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gene expression experiments. Currently, such measurements are taken relative to a control sample (1). However, the problem with this approach is that there are no controls for the probes/genes when they are not expressed in the control sample, which is usually the case. By using internal control samples (e.g., prepared from the DNA used for spotting), defined internal controls can be constructed for all the probes used.

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Multicolor Post-PCR Labeling of DNA Fragments with Fluorescent ddNTPs

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A capillary-based DNA sequencer with multicolor fluorescence detection has high resolution, a broad dynamic range of signal detection, and is capable of automated operation and direct computer-aided data acquisition/handling. Thus, the system is increasingly used for high-throughput fragment analyses other than sequencing, such as microsatellite genotyping and quantitative single-stranded conformational polymorphism (SSCP) analysis.

The DNA fragments to be analyzed by the system, usually PCR products, must be fluorescent. A commonly employed method for labeling the fragments is to amplify the target fragments using fluorescently labeled primers. However, the synthesis of such primers is at least several-fold more expensive than the synthesis of unmodified primers, and the reduction of this cost is a considerable benefit for projects that involve the examination of a large number of fragments.

PCR products can be labeled during amplification by including fluorescent nucleotides in the reaction mixture. Internally labeled fragments are unsuitable for analyses that require the high resolution of fragments because the positions and number of incorporated labeled nucleotides vary from molecule to molecule, and the fragments are chemically heterogeneous (2,4). The products can also be labeled by amplification using primers with 5' tags, followed by the re-amplification of the products using common fluorescent primers that anneal to the tags. This method requires lengthy primers for each target sequence, and the fidelity of amplification is uncertain because of the increased number of primers in the amplification reaction and extended thermal cycles.

We previously developed a post-PCR fluorescence-labeling method in which the target sequences were amplified by PCR using unlabeled primers carrying either 5' ATT or 5' GTT. The

3'-end residues of the products were then exchanged with fluorescent dUTP or dCTP by the terminal exchange activity of the Klenow fragment of DNA polymerase I (2). The procedure is suitable for examining loci by SSCP analysis of SNPs (3,7,8) or for multiplexed microsatellite genotyping (5,6).

We attempted to expand the repertoire of fluorophores for labeling, but some [Alexa Fluor[®] and Oregon Green[®] (Molecular Probes, Eugene, OR, USA), Cy (Amersham Biosciences, Piscataway, NJ, USA), Cy3, Fluorescein Chlorotriazinyl, Lissamine, Naphthofluorescein, and Texas Red[®] (Perkin Elmer Life Sciences, Boston, MA, USA)] were poor substrates for the labeling, as examined by the previously described method (unpublished data and Reference 2). This is presumably because the nucleotides modified with the fluorophores were inefficient polymerization substrates, or the ends with modified nucleotides were too sensitive to the 3' exonuclease activity of the enzyme. We then used fluorescent ddNTPs, which behave differently from their deoxy counterparts, as substrates for various DNA polymerases (1). The Klenow fragment of DNA polymerase I had a very low labeling efficiency (approximately 2%) compared to fluorescent dNTP labeling using the same enzyme, which was expected because the fluorescent ddNTPs are known to be poor substrates for the enzyme.

Genetically modified T7 DNA polymerase or *Taq* DNA polymerase efficiently incorporate ddNTPs (9-11) and are commercially available as enzymes for sequencing by the dideoxy-terminator method. However, these enzymes alone are unsuitable for our end-labeling purposes because they possess weak or no 3' exonucleolytic activity, which is essential for the terminal exchange reaction. Therefore, we used a cocktail of two enzymes, one for end-cleavage and the other for end-filling.

We tested the Klenow fragment of the DNA polymerase I or the T4 DNA polymerase (New England Biolabs, Beverly, MA, USA) as the enzymes for end-cleavage and either Thermo Sequenase[™] (Amersham Biosciences) or Sequenase (version 2.0; USB, Cleveland, OH, USA) for end-filling. The fluorescent nucleotides *N,N'*-diethyl-2',7'-

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dimethyl-6-carboxyrhodamine-ddCTP (R6G-ddCTP) and 6-carboxyrhodamine-ddUTP (R110-ddUTP) were used for the labeling (Perkin Elmer Life Sciences). The products were examined by capillary electrophoresis (CE) using denaturing or SSCP conditions. The best results (i.e., high-fluorescence peaks without spurious peaks)

were obtained with the combination of the Klenow fragment of DNA polymerase I and Thermo Sequenase, while with either of the two enzymes, the labeling efficiency was reduced to approximately one-fiftieth of the combination (Figure 1).

The reaction conditions were further optimized by changing the concentra-

tions of various agents, including fluorescent ddNTPs. The two enzymes have widely different optimal temperatures, but reproducible labeling was observed at 50°C–60°C. We reached the optimized method below in which the target genomic segments are amplified, fluorescently labeled, and analyzed by a CE under denaturing or SSCP conditions. The reduction of the concentration of fluorescent ddNTPs from 1 to 0.2 μM in the labeling reaction resulted in only a moderate decrease of the labeled products (approximately 50%) (Figure 1). Each fluorophore was incorporated in only one strand (Figure 2), which confirmed the specific labeling of the intended strands. The labeling was also successful using TAMRA-ddCTP and ROX-ddUTP in the same concentration range (data not shown). The lowest concentration (0.2 μM) of the fluorescent ddNTPs were still more than 10-fold molar excess of the PCR products in the reaction mixture, and the reduction of the incorporation rate appears to be because of kinetic reasons. We have not attempted to further reduce the concentration of the ddNTPs because the cost for the labeling is sufficiently low for most experiments.

The post-PCR fluorescence-labeling methods mediated by the 3' exchange reaction of DNA polymerases have the advantage of not requiring costly fluorescent primers to obtain DNA fragments suitable for multiplexed or high-throughput analyses by CE with multicolor fluorescence detection. A comparison of the cost of the two methods is not simple because of various

Figure 1. Post-PCR-labeled products with fluorescent dNTPs or ddNTPs. A sequence-tagged site (dbSTS GenBank® accession no. G43316; 106 bp) was amplified using primers 5'-ATTCTGCAAT-CACAAAAGTGA-3' and 5'-GTTCTGATAAAAGGAAGTTGCTAGTG-3' (underlined sequences were added for post-labeling purposes). Fluorescent nucleotides and enzymes used in the labeling are as indicated in each panel. Labeling with fluorescent dNTPs was performed as previously described (2,3). Each labeled product was electrokinetically injected into the capillary at 43 kV/cm for 5 s and run at 319 kV/cm under denaturing conditions (see text for details). Blue and red peaks indicate the fluorescence of R110 and R6G, respectively. Black peaks indicate the TAMRA-labeled internal marker. The horizontal scale represents nucleotide size. The vertical scale represents fluorescence intensity in arbitrary units. K, Klenow fragment; KTS, mixture of Klenow fragment and Thermo Sequenase.

Figure 2. SSCP analysis of post-PCR fluorescence-labeled DNA fragments. Sequence-tagged sites containing SNPs were amplified from homozygotes and heterozygotes, and then labeled with 0.2 μM R110-ddUTP and 0.2 μM R6G-ddCTP. Each labeled product was electrokinetically injected into capillary at 319 kV/cm for 5 s and run at 319 kV/cm under SSCP conditions, as described in the text. (A) Sequence-tagged site G43316 (see Figure 1) containing an SNP (dbSNP_ID rs3469). (B) Sequence-tagged site KG00B0454 containing an SNP, which is located in the upstream region of human PEX12 gene, and amplified using 5'-ATTGTGTTCAAGGATGGAGACCA-3' and 5'-GTTTCGGTGGGAGGAAGGGAATCT-3'; 323 bp. The horizontal scale represents data points. The vertical scale represents fluorescence intensity in arbitrary units.

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pricing systems of the providers of the reagents. The cost also depends on how much of the primers are consumed per PCR, which is widely variable by experimental design. However, the post-labeling method is less costly if the number of PCRs performed per primer set is no more than 200–300 in the method shown below and if the pricing systems available in the US are assumed. The labeled products are homogeneously blunt-ended, as opposed to the products of *Taq* DNA polymerase-based PCR amplification using fluorescent primers, the ends of which are often heterogeneous because of the 3' terminal transferase activity of the enzyme. Homogeneity of the molecule is essential for high-resolution analysis (e.g., microsatellite genotyping or SNP detection by SSCP). Compared to the previous post-PCR fluorescence-labeling method using fluorescent dNTPs, this method using fluorescent ddNTPs requires an additional enzyme, Thermo Sequenase. The total cost for the labeling is similar because the consumption of fluorescent nucleotides is reduced using our method. The commercial availability of a wide spectrum of fluorescent ddNTPs ensures the usage of the method described here in various multicolor DNA fragment analyses by CE and overcomes current limitations in the supply of fluorescent dNTPs.

PCR was performed in a 10- μ L reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂, 0.25 μ M each primer that carries either 5' ATT or 5' GTT, 200 μ M each of the four dNTPs, 50 ng genomic DNA, and 0.25 U AmpliTaq[®] DNA polymerase (Applied Biosystems) premixed with 55 ng TaqStart[™] Antibody (BD Biosciences Clontech, Palo Alto, CA, USA). To amplify fragments with a high GC content, 5% DMSO was added. The thermal cycling profile was 1 min at 94°C for initial heating, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C–60°C, and 1 min at 72°C.

The PCR product was diluted with an equal volume of distilled water, and a 2- μ L aliquot was added to 4 μ L post-PCR-labeling reaction buffer [15 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, 0.3–1.5 μ M each of R110-ddUTP and R6G-ddCTP, 0.075 U/ μ L Klenow fragment, and 0.075 U/ μ L Thermo Sequenase

DNA Polymerase with *Thermoplasma acidophilum* Inorganic Pyrophosphatase (Amersham Biosciences)]. The mixture was incubated at 37°C for 5 min, followed by 57°C for 15 min. The reaction was then stopped by the addition of 6 μ L 20 mM EDTA.

Unincorporated fluorescent nucleotides were removed by the addition of 1 μ L 2 U/ μ L calf intestine alkaline phosphatase (CIP) (Roche Diagnostics, Mannheim, Germany), followed by incubation at 37°C for 30 min. Alternatively, the labeled PCR product was purified from 12 μ L reaction mixture by gel-filtration using Sephadex[™]-G50 (Amersham Biosciences) and Multi-Screen[®]-HV plate (Millipore, Bedford, MA, USA), according to the manufacturer's instructions.

One microliter of the solution treated by CIP or the eluate of gel-filtration was mixed with 0.5 μ L GeneScan[®]-500 TAMRA size standard (Applied Biosystems) and 13.5 μ L 0.5 mM EDTA (final concentration, 1.1–1.8 mM). After heating at 90°C for 2 min, a mixture of the sample and marker was loaded onto an ABI PRISM[®] 310 Genetic Analyzer equipped with a 47-cm capillary (Applied Biosystems). CE was performed under denaturing conditions at 60°C with POP-4[™] polymer (Applied Biosystems) or under SSCP conditions at 30°C with 10% polydimethylacrylamide buffered with 60 mM Tris, pH 6.8, 70 mM MES, 2 mM EDTA, as described previously (7). The resulting electropherogram was analyzed using GeneScan Analysis Software (Applied Biosystems).

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