IN THE LABORATORY

An economic method for the fluorescent labeling of PCR fragments

A poor man's approach to genotyping for research and high-throughput diagnostics.

Markus Schuelke

Genotyping with microsatellite markers was first described by Litt and Luty, and is now a widely used method in molecular biology[1]. It has a firm place in forensic, diagnostic, and scientific applications where haplotyping is employed in linkage analyses.

Most genotyping is performed by polymerase chain reaction (PCR) with defined oligonucleotide primers. In order to analyze the length of the PCR products by electrophoresis and a laser detection system, one of these primers has to carry a fluorescent dye label, which may be 6-carboxy-fluorescine (FAM), hexachloro-6-carboxy-fluorescine (HEX), 6-carboxy-X-rhodamine (ROX), or tetrachloro-6-carboxy-fluorescine (TET)[2]. These fluorescent dyes are very expensive. Labeling one primer in the 50 nmol range can cost US $100–130, depending on the dye. In order to exclude a certain disease-causing locus by haplotyping, it is necessary to test at least three markers on both the telomeric and centromeric sides of the gene, which adds up to US $600–780 per locus. The cost increases substantially if a greater number of loci have to be tested for fine mapping in a large gene hunt project. Above that, unless ordered by large genotyping centers, these expensive primers are used for only 10–30 reactions, whereas the remainder populate the refrigerator until doomsday.

In order to overcome this financial burden, I have tried to label the primers myself with commercially available materials. The results, however, were inconsistent: a considerable amount of dye was wasted, and cleaning of the labeled primers was only successful with HPLC. Thus, drawbacks in the amount of manual work and materials involved far outweighed the financial benefits. I therefore devised a single-tube, nested PCR method that is easy to handle and yields consistent results.

Basics of the approach

Figure 1 shows the general outline of the procedure for fluorescent dye labeling of PCR fragments in one reaction, which is performed with three primers: a sequence-specific forward primer with M13(-21) tail at its 5′ end (Fig. 1A), a sequence-specific reverse primer (Fig. 1B), and the universal fluorescent-labeled M13(-21) primer (Fig. 1C). The amount of the forward primer should be less than half of the reverse primer. The thermocycling conditions are chosen such that during the first cycles, the forward primer with its M13(-21) sequence is incorporated into the accumulating PCR products (Fig. 1D). Later, when the forward primer is used up, the annealing temperature is lowered to facilitate annealing of the universal M13(-21) primer (Fig. 1E). Thus, the universal fluorescent-labeled M13(-21) primer "takes over" as the forward primer and incorporates the fluorescent dye into the PCR product (Fig. 1F).

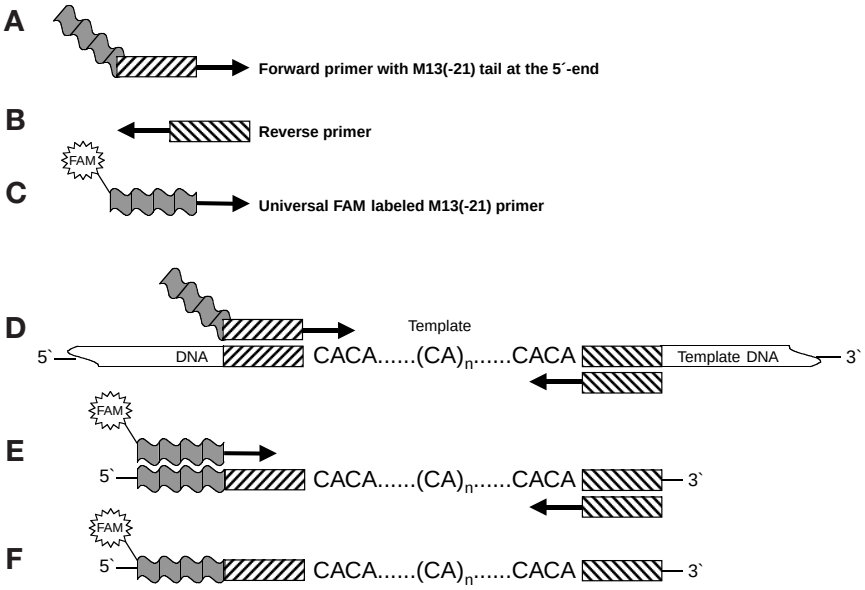

Figure 1. Amplification scheme for the one-tube, single-reaction nested PCR method. (A,B) The hatched boxes indicate the microsatellite-specific primers, (C) the undulating gray box the universal M13(-21) sequence, and the star the fluorescent FAM label. (D) In the first PCR cycles, the forward primer with the M13(-21) tail is incorporated into the PCR products. (E) These products are then the target for the FAM-labeled universal M13(-21) primer, which is incorporated during subsequent cycles at a lower annealing temperature of 53°C. (F) The final labeled product can be analyzed on a laser detection system.

Results

To demonstrate this method, a polymorphic microsatellite marker at the *MaoA* locus[3] was labeled in an informative pedigree (Fig. 2A). Figure 2B depicts the results obtained with the M13(-21) method. The typical stutter peaks can be seen as in the conventional protocol with dye-labeled primers. If referring to the published allelic frequency, one has to subtract from the indicated result 18 bp of the M13(-21) sequence and 1 bp of the A-overhang appended by AmpliTaq DNA polymerase. In family members II.2 and III.1 the DNA quality was bad, thus resulting in low signal strength and an artificial bump in front of the signal proper. The signals, however, can still be clearly outlined and show the expected morphology. This experiment was controlled with a conventionally labeled forward primer (data not shown). The result differed only in that all fragments were 18 bp shorter. The relative differences within the pedigree were reproduced.

Discussion

In this article I describe a reliable single-reaction nested PCR method by which genotyping projects can be performed at a fraction of the cost previously mentioned. All that is necessary is to place a one-time order for a fluorescent dye-labeled M13(-21) primer, and to order the forward primers so that the M13(-21) sequence is appended at its 5′ end. The advantage of the one-time order is that

Markus Schuelke is a scientist and physician in the department of neuropediatrics, Charité University Hospital, Augustenburger Platz 1, D-13353 Berlin, Germany (markus.schuelke@charite.de).

one can choose the best and more expensive energy transfer (ET[4,5]) fluorescent dye primer. The 10-fold improvement in detection sensitivity of these ET primers enables shorter PCR cycles and good results even with minute amounts of template DNA, a feature especially useful in forensic applications.

This protocol cuts the cost by US $540–720 per locus if six microsatellite markers are employed, with no additional manual and pipetting work involved. One single fluorescent dye-labeled primer with the M13(-21) sequence can be used for all fragments. The method can even be multiplexed, if the various PCR products can be size-fractionated sufficiently. Even old, degraded DNA yields enough signal to perform the analysis. For maximum signal strength I have optimized the protocol, and several points have to be kept in mind: (1) Fluorescent-labeled M13(-21) universal primer and reverse primer should be used in equimolar amounts; (2) the forward primer should be used in one-fourth the amount of the reverse primer, so that the M13(-21) universal primer can take over when the forward primer is used up; (3) since the annealing temperature of the fluorescent dye-labeled M13(-21) primer is only 53°C, the last eight PCR cycles should be run with an annealing temperature of 53°C (the same result can be achieved with a touchdown protocol); (4) the final elongation step should not be shorter than 10 min. Otherwise, split peaks by 1 bp may be encountered, due to inhomogeneity of PCR products with only a fraction having the unspecific A-overhang appended by the AmpliTaq DNA polymerase.

In conclusion, the M13(-21) primer genotyping protocol offers an inexpensive alternative to the use of commercially available fluorescent labeled dye primers—an advantage especially for small research groups who perform low-throughput genetic linkage analyses with a high number of microsatellite markers.

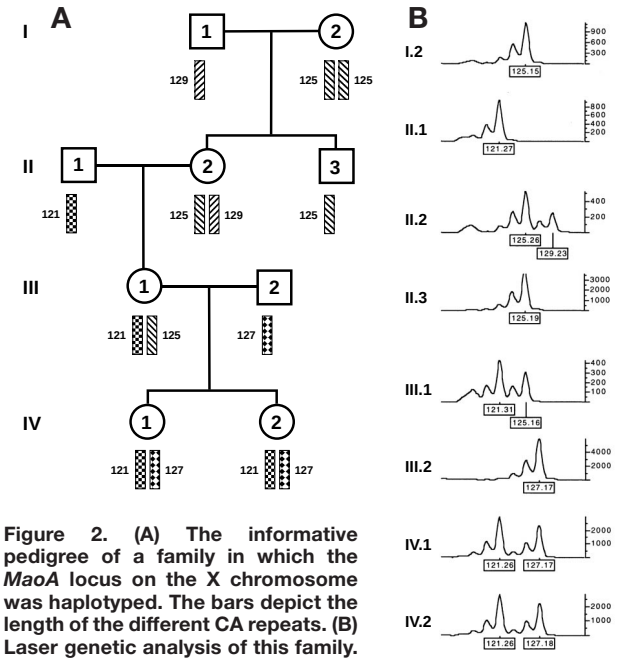

Figure 2. (A) The informative pedigree of a family in which the *MaoA* locus on the X chromosome was haplotyped. The bars depict the length of the different CA repeats. (B) Laser genetic analysis of this family. The relative signal strength is indicated on the right side. In family members II.2 and III.1, the DNA was degraded because of long-time storage. Therefore, the signals are lower and an artifact appears. The signal strength, however, is still sufficient to measure the fragment length confidently.

Methods

In order to test the method in practice, I examined a dinucleotide repeat polymorphism at the *MaoA* locus on the X chromosome (GenBank X55451) in an informative pedigree. The primer sequences of this locus have been published previously[3]. The reverse primer 5′-CAC TAT CTT GTT AGC TCA CT-3′ and the forward primer, a fusion of a leading M13(-21) universal sequence (18 bp) with the originally published primer sequence (19 bp), 5′-TGT AAA ACG ACG GCC AGT AGA GAC TAG ACA AGT TGC A-3′ and a FAM-labeled M13(-21) universal primer: FAM-TGT AAA ACG ACG GCC AGT-3′ were ordered from Life Technologies (Karlsruhe, Germany). The underlining represents the M13(-21) sequence.

The PCR mix contained 8 pmol of each reverse and FAM-M13(-21) primer and 2 pmol of the forward primer in a final 50 µl reaction volume (Perkin-Elmer Standard PCR reaction buffer, 0.2 mM dNTPs, and 50–100 ng template DNA, 1 U AmpliTaq DNA polymerase). Conditions of the PCR amplification are as follows: 94°C (5 min), then 30 cycles at 94°C (30 s) / 56°C (45 s) / 72°C (45 s), followed by 8 cycles 94°C (30 s) / 53°C (45 s) / 72°C (45 s), and a final extension at 72°C for 10 min. Subsequently, 1 µl of the PCR product is added to 22 µl formamide and 0.5 µl ROX standard (Perkin-Elmer) and run on the ABI 310 Prism Genetic Analyzer.

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