

FARG 2002 Study: Comparing Laboratory Protocols for Multiplexing Markers in a DNA Fragment Analysis Application

Doug Bintzler,¹ Pamela Scott Adams², Yongde Bao³, Duane Bartley⁴, Laura Kasch⁴, Robert Keefe⁵, Lynn Pelukova⁶, Caprice Rosato⁷
¹ University of Cincinnati, Chair FARG, ² Truett College, ³ University of Virginia School of Medicine, ⁴ Johns Hopkins University, ⁵ Wadsworth Center/NYS DOH, ⁶ The Rockefeller University, ⁷ Oregon State University
 * to whom correspondence should be directed



Abstract

The Fragment Analysis Research Group (FARG) has organized a study to investigate laboratory protocols for multiplexing fluorescently-labeled markers that amplify microsatellite regions from human DNA templates using PCR. By amplifying more than one marker in a single PCR reaction, laboratories can reduce their reagent expenses, as well as the time spent setting up reactions for DNA fragment analysis projects. Participants who volunteered for this study received two DNA template samples and five fluorescently labeled primer pairs. They were asked to (1) amplify all five markers in a single PCR (pre-PCR multiplex), or (2) PCR the markers individually, and pool the amplicons before analyzing them in a single gel lane or capillary (post-PCR multiplexing). Participants reported both the conditions they used for carrying out their PCR and the allele sizes (in base pairs) for the five microsatellite markers obtained following electrophoresis of the PCR products and software analysis. One aim of this study was to evaluate the methods used by other service facilities to optimize multiplex PCR. Another aim was to present a summary of the survey's results so that other laboratories might learn helpful hints as well as possible pitfalls associated with trying to simultaneously carry out PCR involving multiple primers. Results from the study are reported in this poster.

Methods

Survey participants request and receive DNA (CEPH) samples and PCR primers for multiplexing.
 Web Request for Samples
 Participants are allowed to use their own PCR conditions. The FARG suggested the following reagent concentrations for multiplexing all five markers in a single 10 µl PCR (quantities are final concentrations in PCR tube):
 sPCR Buffer 1X
 sMgCl₂ 2.5 mM
 sdNTPs 0.25 mM
 sPolymerase 0.05 U/µl
 sPrimers (forward & reverse) 0.15 µM DBSS56 & DBSS04, 0.125 µM DBS260 & D75550, 0.25 µM D75517
 sCEPH Template 60 ng
 The PCR thermocycling program depicted below was suggested, but participants were encouraged to use their own protocols.

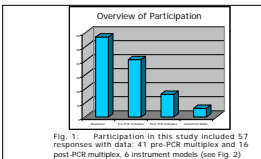
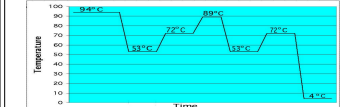


Fig. 1. Participation in this study included 57 responses with data: 41 pre-PCR multiplex and 16 post-PCR multiplex, 6 instrument models (see Fig. 2)

Results

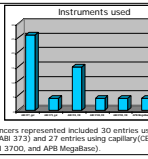


Fig. 2. Sequencers represented included 30 entries using slab gel models (ABI 317 and ABI 373) and 27 entries using capillary (CE) models (ABI 310, ABI 3100, ABI 3700, and ABI MegaBac).

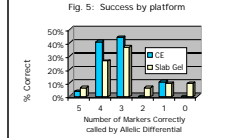


Fig. 5. The success of respondents based on the platform used. CE platforms included 24 respondents, 173 and 271 well Capillary Electrophoresis (CE) models, 310, 3100, 3700 and ABI MegaBac. Slab gel models, 317 and 373. The number of markers correctly identified by each platform is shown. The percentage of respondents who determined the correct allelic differential between the alleles for each marker.

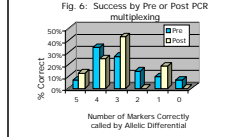


Fig. 6. Success by Pre or Post PCR multiplexing. The number of markers correctly identified using pre or post PCR multiplexing is shown. The percentage of respondents who determined the correct allelic differential between the alleles for each marker.

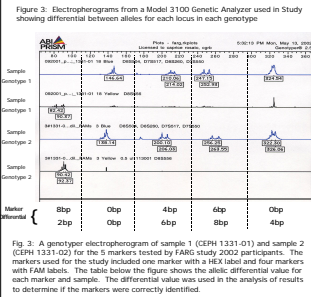


Fig. 3. A genotypic electropherogram of sample 1 (CEPH 1331-01) and sample 2 (CEPH 1331-02) for the 5 markers tested by FARG study 2002 participants. The markers used for the study included one marker with a HEX label and four markers with FAM labels. The table below the figure shows the allelic differential value for each marker and sample. The differential value was used in the analysis of results to determine if the markers were correctly identified.

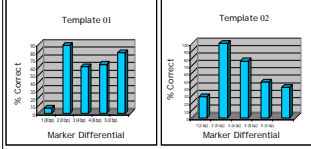


Figure 4. The percentage of respondents who were successful in calling each individual marker for Template 01 (CEPH 1331-01) or Template 02 (CEPH 1331-02). X-axis shows the marker number from smallest to largest and the size of the differential between the markers. Y-axis shows the percentage of respondents who accurately determined the correct differential between the alleles for each marker.

Table 1

| Program | Number of Markers Correctly called by Allelic Differential | % Correct |
|-----------|--|-----------|
| Program 1 | 5 | 100% |
| Program 2 | 4 | 100% |
| Program 3 | 3 | 100% |
| Program 4 | 2 | 100% |
| Program 5 | 1 | 100% |
| Program 6 | 0 | 0% |

Table 1 shows the PCR programs used by the participants of FARG study 2002. Program 1 was the in-house program given to the participants of the study.

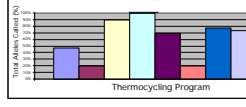


Figure 5. The total number of allelic calls for each thermocycling program. The x-axis shows the thermocycling program number and the y-axis shows the total number of allelic calls.

Conclusions

Fifty-three percent of survey participants used slab-gel based instruments to do their analyses - the remainder used capillary-based instruments. Ninety-three percent of the instruments used were made by Applied Biosystems.
 In general, it appeared that participants using capillary instruments obtained the correct allelic differential values more often than did participants using slab gel instruments.

For PCR template 1331-01, only 1 entry reported the correct allelic differential for all five markers; for template 1331-02, 4 entries were successful in reporting all five marker differentials. High failure rate to correctly call all five markers was directly correlated with failure rate to correctly call alleles for marker DBSS56.

For both CEPH samples, all participants were most successful calling marker DBSS504, and least successful calling DBSS56.

For either CEPH sample, those markers possessing an allelic differential of 0 bp (homozygous) were called more successfully than heterozygous markers.

Nearly three-quarters of the submitted entries were analyzed by pre-PCR multiplexing; the remainder were multiplexed post-PCR. Overall success in determining the correct allelic differentials of the five markers appeared to be similar using either multiplexing method.

Of the 8 thermocycling programs reported, two were single 3-step programs, and six were "back-to-back" 3-step programs. It could not be distinguished that the single 3-step programs were more successful than the "dual" 3-step programs in determining allelic calls. The six dual 3-step programs were similar to one another, and to the program initially suggested by FARG to participants - with one exception. It appeared that those dual 3-step programs (4, 1 & 2) that contained a 12 min initial denature step (necessary to activate "hot start" Tag) resulted in fewer allelic determinations overall than those that had only a 1-2 min initial denature.

Anecdotally, pre-PCR multiplexes require a significant investment of time to optimize, but allow quicker analysis of multiple markers in a single lane/capillary than via post-PCR multiplex analysis. The investment of time could be justified if the same multiplex is carried out frequently and/or for a large number of samples.

Acknowledgments

The Fragment Analysis Research Group would like to thank all of those who participated in this study. Without your help we could not expand our experience and knowledge in the field of fragment analysis. We would also like to acknowledge contributions Laura Kasch (Director, Fragment Analysis Facility, Johns Hopkins University) for working out the conditions for the multiplex PCR referred to in this poster as "in-house" protocol.