Increased efficiency of genetic profiling through quantity and quality assessment of fluorescently labeled oligonucleotide primers

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Optimizing the amount of primer to use in PCR amplification is one of the most important steps when developing protocols for genetic profiling, where subtle changes in primer concentration result in major impacts on the amount of desired product that is amplified. However, there are frequently discrepancies between the reported and actual quantity of primers delivered by suppliers, resulting in a need for re-optimization of conditions between primer orders and limiting the ability to standardize conditions between laboratories. To increase the consistency of genetic profiling protocols, we have developed a simple method to assess the quantity and quality of fluorescently labeled primers and therefore standardize reaction conditions through time and across laboratories. The method is based on analysis by electrophoresis with an automated fluorescent DNA analyzer.

Although there are an increasing number of markers available for use in genetic analyses, microsatellite loci remain the marker of choice for the majority of genetic profiling studies (1). Microsatellite loci were historically analyzed one locus at a time to avoid any ambiguity between alleles of different loci (2). However, with the advent of fluorescent labels of different colors (3), it became possible to simultaneously analyze multiple loci with overlapping size ranges. This type of multiplex PCR now forms the basis for the majority of genetic profiling studies (4–6).

Optimizing the amount of primer to use in PCR amplification is one of the most important steps in multiplex development, with subtle changes in primer concentration resulting in major impacts on the amount of desired product that is amplified (4,7,8). Thus, ensuring that the primer quantity and quality provided by suppliers is accurately quantified becomes essential; reducing the need to re-optimize conditions when new primers are ordered and increasing the universality of multiplex conditions across laboratories. Such a method has previously been developed (9), but requires the use of high-performance liquid chromatography (HPLC) and

mass spectrometry—techniques that are not commonly used or available in modern genotyping laboratories. Therefore, we have developed an inexpensive and simple method to test the quality and quantity of fluorescently labeled primers using standard genotyping equipment.

Fluorescently labeled oligonucleotides will migrate through a slab- or capillary-based polyacrylamide matrix in the same manner as larger PCR products. This technique is based on the electrophoresis of labeled primers, with quality and quantity being assessed based on the characteristics of the emitted fluorescence. Specifically, primer quantity is assessed through evaluation of peak height, and primer quality is assessed through evaluation of peak morphology. Due to the different properties of slab gel and capillary-based electrophoresis, the protocols are slightly different for each, and both are discussed. All slab gel experiments were conducted on an ABI PRISM 377 DNA sequencer with 5% Long Ranger acrylamide gels (both from Applied Biosystems, Foster City, CA, USA), and all capillary-based analyses were conducted on a MegaBACE 1000 with the MegaBACE Long Read matrix (both from GE Healthcare, Piscataway,

NJ, USA). The protocol can be divided into three basic steps: (*i*) development of standards; (*ii*) analysis of primers; and (*iii*) assessment of primer quality and quantity via comparison with the standards.

Development of standards. Observed discrepancies between the reported primer quantity supplied by companies and the actual quantity received were the impetus behind this work. As a result of these discrepancies, the absolute primer concentration desired is unknown (because the optimized estimated quantity may be incorrect), but rather is relative to the primer used for optimization. Therefore, primers that will act as a reference need to be developed, to which all new primers are compared and standardized against. Fluorescent visualization equipment has different sensitivities to different fluorescent labels (10), and thus different standards should be developed for each label.

One primer was chosen as a standard for each fluorescent label. These standards were chosen based on amplification success in previous reactions (for example, they amplified as expected based on the estimated primer amount provided by the suppliers). Several one-time use aliquots of each standard were then made by diluting the primers with water to 0.002 µM (note that using a buffer for dilutions will influence fluorescence on capillary-based equipment due to salt competition during the electrokinetic injection).

Analysis of primers. Primers to be tested were diluted to 0.002 µM in the same manner as the standard aliquots. For each run, two aliquots of the appropriate standard and of each test primer were used, to account for the variation between aliquots and between runs. With the ABI PRISM 377 DNA sequencer, primers were prepared for electrophoresis by mixing 1 µL primer with 1 µL loading dye, which contained a 10:1:1 mixture of deionized formamide:size standard:loading buffer (10). The size standard used for these analyses was the GeneScan 350 ROX (Applied Biosystems). For the MegaBACE 1000, 3 µL each primer aliquot were mixed with 3 µL loading solution, which contained
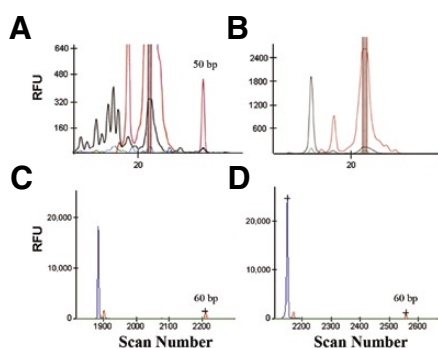
Benchmarks

Figure 1. Electropherograms visualizing the quality and quantity of synthesized oligonucleotides primers. (A and B) Primers labeled with NED as visualized using slab gel electrophoresis, and (C and D) primers labeled with 6FAM as visualized using capillary-based electrophoresis. Panels A and C represent test primers, whereas panels B and D are the standards used for comparison. Note that the primer order used in panel A was clearly synthesized incorrectly and contained oligonucleotides of different length. The ratio of primer fluorescence to size standard fluorescence in panel C is approximately 60% that for the standard in panel D, indicating that the actual primer quantity obtained from the supplier was only ~60% as much as indicated. The red peaks in all panels represent products from the size standard mixture. However, note that only the right-most peak in panel A and the red peaks with a + symbol over them in panels C and D represent actual size standard peaks, whereas the remaining red peaks are shorter than the actual size standard fragments and presumably represent by-products of size standard synthesis. The lengths of the primers used for these analyses are 21, 17, 19, and 19, for panels A, B, C, and D, respectively. The y-axes for all panels are in relative fluorescent units (RFUs), and the x-axes represent the scan number, which can be converted to length (in base pairs) based on comparison with the size standard fragments.

4.2% ROX size standard (MegaBACE ET-550R; GE Heathcare) and 95.8% double-distilled water. Electrophoresis was then carried out as for normal genotyping protocols according to each manufacturer's recommendations.

Assessment of primer quality and quantity. Electrophoresis and visualization of the primers clearly identified batches of poor quality that contain a mixture of oligonucleotides of different lengths (Figure 1). Additionally, the emitted fluorescence also provides information on the quantity of primer provided. With slab gel electrophoresis, a known amount of primer is being visualized, allowing for a direct comparison between the fluorescence of the test primers to the standards. Based on these comparisons, corrections can be made to the estimated amount of the test primers supplied. For example, suppose a stock primer aliquot is made up to a concentration of 200 µM based on information provided by the supplier. However, upon electrophoresis, the peak height produced by the test primer is only half that of the developed standard (even though both were made up to 0.002 µM for electrophoresis). Therefore, the stock solution contains only half as much primer as originally assumed, and the stock concentration can be corrected to 100 µM.

With capillary-based electrophoresis, only a portion of the molecules are electrokinetically injected into the capillaries. Each capillary may have slightly different properties, which compromises the ability to make direct comparisons of primer fluorescence across wells. However, a known amount of size standard molecules is also present in each well, and therefore the ratio of primer-to-size standard fluores-

cence should be constant for primers of the same concentration. Thus, for capillary-based electrophoresis, the ratio of primer-to-size standard fluorescence from the test primers can be directly compared with this ratio for the standards with the same label, and these values can be interpreted and used for correcting primer quantity as described for slab gel electrophoresis (Figure 1). Figure 1, C and D, provides an example of this approach. Both the test and standard primer stocks were made to a concentration of 200 µM and diluted to 0.002 µM for electrophoresis. The height of the standard primer is 24,573 relative fluorescent units (RFU) and that of the first size standard peak is 1258.8 RFU, resulting in a ratio of 19.5. For the test primer, the peak height is 18,256 RFU and that of the first size standard peak is 1558.9 RFU, resulting in a ratio of 11.7. Thus, the ratio for the test primer is only 60% (11.7/19.5) that of the standard primer, and thus the concentration must also be ~60% of the expected value or (200 µM × 0.60) = 120 µM.

This method has been successfully used in our laboratory to assess primer quality and quantity for several years, and all primer supply companies dealt with so far have been willing to replace primers deemed of poor quality or quantity based on this technique. In addition to detecting these large discrepancies, the correction of subtler discrepancies has also greatly reduced variation in amplification between primer batches. This technique does not allow for similar standardization of unlabeled primers, which could also influence amplification, or batches of primer for which the labeling process was incomplete and thus contain a mixture of labeled and

unlabeled oligonucleotides. However, the simplicity of this technique and the resulting marked reduction in amplification variation across primer orders should make it a useful tool for a wide range of laboratories.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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