR System. While the addition of an entire polyacrylamide gel slice ($5 \times 1 \times 0.75$ mm) was not inhibitory to a 50-μL PCR, the re-amplification was much more sensitive to agarose (molecular biology grade), where even small fragments of the gel slice were inhibitory.

The re-amplified bandstab PCR products were purified from unreacted primers and nucleotides using QIAquick™ PCR Spin Columns (Qiagen, Chatsworth, CA, USA) as described by the manufacturer. These templates were sequenced directly from one of the original PCR primers using the PRISM™ Ready Reaction Dye Terminator Cycle Sequencing Kit and a Model 373A DNA Sequencer (both from PE Applied Biosystems, Foster City, CA, USA) essentially as described in the manufacturer's protocol, except that only 50–75 ng of template were used in a 10-μL sequencing reaction with 25 cycles of denaturation, annealing and extension.

Bandstab amplification after fractionation based on shape. SSCP analysis relies on a base change inducing a conformation change in the overall secondary structure of one (or both) of the single strands of a PCR product compared to that of the normal allele. These conformation changes may then be detected as an electrophoretic migration

shift after the heat-denatured and snap-chilled PCR products are fractionated under non-denaturing conditions on SSCP gels (5). Figure 1 shows two distinct SSCPs that were detected in exon 4 of the Cu/Zn superoxide dismutase (*SOD1*) gene from unrelated individuals known to be at risk for familial amyotrophic lateral sclerosis (9). This condition is generally regarded as autosomal dominant. Both at-risk individuals (lanes 3 and 5) were found to be heterozygous where, in addition to the normal allele shown in lane 2, each carried a mutated allele that had been detected as distinct SSCPs (indicated by arrows). Each abnormal band from these patients was re-amplified using the bandstab technique and directly sequenced to determine the precise base change. The SSCP in lane 3 arose from a T to C change (Ile113Thr), a known disease-causing mutation (6). The other SSCP (lane 5) resulted from an A to C change (Asp90Ala), which had been assumed to be a neutral polymorphism (8), although homozygosity for this polymorphism has now been associated with amyotrophic lateral sclerosis (1). The purity of the re-amplified material is also shown on Figure 1 (lanes 4 and 6) where the bandstab products now migrate as an apparent homozygote with a pattern distinct from that obtained for the normal allele. Although traces of the normal single-stranded material were detected (most evident in lane 6), presumably arising from minor contamination with that allele during the bandstab amplification, this contamination was not detectable when the bandstab-purified allele was used directly in a sequencing reaction.

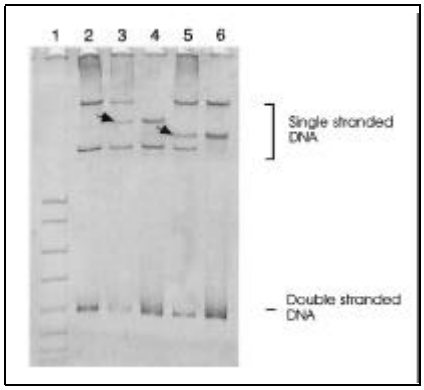

Figure 1. 12% polyacrylamide (99:1 acrylamide:bis) SSCP gel fractionation of amplification products from exon 4 of the *SOD1* gene. Lane 1, marker (pUC19 digested with *Hpa*II); lane 2, normal pattern; lane 3, heterozygous patient with mutation (Ile113Thr); lane 4, mutant allele (Ile113Thr) after bandstab; lane 5, heterozygous patient with mutation (Asp90Ala); lane 6, mutant allele (Asp90Ala) after bandstab.

Bandstab amplification after fractionation based on size. During a study on mouse dystrophin gene transcript processing, two rounds of PCR with nested sets of primers were needed to detect rare, alternately processed dystrophin gene transcripts extracted from dystrophic mouse muscle tissue (2,7). The bandstab technique was then used to re-amplify individual DNA bands after polyacrylamide gel fractionation on the basis of size (Figure 2, lanes 2 and 4) where several PCR products of different lengths were generated. Individual bands were generated after bandstabbing from polyacrylamide gels, purified using QIAquick PCR Spin Columns and directly sequenced for identification.

Of the two products in lane 4, sequence analysis indicated that only the top band (223 bp long) followed the known exon boundaries where exon 21 was spliced precisely to exon 30. This particular dystrophin gene transcript was assumed to be biologically inert because it was out of frame and thus could not be translated into a protein with a carboxyl terminus necessary for the interaction of dystrophin with other cytoskeletal components. The smaller band in lane 4 (181 bp) was a presumed artifact of the PCR since DNA sequencing indicated that this transcript did not follow known exon boundaries but rather, the first 35 bases of exon 22 were joined to approximately the last 85 bases of exon 30. It was not possible to assign the exact contribution from exons 22 and 30 since the sequence 5′CAGGAG3′, present at the fusion site of the transcript, was common to both exons. Two other similar dystrophin transcript artifacts were also identified with 9 bases (5′ATGGAGGAG3′ common to exons 22 and 29) and 5 bases (5′CAGCT3′ common to exons 20 and 30) found at the fusion sites. These PCR products presumably arose from a slippage/reinitiation mechanism during the DNA extension step involving short blocks of sequence common to both exons found at the fusion site. However, not all PCR artifacts arose from this particular mechanism. The single fragment shown in lane 3 of Figure 2 was another presumed artifact where the last few bases of exon 20 were joined to the last 6 bases of exon 25. In this particular fragment, there was no common sequence at the fusion site, so this product presumably arose from more random slippage or reinitiation.

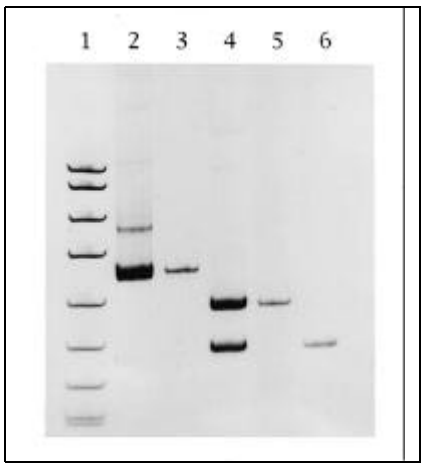

Figure 2. 12% polyacrylamide (39:1 acrylamide:bis) gel fractionation of RT-PCR products from the mouse dystrophin cDNA. Lane 1, marker (pUC19 digested with *Hpa*II); lane 2, smaller than expected PCR products after amplification across mouse dystrophin exons 18–26; lane 3, re-amplification of a single band from the mixture fractionated on lane 2; lane 4, smaller than expected PCR products after amplification across mouse dystrophin exons 21–31; lane 5, bandstab re-amplification of the upper band from lane 4; lane 6, bandstab re-amplification of the lower band from lane 4.

Bandstab re-amplification generally seemed more efficient and reliable after polyacrylamide gel fractionation than after agarose gel fractionation, where there was a tendency for shorter fragments in the lane to be preferentially amplified, sometimes at the expense of the desired target fragment. Presumably, the preferential synthesis of these shorter products arose from trailing of the fragments through the gel and from the tendency of smaller fragments to be amplified more efficiently. Nevertheless, it was possible to reliably carry out bandstab from agarose gels, especially when the desired targets were the smaller bands in the lane. In re-amplification of larger bands from a mixture (fragments in excess of 1000 bp have been synthesized from polyacrylamide gels), there was often an enrichment of

the desired fragment after one round of bandstab. If the desired level of purity had not been achieved from the first round of bandstab, it was possible to re-amplify the target fragment from the gel used to check the yield and purity of the initial bandstab products.

The bandstab approach can bypass the need for cloning PCR products and all associated work required to generate recombinants, check the insert size and prepare recombinant DNA. The bandstab technique may be used to obtain a pure template ready for direct DNA sequencing within hours of first visualizing the band on a gel, with considerable savings in time, labor and reagents.

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Sequential Extraction of DNA and DNA-Binding Proteins from Low Cell Numbers

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It is possible to purify nuclear-binding proteins from a limited supply of cells with relative ease and convenience. More recently, these protocols have improved, resulting in less manual labor and greater flexibility, thereby allowing the analysis of multiple samples (1,2). However, when using these techniques, it is difficult to efficiently purify nuclear proteins and extract genomic DNA from low cell numbers, particularly when dealing with clinical samples. We regularly deal with low lymphocyte numbers from patients with B-cell chronic lymphocytic leukemia (B-CLL) and often do not have enough material to accommodate or provide reasonable yields for nuclear protein and genomic DNA. We now describe a simple protocol that allows for the isolation of genomic DNA following the extraction of DNA-binding proteins. This technique complements existing protocol by Andrews and Faller