BENCHMARKS

Benchmarks are brief communications that describe helpful hints, shortcuts, techniques or substantive modifications of existing methods.

Cycle Sequencing Protocol Using Deep Vent[®] (exo⁻) DNA Polymerase and Reduced dNTP and [α -³⁵S]dATP Concentrations

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Direct sequencing of polymerase chain reaction (PCR) products is a very powerful technique, especially for clinical investigations where quantities of biological material are often very limited and results are rapidly needed. However, numerous researchers still prefer to sequence cloned PCR fragments, a very time-consuming strategy, as most published protocols for direct sequencing of PCR products suffer from fairly serious drawbacks. Manual sequencing using exonuclease-deficient bacteriophage T7 derived DNA polymerases gives good results but requires large amounts of material: 0.5–1 pmol (corresponding to ± 325 to 650 ng of a 1-kb fragment) for one set of reactions, generally providing no more than 200-bp information. In contrast, protocols using PCR technology allow the sequencing of femtomolar quantities of fragment due to the fact that template molecules are released at the beginning of each cycle and thus are again available for the synthesis of new complementary strands. However, most commercial thermostable DNA polymerases for PCR sequencing are supposed to require relatively high nucleotide concentrations, in the range of 100–200 μ M. Therefore, the high concentration of nonradioactive dATP strongly competes with the commonly used [α -³⁵S]dATP nucleotide, which consequently is poorly incorporated. The synthesized strands are thus weakly labeled, imposing long autoradiography exposure times. This is often compensated for by increasing the input of expensive radioactivity (up to 20 μ Ci), which has an adverse effect on waste disposal. It is, therefore, often preferable to label the sequencing primer, but this dictates the use of high-exposure (³²P) or very expensive (³⁵P) radionu-

cleotides, and this may be very tedious if different primers are needed for the sequencing of long fragments.

Here, I propose an easily implemented modification that eliminates most drawbacks of current protocols for cycle sequencing with [α -³⁵S]dATP incorporation. This modification is based on the observation that Vent[®] (exo⁻) or Deep Vent[®] (exo⁻) DNA polymerases (New England Biolabs, Ozyme, France), at least in this application, do not require very high dNTP concentrations. These two enzymes work perfectly with dNTP concentrations 10 times lower than those currently recommended for sequencing. I have, therefore, determined conditions where, despite a 2.5 times reduction in radioactivity input, the ratio between labeled and nonradioactive dATP is much more favorable to [α -³⁵S]dATP incorporation.

The fragment to be sequenced is purified after agarose gel electrophoresis, using JetSorb[™] (Bioprobe Systems, Paris, France) or GENE CLEAN[®] (Bio 101, Ozyme, France) kits. Ten to one hundred fmol of purified PCR fragment (corresponding to 6.5–65 ng of a 1-kb fragment) in 2 μ L Tris-EDTA (TE) or water are mixed with 10 μ L of the following solution: 6 μ L water, 1.2 μ L 10 $\times$ sequencing buffer, 1 μ L 2.4% Triton[®] X-100, 0.5 μ L of 10 μ M sequencing primer, 0.8 μ L [α -³⁵S]dATP (1000 Ci/mmol, 10 mCi/mL; ICN Biomedicals, Orsay, France) and 1.0 U Deep Vent[®] (exo⁻) DNA polymerase (0.5 μ L). After careful homogenization, 2.5 μ L of this template-primer-enzyme solution are then added to each of four 0.5-mL microcentrifuge tubes, each containing 2 μ L of one 1 $\times$ dNTP/ddNTP mixture. In these mixtures, dNTP and ddNTP concentrations are about ten times lower than in the mixtures recommended by the enzyme manufacturer. After addition of 10 μ L PCR oil and brief centrifugation, tubes are placed in a TR3 Omnigene thermal cycler (Hybaid, Teddington, England, UK) and submitted to the following program: one cycle at 95°C for 2.5 min and then 22 cycles at 95°C for 20 s, 60°C for 5 s and 72°C for 20 s. Of course, the annealing temperature may be modulated according to the melting temperature (T_m) of the primer used.

After addition of 3 μ L stop dye and a 3-min denaturation step at 85°C, 1.8–2 μ L of each sample are loaded on a sequencing polyacrylamide gel, pre-run

Figure 1. Partial sequences of the third exon of the Epstein-Barr virus BNLF1 gene, in strains associated with T lymphoma (L) and with Burkitt's lymphoma (B). Sequencing reactions were performed either directly on the purified PCR fragment (L) or on the double-stranded plasmid after cloning of the PCR fragment in the pGEM-7 vector. Reactions were loaded on a 6% homogeneous polyacrylamide gel and run for 4.5 h in 0.5× TBE buffer. Under these conditions, the readable sequence easily extends to 400 nucleotides from the primer.

in 0.5× Tris borate EDTA (TBE) buffer. After electrophoresis, the gel is fixed for 15 min in a bath of 20% ethanol/10% acetic acid, dried and exposed at room temperature on a Kodak BioMax MR Film (Eastman Kodak, Rochester, NY, USA) for 16 h. The dNTP/ddNTP mixtures are prepared and stored as 10× concentrated solutions that contain 45 μ M dATP, 100 μ M dCTP, 100 μ M 7-deaza-GTP and 100 μ M dTTP; moreover, the 10× dNTP/ddATP, dNTP/ddCTP, dNTP/ddGTP and dNTP/ddTTP mixtures, respectively, contain 2.5 mM ddATP, 1.2 mM ddCTP, 1.8 mM ddGTP and 2 mM ddTTP. Before use, the dNTP/ddNTP mixtures are made 1× in 1× sequencing buffer: 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl, pH 8.8, 7 mM MgSO₄. dNTP and ddNTP were purchased from Pharmacia Biotech (Orsay, France) and Boehringer Mannheim (Meylan, France), respectively. In some cases, artefactual bands across the four lanes are observed, which are clearly sequence specific and dependent on fragment purity. When encountered, this problem is solved by treating the samples with deoxynucleotidyl terminal transferase (TdT) as described by Fawcett and Bartlett (2). At the end of the cycle sequencing program (and before addition of the stop dye), 2 μ L of a solution containing 1 mM each dNTP and 1 U TdT (Life Technologies, Eragry, France) in 2× TdT buffer are added to the reactions that are incubated 30 min at 37°C. In this case, the quantity of stop dye is increased to 4 μ L.

By using this protocol, I routinely read 220–240 nucleotides (and sometimes up to 300), starting at 10–15 nucleotides from the primer, on a single buffer gradient gel (3). However, the sequences may be read up to 400 nucleotides, if the same reactions are loaded on a second gel and run for a longer time (Figure 1).

This protocol strongly improves the efficiency of [α -³⁵S]dATP incorporation, eliminating the most serious drawback of this sequencing technique. The procedure is simple and short, and six series of ten templates may be performed easily in one day, provided the capacity of the thermal cyclor is sufficient. The technique is reliable. When sequencing the BNLF1 gene of differ-

ent Epstein-Barr virus strains whose sequences were already determined, the sequences obtained with this protocol were always identical to those already published.

This protocol is also universal. I have used it without modification for the sequencing of double-stranded PCR fragments in the range between 165 bp and 4.3 kb and of double-stranded plasmids prepared either by centrifugation on CsCl gradients or by the "miniprep" technique of Del Sal et al. (1). Finally, this protocol provides good quality sequences at an appreciably lower cost due to the reduction in nonradioactive and labeled nucleotide concentrations and the use of the relatively inexpensive and efficient Deep Vent_R (exo⁻) DNA polymerase.

REFERENCES

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