An Efficient Multiplex PCR Suitable for Large Scale Typing in Linkage Mapping

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ABSTRACT. The dissection of polygenic traits is made possible with the development of microsatellite markers. Linkage study of this kind involves many markers with tens of hundreds of samples. Although typing essentially contains only two steps: PCR amplification and gel electrophoresis. Such work is still heavy when a large number of samples had to be genotyped. Multiplex PCR may reduce the work, but one has to optimize the conditions from marker to marker. Here we describe a dye-compatible multiplex PCR that works under standardized condition without the need to pre-determine the combinational primer concentration and the time-consuming step to mix many samples with gel loading dye before electrophoresis. This successful protocol should greatly reduce the cost and labor for genetic study of polygenic traits.—KEY WORDS: gel loading dye, linkage mapping, multiplex PCR.

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Microsatellites or simple sequence length polymorphisms (SSLPs) are abundant in the mammalian genome, highly polymorphic among individuals or common laboratory strains of animals, and can be typed by polymerase chain reaction [9, 11]. Linkage maps contained thousands of such markers were developed in mouse and man [4, 5]. In rat, the number of microsatellite markers is rapidly increasing. Genetic dissection of polygenic traits has the advantage of starting from such maps, but the sample size in linkage study is usually large and hence will keep several people to work for several months to scan the whole genome. Multiplex PCR may reduce the work, but one has to optimize the conditions from marker to marker [1, 6–8]. To find a method for fast genotyping, we begin to search for a standardized multiplex condition that works with components of gel loading dyes.

First we tested whether loading dyes were compatible with PCR. Two traditional gel loading dyes were prepared in 6x stock concentration [Loading dye 1 contains 0.25% bromophenol blue (Wako Pure Chemical Industry, Osaka, Japan), 0.25% xylene cyanol FF (Wako Pure Chemical Industry, Osaka, Japan), and 30% glycerol in water; and Loading dye 2 contains 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 15% Ficoll (Mol. Wt. 400,000; Sigma Chemical Co., St. Louis, MO) in water] [10]. PCR was conducted in 10 μl volume, which contained 10 mM Tris-HCl/pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001%(wt/vol) gelatin, 200 μM dNTPs, 0.25 u AmpliTaq Gold™ (Perkin-Elmer Corporation, Foster City, CA), 0.33 μM of primers, 25 ng of genomic DNA, and 1x loading dye (5% glycerol or 2.5% Ficoll with approximately 0.04% Bromophenol blue and 0.04% Xylene cyanol FF). The reaction mixture was covered with 15 μl mineral oil (Sigma Chemical Co., St. Louis, MO) and then thermal-cycled in HYBAID Omn-E thermal cycler using tube mode with the following conditions: 94°C, 10 min for 1 cycle; 94°C, 45 sec, 55°C, 1 min, and 72°C, 30 sec for 40 cycles; and 72°C, 5 min for 1 cycle. As shown in Fig. 1, whole dye of such composition did not support amplification (lane 2 and lane 3). This indicated that inhibitors in the gel loading dyes exist. To determine which ingredient inhibits the reaction, components of whole dye were separately tested. Instead of 1x loading dye, dye component at a final concentration of 5% glycerol, 2.5% Ficoll, 0.04% Bromophenol blue (BPB), or 0.04% Xylene cyanol FF (XCFF) was separately introduced into the above reaction mixture at the same reaction condition. The results showed that 0.04% XCFF completely inhibited the reaction (Fig. 1, lane 4), on the other hand 0.04% BPB yielded product level similar to control (Fig. 1, lane 5), although 0.04% BPB sometimes reduced the product yield to some extent (data not shown). These data indicated that 0.04% XCFF and 0.04% BPB can suppress DNA amplification and that XCFF have a much stronger inhibitory effect than BPB. In contrast, 5% glycerol or 2.5% Ficoll did not have demonstrable inhibition, with product yield similar to control (Fig. 1, lane 6 and lane 7). The inhibitory effect of XCFF could not be relieved when its concentration was reduced to 0.02% (Fig. 1, lane 8), whereas 0.02% BPB worked well in PCR (Fig. 1, lane 9). Moreover, no additional inhibition was observed when the combination of 5% glycerol and 0.02% BPB or 2.5% Ficoll and 0.02% BPB was used in PCR (Fig. 1, lane 10 and lane 11).

Taken together, these results indicate that certain components of gel loading dye, either alone (0.02% BPB, 5% glycerol and 2.5% Ficoll) or in combination (0.02% BPB + 5% glycerol and 0.02% BPB + 2.5% Ficoll) can be incorporated into PCR reaction. In addition, the inclusion of 0.02% BPB and 5% glycerol did not affect the amplification specificity as reproducible results were obtained in PCR without both components (data not shown). The validity of the modified method had been intensively tested and proved with more than 500 individual PCR amplification reactions [results not shown, 12]. The combination of 0.02% BPB and 5% glycerol was explicitly chosen to meet the two basic requirements of gel loading dye in that glycerol facilitates DNA loading by increasing the density of the sample, while BPB helps tracking the

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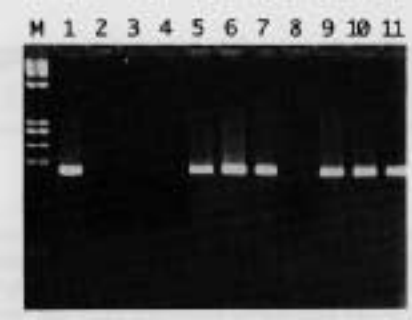

Fig. 1. The compatibility of gel loading dyes in PCR. PCR was conducted with D2Wox6 and DNA from F344 rat as described in the text. Lane M: molecular weight standard ØX 174 Hae III digest. Lanes 1-11 showed that the final PCR reaction mixture contained the indicated amount of the following components. Lane 1 (control): PCR without any components of gel loading dye; Lane 2: 1x loading dye 1 (5% glycerol with approximately 0.04% Bromophenol blue and 0.04% xylene cyanol FF); Lane 3: 1x loading dye 2 (2.5% Ficoll with approximately 0.04% Bromophenol blue and 0.04% xylene cyanol FF); Lane 4: 0.04% XCFF; Lane 5: 0.04% BPB; Lane 6: 5% glycerol; Lane 7: 2.5% Ficoll; Lane 8: 0.02% XCFF; Lane 9: 0.02% BPB; Lane 10: 5% glycerol and 0.02% BPB; and Lane 11: 2.5% Ficoll and 0.02% BPB.

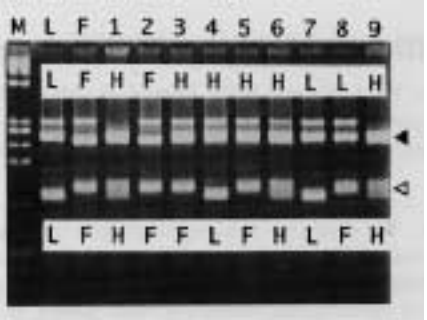

Fig. 2. Clear and easy-to-read genotypes were simultaneously generated using multiplex PCR with two markers D5Rat85 and D3Rat44 and DNAs from LEC rat (lane L), F344 rat (lane F) and 9 (F344 x LEC)F2 rats (lanes 1-9). PCR was conducted with 5% glycerol and 0.02% BPB as described in the text. PCR products were directly loaded into 3% MetaphorTM agarose gel and photographed after Et-Br staining. D5Rat85 detected large fragments on top (filled arrowhead) and D3Rat44 amplified small fragments below (open arrowhead). L, F and H represented homozygotes of LEC allele, homozygotes of F344 allele and heterozygotes of both alleles at the loci respectively.

sample during electrophoresis. We did not test Xylene cyanol FF (XCFF) further because 0.02% XCFF inhibited PCR reaction completely. For analysis involving Xylene dye, one can formulate the PCR reaction as described and load the samples directly onto gel, with the addition of 1x Loading dye 1 or Loading dye 2 in a separate well without sample to monitor large PCR fragments.

The combination of 5% glycerol and 0.02% BPB was also tested in our multiplex protocol. Multiplex PCR was again conducted in 10 μl volume, which contained 10 mM Tris-HCl/pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.35 u AmpliTaq Gold™, 0.33 μM of primers (marker 1 and marker 2), 25 ng of genomic DNA, 5% glycerol and 0.02% Bromophenol blue. The reaction mixture was covered with 15 μl mineral oil and then thermal-cycled in HYBAID Omn-E thermal cycler using tube mode with the following conditions: 94°C, 10 min for 1 cycle; 94°C, 45 sec, 55°C, 1 min, and 72°C, 40 sec for 45 cycles; and 72°C, 5 min for 1 cycle. As shown in Fig. 2, both components again did not interfere with PCR amplification. The feasibility to use dye componenets in multiplex PCR were shown in several mapping projects in our laboratory. We have applied this dye-compatible multiplex PCR to genotype ~300 markers in several crosses. Ninety % of our multiplex protocol resulted in clear two-marker genotypes in a single PCR [similar to result shown in Fig. 2, 12]. This successful modification has important implication in linkage mapping because linkage study involves genotyping tens of hundreds of samples with many markers. Direct sample loading coupled with two-marker multiplex PCR under standardized conditions provides a fast and efficient genotyping method by reduction of work into half.

Multiplex PCR was first described to simultaneously amplify multiple exons in the Duchenne muscular dystrophy locus with several pairs of primers in a single PCR reaction [3]. Soon the method gained application to microsatellite markers [1, 7]. General use of multiplex PCR in linkage study has certain difficulties for at least two reasons: First, linkage study requires genotyping a large number of samples. The preparation of many samples for PCR takes considerable time, and the existence of several primers in the PCR reaction mixture allows mispriming to occur during room temperature reaction setup [2, 8]. Second, multiplex PCR with several markers in general needs to pre-determine the combinational primer concentration [1, 6–8] because unequal amplification in multiplex PCR is often observed. In some cases the amplification of the efficient pair of primers is so favored that the less efficient one yields products not enough to be detected. In order to increase the probability of having successful multiplex PCR, many people employed a higher detection sensitivity with radiolabeled primer or dNTP [1, 7]. The success of our

multiplex protocol may benefit from an automated Hot Start PCR furnished by AmpliTaq Gold™ and a less complex reaction system with only two markers. AmpliTaq Gold™, which is inactive in room temperature and must be activated through incubation at 94°C for about 10 min [2, 8], ensured that non-specific amplifications were kept to minimum. Non-specific amplifications were often caused by mispriming during room temperature reaction setup and they consumed primers and other resources and hence were one of the major factors of unsuccessful PCR. We observed more non-specific bands or sometimes even failure of multiplex PCR using Gene Taq (data not shown). Unequal amplification was less common with our protocol probably because the competition for common resources in the reaction was less intense to amplify two markers than to multiplex with more than two markers.

In summary, we had developed a dye-compatible multiplex PCR. The successful introduction of dye components (5% glycerol and 0.02% BPB) into PCR reaction greatly facilitates post-PCR analysis, eliminating the time-consuming step to mix many samples with gel loading dye before electrophoresis. Moreover this multiplex procedure works under standardized condition without the need to determine the combinational primer concentration. This modified method had been intensively tested and proved to be highly successful with microsatellite markers for use in linkage mapping. It should find applications in others as well.

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