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Efficient Detection of DNA Polymorphisms by Fluorescent RAPD Analysis


ABSTRACT

A method is presented for the analysis of fluorescently labeled random amplified polymorphic DNA (FRAPD) fragments. A DNA sequencer and collection and analysis software were used to estimate the sizes of DNA fragments based on their mobilities relative to in-lane size markers. This allowed confident identification and comparison of FRAPD markers both within and between polycrylamide gels. In comparison with analysis of RAPD products using ethidium bromide-stained agarose gels, fluorescent analysis improved the sensitivity, resolution and precision of sizing of RAPD products of about 50–2100 bp. FRAPD fragments produced from amplification of zebrafish DNA are informative as genetic markers that segregate with Mendelian inheritance. FRAPD analysis was found to be very efficient for identifying new DNA polymorphisms.

INTRODUCTION

Detection of sequence differences in DNA samples is useful for many applications including positional cloning, the generation of high-resolution genetic maps and inheritance analysis. Random amplified polymorphic DNA (RAPD) allows the generation of many potentially polymorphic fragments from a single short primer (usually a decamer) of arbitrary sequence in a polymerase chain reaction (PCR). These fragments are usually detected using agarose gel electrophoresis and ethidium bromide (EtBr) staining (7,10). We present a technique using polycrylamide gel electrophoresis (PAGE), fluorescence detection and in-lane size markers that increases the sensitivity and reliability of identification of RAPD products.

The Model 373A DNA Sequencer and GENESCAN™ 672 collection and analysis programs (both from PE
Applied Biosystems, Foster City, CA, USA) are routinely used for analysis of polymorphic microsatellites and other simple sequence repeats (SSR) (5). However, we are unaware of any reports of their application to the analysis of RAPD products. Analysis of fluorescent RAPD (FRAPD) products introduces some technical difficulties compared to analysis of microsatellites, since FRAPD fragments are fluorescently labeled on both ends, and some are larger than fragments normally used for microsatellite analysis. We found it difficult to discern if two closely spaced bands on a denaturing polyacrylamide gel represented two different FRAPD fragments or two strands of the same fragment resulting from single-strand conformation polymorphism. Thus, we

![Figure 1. Anomalous mobility of the GENESCAN-2500 ROX in-lane size standards. (A) GENESCAN electropherogram of size markers separated on a 4% native polyacrylamide gel under the conditions described in Materials and Methods. The x axis indicates mobility (scan number; scans every 6 s). The y axis indicates arbitrary units of fluorescence. Sizes indicated above peaks were assigned according to PE Applied Biosystems (2). (B) Plot of peak sizes assigned by PE Applied Biosystems against scan number, which shows the anomalous mobility of the 508-bp marker.](image)

![Figure 2. Comparison of methods for analysis of RAPD products. Two separate PCRs were prepared using the same template DNA and fluorescent primer. An aliquot of each reaction was analyzed on a 1.8% agarose gel with EtBr staining (Agarose Technique Panel) and on a 4% native polyacrylamide gel using fluorescence detection (Fluorescent Technique Panel). Agarose Technique Panel: Agarose gel and densitometric comparisons of the two samples in lanes 2 and 24; the densitometric plots are shown as output from the GelReader. The gel had 26 lanes, with 230 ng of 100-bp ladder (Life Technologies, Gaithersburg, MD, USA) loaded in lanes 1, 12, 17 and 26; the bright band is the 600-bp marker. Lane 25 had a different PCR sample to provide spacing. Fluorescent Technique Panel: Traces shown are output from the GENESCAN Analysis program. The asterisk (*) indicates a corresponding region of the agarose and polyacrylamide gels. The single peak on the agarose gel was resolved into 7 peaks by GENESCAN.](image)
used native conditions for electrophoresis. We present methods, including an adjustment of the size standards, that result in reproducible and sensitive detection and precise identification of FRAPD products.

METHODS AND MATERIALS

PCR and Gel Conditions

Tissue samples were collected from zebrafish (*Danio rerio*), and the DNA was prepared by phenol/chloroform extraction. Quantification of DNA was done by spectrophotometry using PicoGreen® (Molecular Probes, Eugene, OR, USA). We synthesized and fluorescently labeled the RAPD decamer oligonucleotide primers; 6-FAM (PE Applied Biosystems) was attached to the 5’ end (1). For simplicity, we focus on results obtained using a single RAPD primer (sequence of RAPD primer No. 210 produced by the Nucleic Acid-Protein Service Unit, University of British Columbia, Vancouver, BC, Canada).

For RAPD amplifications, 4 ng of each template DNA were suspended in PCR cocktail containing 3 mM MgCl₂, 50 mM Tris-HCl, pH 8.3, 0.25 mg/mL crystalline bovine serum albumin, 100 µM of each dNTP (Pharmacia Biotech, Uppsala, Sweden), 0.06 U/µL of Taq DNA Polymerase (in storage buffer B; Promega, Madison, WI, USA) and 1.6 µM of a fluorescently labeled (6-FAM) RAPD decamer primer. Thermal cycling was performed in heat-sealed glass capillary tubes containing a total volume of 10 µL using a Model 1605 Air Thermo-Cycler™ (Idaho Technologies, Idaho Falls, ID, USA). Two cycles of 91°C for 60 s, 42°C for 7 s and 72°C for 70 s were followed by 38 cycles of 91°C for 1 s, 42°C for 7 s and 72°C for 70 s, which was followed by a 3-min hold at 72°C.

For each FRAPD reaction, an 8-µL aliquot was loaded onto a 1.8% agarose gel (Bio-Rad, Hercules, CA, USA) in 0.5× TBE buffer (0.09 M Tris base, 0.09 M boric acid, 0.002 M EDTA) containing 0.5 µg/µL of EdBr. DNA products were visualized with a 300-nm transilluminator, and the images were digitized using a Gel Documentation System (Ultraviolet Products, San Gabriel, CA, USA). Digitized images were analyzed using the GelReader program, Macintosh® Version 2.0.5 (National Center for Supercomputing Applications, University of Illinois at Urbana-Champaign). The GelReader program compensates for variability among lanes by using isomolecular weight lines connecting DNA size standards in multiple lanes.

An aliquot of each FRAPD reaction was diluted 5 times, and 2 µL of this solution were mixed with 3 µL of agarose loading buffer containing 4 fmol of fluorescent size standards (Model GS-2500 ROX; PE Applied Biosystems). This mixture (5 µL/well) was loaded onto a 4% native polyacrylamide gel (Bio-Rad Catalog No. 161-0144) in TBE and electrophoresed on the Model 373A DNA Sequencer. Data were collected using the GENESCAN Collection Software (Version 1.1) and analyzed with the GENESCAN PCR Analysis Software (Version 1.2.2-1). RAPD markers are dominant markers and were scored as either present or absent; this was facilitated by the low baseline observed. Markers were named according to the convention adopted for the zebrafish RAPD linkage map (4,8).

Reassignment of Size-Standard Values

The GENESCAN program identifies and sizes peaks based on internal algorithms and specifications entered by the user, including a list of sizes to be assigned to the in-lane size standards. Initial separations of FRAPD fragments, performed according to PE Applied Biosystems specifications and in collaboration with the PE Applied Biosystems Core Facility, resulted in only some of the 24 lanes per gel being analyzable. The lanes could have been analyzed separately by changing user-defined specifications, but this would have prevented comparisons of data from lane to lane within a gel, undermining confident peak identification. The problem was associated with the anomalous mobilities observed for some of the fragments of the size standards, as shown in Figure 1.

The peaks of the size standards, fragments of λ phage DNA fluorescently end-labeled with ROX, corresponded to PE Applied Biosystems specifications (Reference 2, and insert supplied with the GENESCAN-2500 ROX kit): 55, 112, 127, 134, 190, 204, 251, 256, 287, 304, 379, 488, 508, 554, 845.
1133, 1199, 1740 and 2026 bp. Using the SIZE program (6), adapted to accept input of mobility as scan number, we reassigned size values to these peaks based on the best fit curve of peak size plotted against scan number: 58, 112, 125, 132, 187, 202, 237, 249, 254, 290, 308, 378, 494, 553, 856, 1128, 1194, 1741 and 2100 bp. Note that, as shown in Figure 1, the 508 and 554 peaks are poorly resolved and have been reassigned a single value of 553. These reassigned size-standard values were used in all subsequent analyses with the GENESCAN software and allowed reproducible assignment of sizes for all 24 lanes in the range from approximately 50 to 2100 bp.

RESULTS AND DISCUSSION

In Figure 2, products from two separate PCR amplifications using the same primer-template combination were compared after separation on agarose and polyacrylamide gels. The number of markers detected by each method is an indication of sensitivity. Agarose analysis detected 21 bands, and FRAPD analysis detected at least 43 products. The sensitivity of detection of FRAPD fragments (2 fluorescent labels/fragment) is constant over the size range of fragments in comparison to agarose analysis, where size of fragment influences intensity of fluorescent signal. Thus, the sensitivity of detecting small fluorescent DNA fragments using the fluorescence method is especially improved compared to EtBr staining. What appeared as a single RAPD product on the agarose gel (sized as 336 bp by the GelReader program in lane 2) was resolved as seven products using fluorescence analysis. Improper identification of two or more markers as a single marker can confuse interpretation of inheritance data.

Identification of RAPD markers is based on their sizes calculated from their mobilities relative to size standards. Replicates of the same primer-template combination were analyzed on different, widely separated lanes of the same gel (Figure 2). The coefficient of variation (12) was less for GENESCAN-calculated sizes (0.07%) than for GelReader-calculated sizes.
(2.09%). For a given template-primer combination, even when FRAPD reactions were performed several days apart and analyzed on different polyacrylamide gels, all products were assigned reproducible sizes that varied by less than 0.22% and showed similar peak heights relative to other peaks (data not shown). Thus, corresponding FRAPD peaks could be identified with confidence between gels.

Although the densitometric scans of replicates run on an agarose gel are similar, the peaks did not coincide precisely nor were they sized precisely when compared with size markers in nearby lanes (e.g., the peak sized at 1061 and 1081 bp in different lanes in Figure 2). When the template is the same, it is easy to identify corresponding markers by counting peaks. However, this is less reliable when comparing RAPD products amplified from different DNA samples that do not share all markers. FRAPD analysis allows more confident identification of markers in comparing different DNA samples.

Inheritance analysis requires reproducible detection and confident identification of markers from different DNA samples, an example of which is shown in Figure 3. In extensive pedigree analyses on zebrafish (Reference 4; our unpublished observations), FRAPD markers were found to be informative for inheritance analysis for the following reasons: (i) they are highly reproducible; (ii) all scorable markers observed in progeny to date have been observed in one or both parents; (iii) segregation of markers is consistent with Mendelian inheritance according to Chi-square analysis; and (iv) of 15 polymorphic FRAPD markers analyzed for segregation, none was found to be closely linked, indicating that they are not length variants of the same locus and that these markers appear to be randomly distributed throughout the genome of zebrafish. Peaks for homozygous markers are consistently larger than for heterozygotes. For example, in Figure 3, two parental-specific markers (210bcf.452 and 210bcf.799) were both apparently homozygous in that parent since they were present in all 12 progeny tested, but as smaller peaks.

RAPD technology has sometimes been criticized for a lack of reproducibility and the generation of artificial products. Inheritance studies of RAPD markers indicate that both of these reported problems have been mitigated (4,7,8). Reproducibility can be assured by careful quantification of all thermal cycling reaction components, particularly template DNA concentration (10) (we recommend fluorescence rather than UV quantification because impurities have less effect), combined with stringent thermal cycling conditions. A slow ramp speed between the
denaturing and annealing temperatures may allow annealing of noncomplementary template strands to each other, thus resulting in heteroduplex (nonparental) band formation (3, 9). This can be mitigated by a low concentration of DNA template relative to primer, fast ramp speeds (as achieved in capillary tube electrophoresis) and sufficient stringency for primer annealing.

An average of 7.7 polymorphisms per primer were detected using three FRAPD primers applied to 10 pairwise comparisons between fish of the SFU and *AB lines, which are substantially inbred (4). Polymorphic markers were also detected within the SFU line, but less frequently. Other methods for detecting polymorphic DNA markers that can use fluorescent labels include SSR/microsatellite analysis and amplified fragment length polymorphisms (AFLP) (11). The former requires cloning and sequence analysis to create custom primers before being able to scan for new polymorphic markers, while the latter requires several operations besides PCR amplification. FRAPD technology is much simpler to apply in scanning for new polymorphic markers and is more random in sampling the genome than SSR analysis, which we have found to be considerably less efficient (4). While an automated DNA sequencer is expensive to acquire and operate, the cost per sample analyzed can be decreased and throughput increased by the multiplexing of three FRAPD samples per lane. The cost of producing a fluorescent primer is $75–$150 more than for a standard RAPD primer (<$0.04/reaction). The efficiency and precision of detection of new FRAPD polymorphisms may justify the increased cost, especially if kits of fluorescent primers become commercially available.

REFERENCES


2. Applied Biosystems. 1993. Electropherogram of GENESCAN-2500 run under native conditions [Figure 2-4; p. 2-9]. In GENESCAN 672 Software User’s Manual (Sept.). Foster City, CA.


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