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Simplified Template Preparation and Improved Direct Sequencing Using *Taq* Polymerase

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A streamlined version of direct dideoxy sequencing is presented that includes template preparation as well as sequencing protocols. The method is used routinely to sequence double-stranded PCR products after minimal purification with one of the primers used in amplification. Either ^{35}S or ^{32}P labeling can be used with equally good results.

The polymerase chain reaction (PCR) has provided an effective way to bypass subcloning steps in the preparation of templates and sequencing.[1] We have applied an amplification-driven sequencing procedure (a combination of PCR-based template preparation and direct temperature-cycled sequencing) to a variety of tasks (see below) including direct sequencing of double-stranded PCR products.

The methods described here are highly dependable when rapid sequencing of large numbers of templates is needed. The advantages of this protocol over previously published methods of direct sequencing include: (1) simple template preparation (see below) with minimal manipulation of the sequencing template (tiny quantities of template DNA present in agarose are sufficient, and further DNA purification is not required); (2) efficacy with both double-stranded and single-stranded templates; (3) routine application, with either primer used for the template preparation or with an internal primer; and (4) use of either ^{35}S or ^{32}P as label. The quality of the gels is high enough (with less background) to read up to 300 nucleotides in a single run. Even longer sequences can be read by double loading.

METHODS

Genomic or cloned DNA is amplified in a thermal cycler (Perkin-Elmer Cetus), typically for 30–35 cycles at 94°C for 1 min, 55–65°C for 2 min, and 72°C for 2 min in a reaction volume of 25 µl containing TNK 50 buffer (10 mM Tris-HCl, pH 8.6, 50 mM KCl, 1.5 mM $MgCl_2$, 5 mM NH_4Cl; Nowotny et al., pers. comm.), 10 pmoles of each primer, 100 µM dNTPs, and 0.5 units of AmpliTaq (Perkin-Elmer Cetus). The reaction products are separated in 1% agarose gels (SeaPlaque), and a piece of agarose containing the specific product to be sequenced is excised and melted before adding directly to sequencing reactions (gel pieces can also be stored at 4°C). (In some problem cases, reamplification and excision from agarose gel or high-resolution separation of the specific band on 6–8%, wt/vol, polyacrylamide/bisacrylamide gels help to reduce background. DNA is recoverd by electroelution, and then it is phenol: chloroform extracted once, or extracted in 0.5 M ammonium acetate/1 mM EDTA from a piece of the gel, precipitated with ethanol, and suspended in 15 µl of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.) Reaction conditions for preparing PCR products can be varied depending on the primers. In our hands, varying KCl concentration from 25 to 100 mM covers most of the primer pairs tested.

Two variations of the basic sequencing reactions have been used to label sequencing products. In one approach, unlabeled sequencing primer (0.5 pmole per mix) is used with either [α-^{35}S]dATP or [α-^{32}P]dATP in the sequencing reaction. Labeled dCTP can be substituted for labeled dATP. Alternatively, 5 pmoles of sequencing primer is kinase labeled at its 5′ end with [γ-^{32}P]ATP[2]; and one-tenth of the end-labeling reaction mix is added to the mix without any purification

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(see below). In at least some cases, α-labeled nucleotide provided more uniformity of signal strength across the gel. Presumably, the method could be adapted to fluorescence-based sequencing.[3]

A sequencing mix is prepared in a final volume of 36 μl containing 8 μl 5x TNK 50 buffer, 4.5 μl dNTPs (25 μM each), 1.0 unit of AmpliTaq, and 100–200 ng of template in agarose or suspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, 0.5 pmole of unlabeled primer with [α-^{35}S]dATP (7.5–15 μCi of 1 mCi/nmole sol., Amersham) or [α-^{32}P]dATP (2 μCi of 3 mCi/nmole sol., Amersham) or 0.5 pmole of end-labeled sequencing primer. (With unlabeled sequencing primers, template DNA should be free of any contaminating primers or oligonucleotides.) Nine-microliter portions of the reaction mix are added to each four tubes (marked G, A, T, and C) containing 1 μl of 0.25 mM ddGTP, 2.5 mM ddATP, 5.0 mM ddTTP, or 2.5 mM ddCTP, respectively. Each reaction mix is overlaid with 20–30 μl mineral oil, and sequencing cycling is performed for 15–25 rounds, in the same conditions used for template preparation (see above). Four microliters of stop solution (95% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue) is added at the termination of reaction, and the content is heated at 75°C for 4 min before loading 3–4 μl of each reaction mix on a 6% (wt/vol) polyacrylamide-urea or a 5% (vol/vol) Hydrolink (AT Biochemicals)-urea sequencing gel. The autoradiograms are exposed at room temperature without an intensifying screen. If pauses or stops are encountered, they can usually be overcome by elevating the annealing temperature in the sequencing protocol or by using 10% DMSO in the sequencing reaction.

RESULTS AND CONCLUSIONS

Typical results are shown in Figure 1. All 20 different sequencing primers used thus far have yielded useful sequence.

We have successfully used this procedure in the analysis of Alu-PCR products,[4] determination of terminal sequences of inserts in yeast artificial chromosomes (YACs) for chromosomal walking,[5] design of sequence tagged sites (STSs,[6]) comparison of homologous regions from different species (Mazzarella et al., in prep.) and detection of point mutations.

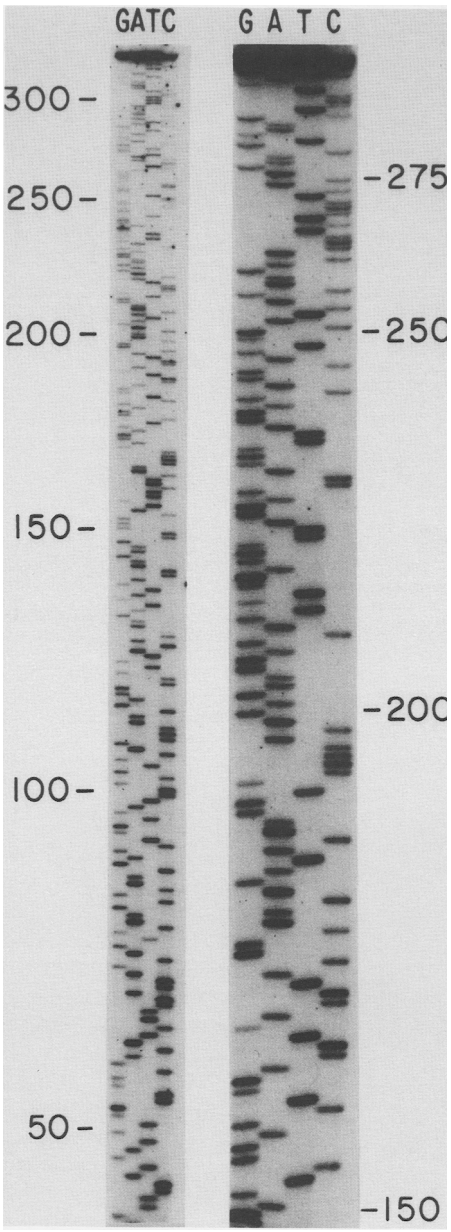

FIGURE 1 Full-length view of a sequencing autoradiogram obtained using a 20-mer oligo-primer and [α-^{35}S]dATP. The gel was fixed in 10% methanol/acetic acid solution, dried, and exposed for 14 hr at room temperature (*left panel*). (*Right panel*) Upper portion of a sequencing autoradiogram obtained using a ^{32}P kinase-labeled oligo-primer (20 nucleotides) specific for the glucose-6-phosphate-dehydrogenase gene. The gel was exposed for 3 hr at room temperature.

Simple and generalized template preparation with direct sequencing of double-stranded PCR products makes this procedure rapid without any loss in gel quality as compared to other methods using Sequenase or *Taq* polymerase.

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