BENCHMARKS

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

Manual DNA Sequencing Using Fluorescent-Labeled Primers and a Fluorescence Scanner

BioTechniques 27:646-648 (October 1999)

DNA sequence information is required for many routine applications of current molecular biology. Although sequence data may be generated quickly and efficiently using dedicated automated DNA sequencers, many small laboratories do not have the sequencing demands or finances to justify their purchase. For instance, a primary objective of the U.S. Fish and Wildlife Service's Fish Genetics Laboratory (Anchorage, AK, USA) is to determine the population structure of fish on micro- and macro-geographic scales. Allele frequency data are generated by determining size differences of polymerase chain reaction (PCR) amplification products from microsatellite or other polymorphic loci (1). Therefore, our primary requirement is the ability to size thousands of amplification products in a short period of time. Secondary to this requirement is the need to readily generate DNA sequence data to develop primer sets for novel loci. The FMBIO®II Fluorescent Image Scanner (Hitachi Instruments, San Jose, CA, USA) was determined to be the preferred equipment to fulfill these needs.

The FMBIO II came with software for high-resolution gel scanning and for DNA analysis, but DNA sequencing reaction conditions had not been defined. Initially, we followed the manufacturer's protocol for cycle sequencing using the Sequitherm® EXCEL II DNA Sequencing Kit (Epicentre Technologies, Madison, WI, USA). Rather than the ^{32}P-labeled primers the protocol was originally developed for, HEX-labeled primers were used. Although sequencing products were detected, they were not of sufficient intensity to be useful. Therefore, we varied the amount of DNA and HEX-labeled primer in the reactions and defined conditions (Table 1) that produced sufficient fluorescent signal to routinely and reproducibly sequence double-stranded DNA templates (Figure 1).

Table 1. Protocol for Manual DNA Sequencing Using End-Labeled Primers Tagged with the Fluorescent Dye, HEX

1. **DNA Isolation.** The DNA fragments to be sequenced had been ligated into the *Bam*HI site of pBS-SKII (Stratagene, La Jolla, CA, USA) and transformed into *Escherichia coli* strain JM105. Single isolated colonies were inoculated into 3 mL of LB medium supplemented with 100 μg/mL of ampicillin and grown overnight (ca. 16 h) at 37°C, 225 rpm. Plasmid DNA was purified using the Wizard® *Plus* Miniprep DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's recommended protocol and was eluted from the resin with 50 μL TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).
2. **Primers and DNA Sequence Reactions.** The SK and KS primers (Stratagene) were synthesized commercially with a HEX-label at the 5′ end. HEX-labeled M13 forward primer was used to read sequence close to the cloning site on the SK-primed side of the inserts. Sequitherm EXCEL II cycle sequencing kits were used according to the manufacturer's recommendations for ^{32}P end-labeled primers with minor modifications: (*i*) the amount of labeled primer was increased to 30 pmol per reaction; (*ii*) the amount of DNA was increased to approximately 200–300 ng (100–150 fmol) per reaction; and (*iii*) the ddNTP termination mixes were mixed 1:9, long read:standard.
3. **Cycle Sequence Regimen.** One cycle at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 56°C for 30 s, 70°C for 60 s and finally 1 cycle at 70°C for 5 min. More recent experiments (data not shown) indicate that annealing at 52°C (or lower) can provide higher sensitivity. The reactions were run under mineral oil in polypropylene 96-well plates using a RoboCycler® Gradient Temperature Cycler (Stratagene).
4. **Gel Electrophoresis and Scanning.** Five microliters stop/loading buffer (99.5% formamide, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 2 mg/mL orange G or 1 mg/mL BPB[a]) were added per reaction and mixed well. After heating (95°C BPB[a] for 2 min) and quick cooling on wet ice, 5-μL aliquots were electrophoresed through 6% polyacrylamide (19:1, 5%C; Bio-Rad, Hercules, CA, USA) gels (0.4 mm × 25.5 cm × 32 cm) cast in 1× TBE. The gels were preheated for 1 h at 60 W (constant power) before loading. Following an initial short run of 1 h 30 min, the lower ca. 2/3 of the gel was scanned using the FMBIO II with ReadImage® 1.1 software (Hitachi Software Engineering America Ltd., South San Francisco, CA, USA) and a 585-nm emission filter. The gel was then placed back in the electrophoresis unit with fresh buffer in both upper and lower reservoirs, run at 60 W for another 2–3 h and scanned again. The digitized images were opened, and the DNA sequences were analyzed using FMBIO Analysis software version 8.0 (Hitachi Software Engineering America Ltd.).

[a]See text regarding the use of tracking dyes.

In addition to the many advantages of not working with radioactivity (e.g., exposure and waste disposal), there are other benefits of fluorescence-based sequencing technology (2). Data acquisition was relatively rapid (ca. 1 h to scan a gel) and "overexposed" or "underexposed" areas of the gel were read by adjusting the gray-scale correction of the computerized image (Figure 1). Also, a single gel was run and scanned multiple times, thereby providing longer reads of sequence from a single aliquot of sequencing reaction than would otherwise have been possible (Figure 1). This was feasible because the gel did not have to be disassembled to be scanned. After electrophoresis was complete, the gel was removed from the electrophoresis unit, and the plates were rinsed and cooled under a stream of water, wiped dry and scanned. Attempts to reuse the buffers for subsequent runs resulted in distorted and poorly resolved DNA bands. However, if the used buffer in both reservoirs was discarded and replaced with new buffer, the bands remained sharp and well resolved after further electrophoresis (Figure 1).

The stop/loading buffer provided with the sequencing kit was not used because fluorescence of the xylene cyanol would have obscured part of the DNA sequence. Therefore, we made a similar loading buffer that lacked xylene cyanol but still contained bromophenol blue (BPB) as a tracking dye. Although the BPB fluoresced, it migrated faster than any DNA bands of interest, and so this buffer was used routinely. However, intermittent background at approximately 145–170 nucleotides (nt) from the primer (Figure 1) were encountered. Individual components of the stop buffer were electrophoresed and scanned (data not shown) to determine the cause. The data indicated that a contaminant(s) or degradation product(s) of BPB was responsible for the background. A comparison of BPB from several sources (data not shown) suggested that the BPB in the stop buffer was degrading, and the degradation product(s) migrated slower but still fluoresced. To overcome this problem, we evaluated the use of orange G as a tracking dye and determined, at least in initial experiments, that it appeared to be a suitable substitute.

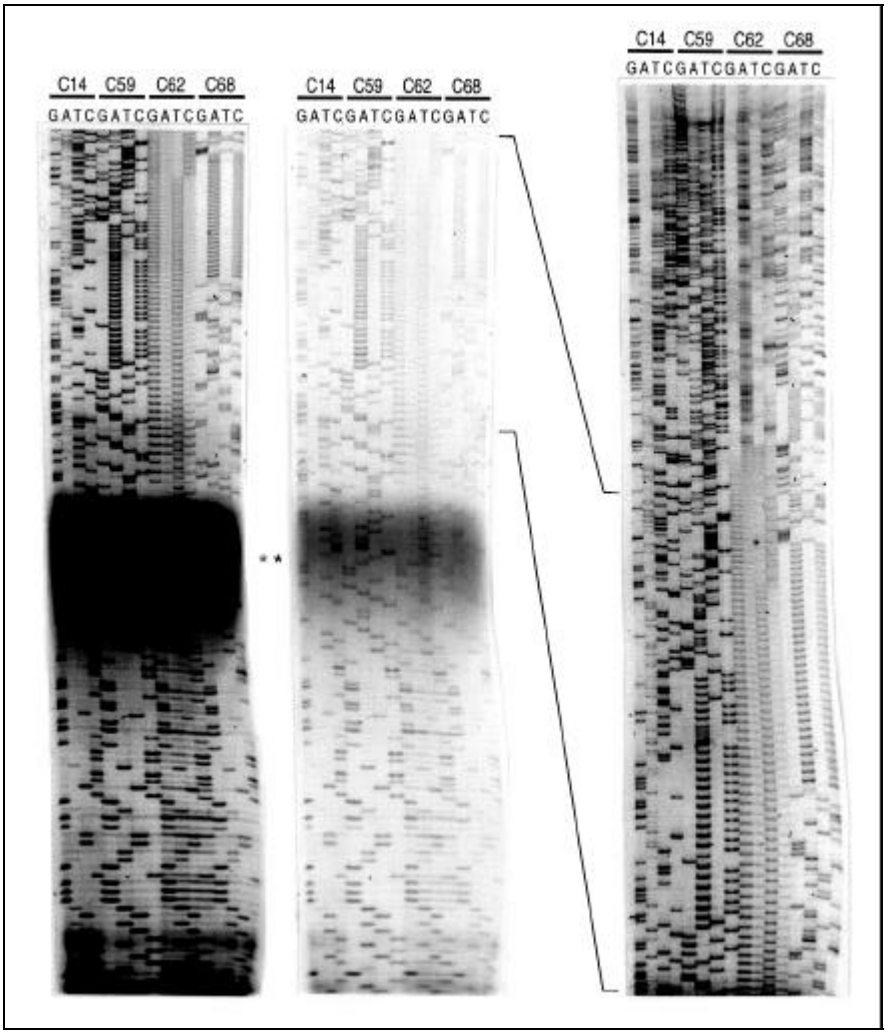

Figure 1. DNA sequence gel images. Four cloned DNA fragments were sequenced according to the accompanying protocol. The left and center panels are the same image viewed with different gray-scale adjustments to allow visualization of fainter bands (e.g., the "A" lane of clone C14) and to allow resolution of bands masked by the diffuse dark band (**). The latter is apparently due to degradation of the tracking dye, BPB (see text). The right panel shows the same gel after running for approximately 2 additional h and re-scanning.

Budgetary constraints seldom allow a researcher to purchase the ideal equipment for every experimental application. Whereas we purchased the FMBIO II fluorescent scanner primarily to scan images for fragment-length polymorphism analysis, we also needed to conduct DNA sequence analysis for PCR primer development. To take advantage of the versatility of the FMBIO II fluorescent scanner, we defined DNA sequence reaction conditions that produce sufficient quantities of sequencing products to be readily detectable by the fluorescent image scanner. Gels were electrophoresed and scanned multiple times, which allowed for longer reads of DNA sequence. Using our apparatus, two scans usually resulted in 350–400 bases of readable sequence with an overlap of approximately 50 bases. The resolving power of the polyacrylamide gel electrophoresis (PAGE) system appeared to be the primary limitation in sequence-read length with fluorescent-labeled DNA. The high sensitivity of detection associated with fluorescence technology required the use of an alternative tracking dye to avoid background problems.

This protocol is adequate for small sequencing projects, but should be viewed as a starting point to further optimize conditions for larger scale projects.

REFERENCES

1. **Bentzen, P., A.S. Harris and J.M. Wright.** 1991. Cloning of hypervariable minisatellite and simple sequence microsatellite repeats for DNA fingerprinting of important species of salmonids and tilapias, p. 243-262. *In* T. Burke, G. Dolf, A.J. Jeffreys and R. Wolff (Eds.), DNA FingerPrinting: Approaches and Applications. Birkhaeuser Verlag, Basel, Switzerland.
2. **Griffin, H.G. and A.M. Griffin.** 1993. DNA sequencing. Recent innovations and future trends. Appl. Biochem. Biotechnol. *38*:147-159.

This work was supported by a grant from the U.S. Fish and Wildlife Service's Fisheries Stewardship Program. The mention of trade names or commercial products in this report does not constitute endorsement or recommendation for use by the federal government. *Address correspondence to, Dr. Wallace G. Buchholz, U.S. Fish and Wildlife Service, 1011 E. Tudor Rd., Anchorage, AK 99503, USA. Internet: wally_buchholz@fws.gov*

Received 19 April 1999; accepted 12 July 1999.

Wallace G. Buchholz, Steve J. Miller and William J. Spearman
U.S. Fish and Wildlife Service
Anchorage, AK, USA

Sequencing Errors in Reactions Using Labeled Terminators

BioTechniques 27:648-650 (October 1999)

Premature chain termination is a common problem in sequencing reactions and can result in multiple co-migrating bands that make accurate sequence determination difficult. The use of labeled dideoxynucleotides is generally assumed to alleviate this problem because only dideoxynucleotide-terminated chains are detected (1). However, we have found that under some circumstances troublesome artifacts can still be seen when this class of terminators is used. In particular, we have observed such artifacts in sequencing G+C-rich templates when dITP is used in place of dGTP.

Templates that contain G+C-rich regions are prone to compression artifacts in standard reactions containing dGTP. These compressions are due to the formation of secondary structures on the nascent strand. The most stable, and therefore the most troublesome, structures are those that involve a number of G·C or G·G pairs. Figure 1 shows an example of compressions seen in standard reactions with the Thermo Sequenase™ Radiolabeled Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Deoxyinosine triphosphate, an analog of dGTP, is commonly used to relieve compressions. Since dITP lacks an N^2 amino group, it forms a less stable bond with cytosines and other guanines. Incorporation of inosine destabilizes the secondary structures that can form in the nascent DNA strand, thereby reducing compression artifacts. The use of the dITP master mixture, supplied with the Thermo Sequenase kit, eliminated the compressions but also produced a number of instances of two co-migrating bands terminated by a different dideoxynucleotide, (Figure 1, 1–9).

Because these bands are not seen when dGTP is used, nor are they seen at every position, they do not result from the presence of incomplete synthesis products in the primer. Most of the spurious products are not seen when a more distal primer is used (Figure 1, primer 2). Therefore, the sequencing artifacts observed with dITP and primer 1 probably do not result from a mixture of sequences, a problem associated with the preparation of the

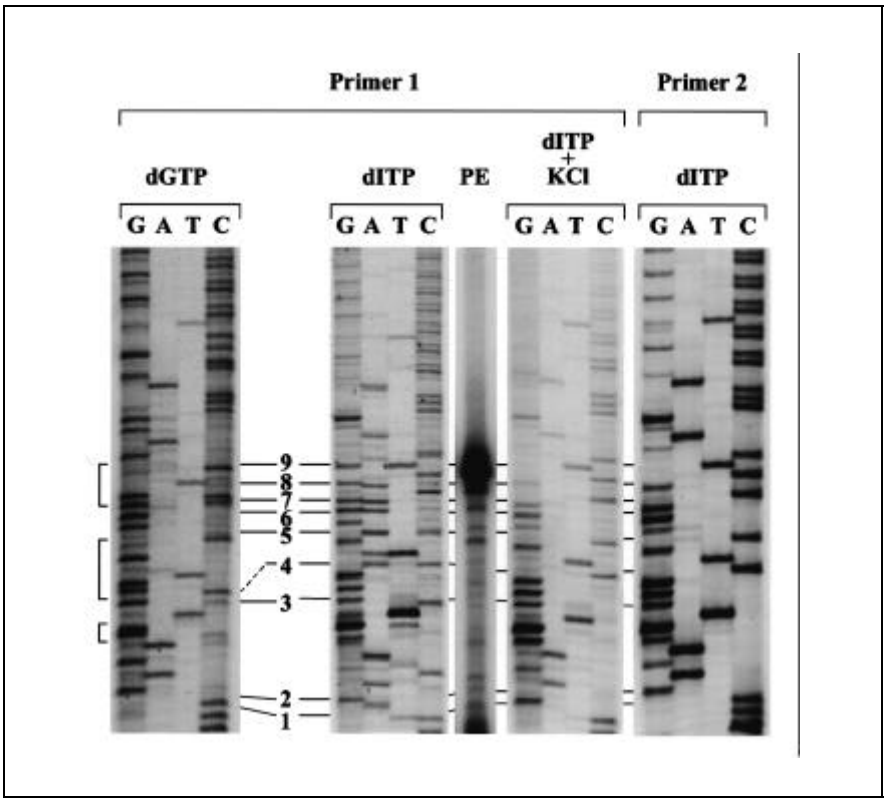

Figure 1. Sequencing artifacts in cycle sequencing reactions containing radiolabeled terminators and dITP. Sequencing of a PCR fragment was carried out using the Thermo Sequenase radiolabeled terminator cycle sequencing kit using the conditions and buffers provided by the supplier, either without further modification or after the addition of 50 mM KCl. The brackets on the left-hand side of the figure indicate the region of compressions seen when dGTP is used. The solid lines numbered 1–9 indicate the location of the spurious products seen when dITP and primer 1 are used. The 5′ end of primer 1 is located 40 bases 5′ of the spurious product labeled 1, while the 5′ end of primer 2 is 68 bases 5′ of this product.