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Capillary electrophoresis as a tool for optimization of multiplex PCR reactions

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Abstract Copying multiple regions of a DNA molecule is routinely performed today using the polymerase chain reaction (PCR) in a process commonly referred to as multiplex PCR. The development of a multiplex PCR reaction involves designing primer sets and examining various combinations of those primer sets and different reaction components and/or thermal cycling conditions. The process of optimizing a multiplex PCR reaction in order to obtain a well-balanced set of amplicons can be time-consuming and labor-intensive. The rapid separation and quantitation capabilities of capillary electrophoresis make it an efficient technique to help in the multiplex PCR optimization process.

Introduction

Since it was first described in 1985, the polymerase chain reaction (PCR) has greatly aided molecular biology and gained widespread use in rapid and sensitive measurements of DNA information [1, 2]. One variation on PCR that has become popular more recently is the simultaneous amplification of two or more regions of DNA in what is commonly referred to as multiplexing or multiplex PCR [3, 4].

The process of multiplex PCR is initiated by adding more than one primer set to the amplification reaction mixture (Fig. 1). These primers need to be compatible with one another in order for the multiplex to work well. First described over a decade ago [5], multiplex PCR has become an important facet of modern molecular biology because it offers several important advantages over amplifications involving one DNA marker at a time or "singleplexes". First, the cost of analysis and labor to obtain a set of results from multiple markers can be reduced. Second, the amount of information obtained per unit time of investment improves. Third, the amount of template DNA required to obtain results is reduced. Such multiplexed reactions are carefully balanced by optimizing the reaction conditions and primer sequences so that one locus with its respective primer set does not preferentially amplify over the others.

The variables that are examined when trying to obtain optimal results for a multiplex PCR amplification include reagents such as magnesium chloride as well as the thermal cycling temperature profile [6]. Primer sequences and concentrations along with magnesium concentrations are usually the most crucial to multiplex PCR. Extension times during thermal cycling are often increased for multiplex reactions in order to give the polymerase time to fully copy all of the DNA targets. Obtaining successful coamplification with well-balanced PCR product yields some-

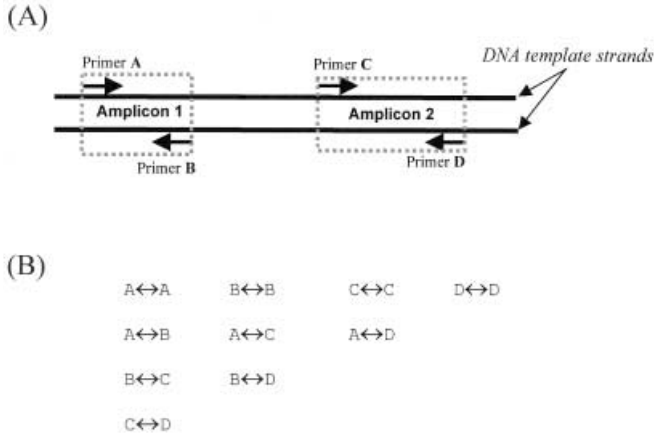

Fig. 1 (*A*) Schematic of multiplex PCR illustrating that multiple primer pairs target separate regions of the DNA template for simultaneous amplification. In this example, two amplicons are produced that differ in size with amplicon 1 being smaller than amplicon 2 because primers A and B bind in closer proximity to one another on the DNA template than primers C and D. (*B*) Multiplex PCR primer design involves pair-wise comparisons of all primer pairs to verify that primer sequences do not interact significantly. For the 4 primers illustrated in this example, a total of 10 pair-wise comparisons are required

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times requires redesign of primers and tedious experiments with adjusting primer concentrations [17].

Well-designed primers are probably the most important components of a good PCR reaction [22]. These primers define the target region(s) to be amplified from a DNA sample. PCR yield is directly affected by the annealing characteristics of the primers. For the PCR to work efficiently, the two primers must be specific to the target region, possess similar annealing temperatures, and not interact significantly with each other or themselves to form "primer dimers". Likewise, the sequence region to which the primers bind must be fairly well conserved because if the sequence changes from one DNA template to the next then the primers will not bind appropriately in all samples.

The traditional method for analysis of PCR products has been agarose or polyacrylamide gel electrophoresis [6]. The development of capillary electrophoresis (CE) has led to a number of advantages including the ability to rapidly separate the DNA amplification products in an automated fashion with quantitative results [7, 8]. A high degree of resolution is achievable which aids in separating similar-sized amplicons.

In this work, we describe the use of CE to measure two multiplex PCR reactions: A pentaplex from unique locations on the Y chromosome containing single nucleotide polymorphism (SNP) markers and a triplex that amplifies short tandem repeat (STR) markers from three autosomal chromosomes. STRs and SNPs are both important genotyping markers used for measuring genetic variation [9].

Materials and methods[1]

Primer design and synthesis. Several PCR primer design programs were used including Gene Runner (Hastings Software Inc., Hastings, NY), Oligo 6.0 (Molecular Biology Insights, Inc., Cascade, CO), and Primer 3 version 0.2 over the World Wide Web [10]. Potential primer cross-reactions were examined via Oligo or a custom-designed program written in Visual Basic 6.0.

PCR primer oligonucleotides were purchased from Operon Technologies (Alameda, CA) or MWG Biotech (High Point, NC). Primers were quality control tested via mass spectrometry prior to further testing to confirm proper synthesis and to determine the presence or absence of failure products [20]. The primer sequences for the Y chromosome SNP loci tested in this study are listed in Table 1 along with their calculated annealing temperatures [11, 12] and the expected PCR product sizes produced for each locus. The STR loci D351358, VWA, and FGA were amplified with a commercially available kit, AmpFISTR® Blue (Applied Biosystems, Foster City, CA) [16].

PCR reaction and thermal cycling conditions. Separate PCR reaction conditions were performed for samples amplified with the Y chromosome SNP pentaplex and the autosomal STR triplex primer sets. The Y SNP pentaplex PCR reaction components included 2 units of AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5.0 mM $MgCl_2$, and 250 μM deoxynucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP) in a reaction volume of 20 μL. Thermal cycling was performed on a 96 block PE9700 thermal cycler (Applied Biosystems). Thermal cycling conditions were as follows:

95 °C for 10 min
50 cycles:
  94 °C for 10 s
  55 °C for 30 s
  68 °C for 60 s
72 °C for 5 min
25 °C forever

The multiplex amplifications of the 3 STR loci were performed in reaction volumes of 10 μL with 1 unit of AmpliTaq Gold® DNA polymerase, 4 μL AmpFlSTR® PCR Reaction Mix (Applied Biosystems), 2.2 μL AmpFISTR® Blue primer mix (Appl. Biosyst.), and 4 μL DNA template at a concentration of ~ 1.5 ng/μL (Table 1). Thermal cycling conditions were as follows:

95 °C for 10 min
25 cycles:
  94 °C for 1 min
  59 °C for 1 min
  72 °C for 1 min
60 °C for 45 min
25 °C forever

Anonymous DNA samples from healthy blood donors were purchased as buffy coats from QC Products Inc. (Pompano Beach, FL) and extracted using an organic extraction procedure as described elsewhere [13].

Capillary electrophoresis. A Beckman P/ACE™ 5510 System capillary electrophoresis instrument (Beckman Coulter, Fullerton, CA) with argon ion laser was used for separation and detection of PCR products. Fluorescent detection of unlabeled DNA fragments was accomplished with the intercalating dye YO-PRO™-1 (Molecular Probes, Eugene, OR). This dye was present in the running buffer at a concentration of 500 ng/mL. The running buffer also consisted of 1% hydroxyethyl cellulose (Aldrich Chemical Company, Milwaukee, WI) and 100 mM Tris-borate and 1 mM EDTA as described previously [14]. A 27 cm × 50 μm i.d. eCAP™ Neutral Capillary (Beckman Coulter) was used to perform most of the separations. DNA samples were prepared for the CE by adding 1 μL of PCR product(s) to 50 μL deionized water. The samples were then injected at 2,000 volts for 10 s and separated at 5,000 volts for 12 min with a run temperature of 25 °C. Data collection and analysis were performed with P/ACE Station version 1.0 software (Beckman Coulter). Data points were plotted in SigmaPlot version 6.0 (SPSS Inc., Chicago, IL) or Microsoft® Excel.

Some DNA separations were also accomplished with the ABI Prism® 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) using the POP-4™ sieving polymer matrix, 310 Genetic Analyzer Buffer with EDTA, and a 47 cm × 50 μm capillary (J & W Scientific, Folsom, CA). Samples were injected for 5 s at 15,000 V and separated at 15,000 V for 24 min with a run temperature of 60 °C. DNA sizing was performed with ROX-labeled GS500 (Applied Biosystems) as the internal standard. Samples were prepared by adding 1 μL PCR product(s) to 20 μL deionized formamide containing 0.75 μL ROX-GS500 standard. The samples were heat-denatured at 95 °C for 3 min and then snap cooled on ice prior to loading them into the autosampler tray. Following data collection, samples were analyzed with Genescan® 3.1 and Genotyper® 2.5 software programs (Applied Biosystems).

[1] Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology (NIST) nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

Table 1 Primer sequences used in this study. "F" refers to the forward primer and "R" to the reverse primer for each locus. "FAM" is a commercially available fluorescent dye

	Locus amplified		Primer sequences (5′–3′)	T_m (°C)[a]	PCR Product size (bp)
SNP Pentaplex	Y-M96	F	TGCCCTCTCACAGAGCACTT	70.4	151 bp
		R	AGATTCACCCACCCACTTTG	67.2	
	Y-M45	F	CATCGGGGTGTGGACTTTAC	67.5	183 bp
		R	ACAGTGGCACCAAAGGTCAT	69.1	
	Y-M9	F	ACTGCAAAGAAACGGCCTAA	67.1	196 bp
		R	TTTTGAAGCTCGTGAAACAGA	65.1	
	Y-M89	F	CCAAACAGCAAGGATGACAA	65.6	228 bp
		R	TGCAACTCAGGCAAAGTGAG	68.0	
	Y-M42	F	AGATCACCCAGAGACACACAAA	68.0	253 bp
		R	GCAAGTTAAGTCACCAGCTCTC	68.0	
STR Triplex	D3S1358		AmpFISTR® Blue kit primers		97–145 bp
	VWA		AmpFISTR® Blue kit primers		152–212 bp
	FGA		AmpFISTR® Blue kit primers		196–348 bp

[a] Predicted primer melting temperatures (T_m) were calculated using a total primer concentration = 1.0 µmol/L and [Na^+] = 0.3 mol/L and equations from ref. [11, 12]

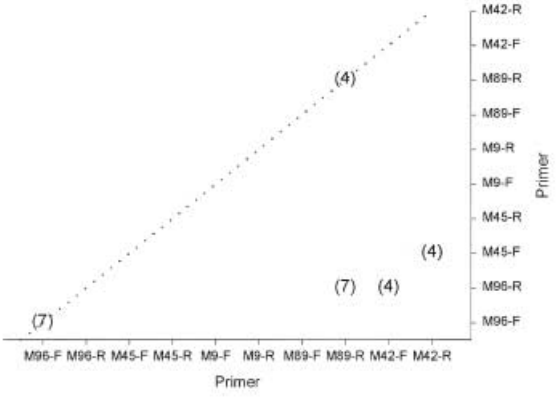
Results and discussion

Primer design

For a multiplex reaction to work properly the primer pairs for all amplified regions need to be compatible [4, 21–23]. Two criteria are highly desirable for successful and balanced multiplex PCR amplifications. First, the primer annealing temperatures should be comparable so that they all anneal at similar times during PCR thermal cycling. Second, excessive regions of complementarity between primers should be avoided to prevent the formation of primer-dimers that will cause the primers to bind to one another instead of the template DNA. If the primers anneal to the DNA template in a similar fashion then they have a greater chance of producing similar quantities of each PCR product being amplified. Likewise if primers are free to bind to the template instead of another primer in the reaction, then the primer mix has a greater chance for successful amplification of all targeted DNA regions.

Table 1 shows the primers used in this study. Note that the primer annealing (or melting) temperatures (T_m) listed in this table are very similar, which simplifies achieving more uniform amplification results across the loci tested [11, 12]. The primers across each multiplex set have T_ms within 5 °C of each other. The first five primer pairs listed are for a pentaplex amplification of 5 different regions from the Y chromosome. Each of these regions contains a single nucleotide polymorphism that can be used to form a Y chromosome haplotype for human identity testing purposes or human evolution studies [15]. Three STR markers located on human chromosomes 3, 4, and 12 were also amplified using a commercially available STR kit [16]. Each of these STRs contains a tetranucleotide repeat with allele ranges that are indicated in the PCR product size column of Table 1. For example, the STR marker D3S1358 contains alleles with repeat sizes of 9 to 21 that with the PCR primers listed produce amplicons 97–145 bp in size. One member of the primer pair for each STR locus has been end labeled with the commercially available fluorescent dye "FAM" for detection purposes under a denaturing separation environment.

(A)



(B)

Sequence Information	Potential Interaction
M96-F vs M96-F Matches = 12 Blast = 7	5-TGCCCTCTCACAGAGCACTT-3 ||| || | | || ||| 3-TTCACGAGACACTCTCCCGT-5
M89-R vs M96-R Matches = 8 Blast = 7	3-GAGTGAAACGGACTCAACGT-5 | |||||| 5-AGATTCACCCACCCACTTTG-3
M42-F vs M96-R Matches = 4 Blast = 4	3-AAACACACAGAGACCCACTAGA-5 |||| 5-AGATTCACCCACCCACTTTG-3
M42-R vs M45-F Matches = 11 Blast = 4	3-CTCTCGACCACTGAATTGAACG-5 | | || |||| || 5-CATCGGGGTGTGGACTTTAC-3
M89-R vs M89-R Matches = 4 Blast = 4	5-TGCAACTCAGGCAAAGTGAG-3 |||| 3-GAGTGAAACGGACTCAACGT-5

Fig. 2 Cross-checking performed on the Y chromosome pentaplex primers. (*A*) Primer pairs with the highest complementarity are shown by the level of interaction based on a "blast" score (see text for details). (*B*) Potential primer pair interactions are represented in more detail

Once primers are selected for a potential multiplex set they should be compared with one another to look for possible cross reactivity. We have designed a computer program that enables a pair-wise comparison of each primer to be performed. The results of this type of analysis may be plotted as shown in Fig. 2. The number of primer checks that need to be completed is equal to

$(2n^2 + n)$ where n = number of primer pairs (i.e., loci tested).

The addition of each new primer in a multiplex PCR reaction exponentially increases the complexity of possible primer interactions. Thus, there are 55 possible primer interactions when there are only 5 loci involved. Figure 2 shows the primer pairs with the highest degree of cross-reactivity. Of the 55 interactions checked, only 5 had alignment scores that were above the selected threshold of 4 contiguous bases (Fig. 2 B).

The "blast" score shown in Fig. 2 was developed as a method for computer algorithms to rank possible primer problems [10, 23]. For our purposes the "blast" score reflects the number of complementary base pairs minus the number of mismatched base pairs between two primers. Typically, a blast score of greater than 8 (the default value in the Primer 3 program [10]) can lead to significant primer dimer formation depending on the PCR conditions used for amplification [22]. While careful primer design can greatly aid PCR optimization, empirical studies are still necessary to obtain an even balance between multiplex PCR amplification products.

Each new PCR application or set of primers tested is likely to require some degree of optimization in either the reagent components or thermal cycling conditions. Multiplex PCR optimization is even more challenging than singleplex reactions because so many primer annealing events must occur simultaneously without interfering with each other. Extensive optimization is normally required to obtain a good balance between the amplicons of the various loci being amplified [6, 17].

Quantitative information generated with capillary electrophoresis

The ability to obtain quantitative information in the form of peak areas or peak heights is important when trying to balance the amplification yield from multiple PCR products. A visual inspection of the CE electropherogram data, such as is seen in Fig. 3, permits the empirical adjustment of primer concentrations in order to balance the amplification yields. When comparing the multiplex amplification yields from several different PCR conditions, relative peak areas or peak heights of the amplicons can be used to estimate primer concentration adjustments that need to be made. If one PCR product is lower in peak height relative to the other amplicons in the multiplex, the appropriate primer pair concentration can be increased to generate a more balanced multiplex (Fig. 3 B). High precision of migration time between runs is not necessary for evaluation

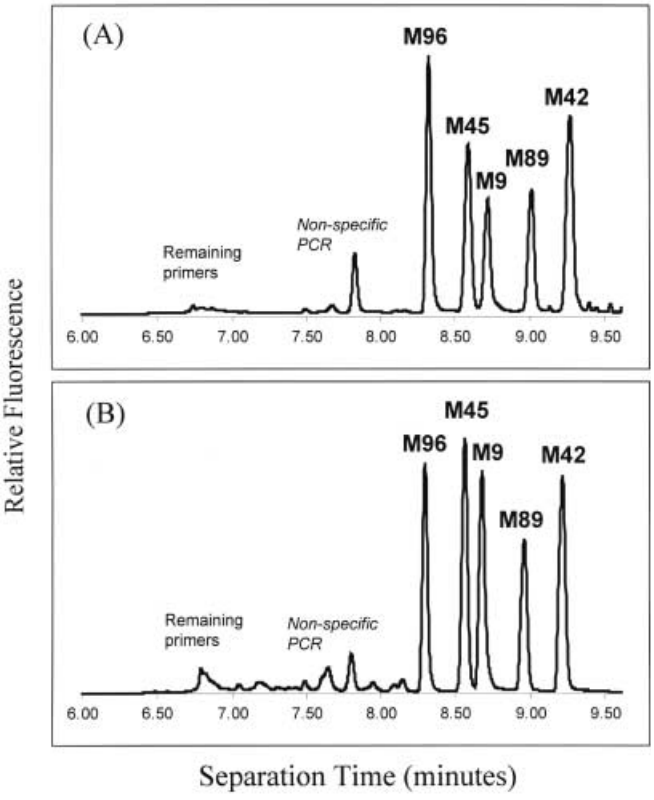


Fig. 3 Electropherogram of Y chromosome pentaplex PCR products. (*A*) Primers in equal concentrations at 1 μM. (*B*) Primers adjusted empirically to yield a better balance of PCR products. Conditions: Capillary: 50 μm i.d. coated × 20 cm to detection; Buffer: 1% HEC, 100 mM Tris-borate-EDTA, 500 ng/mL YO-PRO-1 intercalating dye; Temperature: 25 °C; Sample: 1 μL native sample in 50 μL deionized water; Injection: 10 seconds at 2 kV; Separation: 5 kV for 12 min

of multiplex PCR product yields although the use of internal sizing standards can be used to correct for mobility differences in CE separations [14].

Fluorescence detection of DNA molecules is primarily accomplished by either labeling the PCR products during amplification with dye-labeled primers [3] or via post-PCR intercalation during the CE separation [7]. End labeled PCR products may be separated under denaturing electrophoretic conditions since one of the DNA strands is labeled and can be detected. On the other hand, intercalating dyes work best with double-stranded DNA and therefore require a nondenaturing or native separation environment. However, results between the two dye labeling approaches are comparable as demonstrated with a STR triplex sample (Fig. 4). The separation in Fig. 4A is faster because the capillary is shorter and a less viscous polymer solution is used. Note that the resolution between the stutter products (indicated by arrows) [18] and the STR alleles is improved with denaturing separation conditions and the longer capillary (Fig. 4 B).

While the same peak pattern exists between the two separation/detection approaches, the relative peak intensities are slightly different. Table 2 contains a more detailed

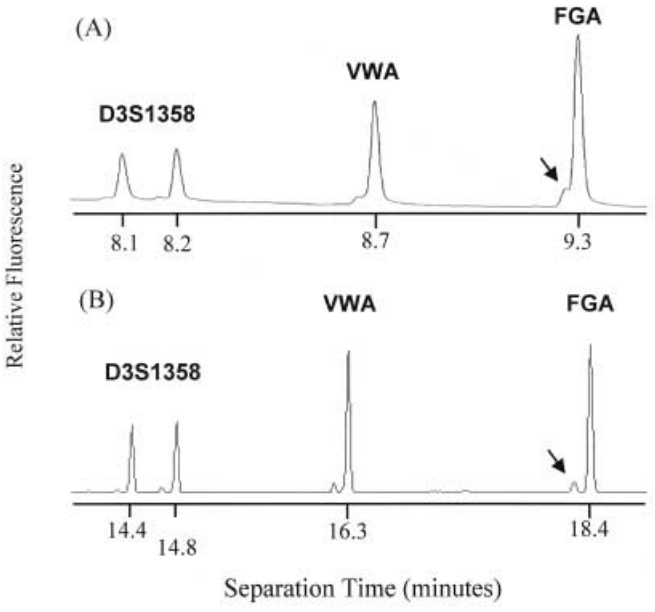


Fig. 4 Comparison of a DNA sample amplified with the STR triplex detected (*A*) with an intercalating dye in a non-denaturing separation environment and (*B*) with a 5′-end fluorescent dye label under denaturing conditions. The D3S1358 locus has two peaks because this sample contains heterozygous alleles. The arrow illustrates a stutter product that may be seen prior to all of the STR alleles. Conditions: (*A*) same as Fig. 3, (*B*) Capillary: 50 μm i.d. uncoated × 36 cm to detection; Buffer: POP-4 polymer, 1X Genetic Analyzer Buffer; Temperature: 60 °C; Sample: 1 μL heat-denatured end-labeled PCR products in 20 μL deionized formamide; Injection: 5 s at 15 kV; Separation: 15 kV for 24 min

Table 2 Comparison of peak information shown in Fig. 4. The intercalating dye approach (Fig. 4 A) yields a greater fluorescent signal for longer DNA molecules where more dye molecules may be attached

Peaks	Calc. Size[a]	Relative peak areas		Relative peak heights	
		Intercalating	End-labeled	Intercalating	End-labeled
D3 allele 1	120 bp	1.0	1.0	1.0	1.0
D3 allele 2	132 bp	1.2	1.0	1.1	1.1
VWA allele	179 bp	2.4	2.0	2.2	2.1
FGA allele	246 bp	3.9	2.3	3.7	2.2

[a] Allele sizes are calculated on the ABI 310 relative to the GS500 ROX-labeled internal sizing standard

analysis of the peak areas and heights from Figs. 4A and 4B. Larger DNA fragments in Fig. 4A have a higher fluorescent signal because more intercalating dye molecules bind to the longer PCR products. This particular STR multiplex has been optimized for an even balance between loci when the PCR products are end-labeled with a single dye molecule per amplicon. Homozygous alleles, where alleles from both chromosomes are the same size, should thus possess twice the fluorescence peak intensity as separate heterozygous alleles, which is in fact what is observed in Table 2 for the end-labeled amplicons.

We have found that the intercalating dye approach is quite useful for primer screening and rapid PCR optimizations because unlabeled primers may be used. Dye-labeled primers are more expensive and thus increase the cost of multiplex development. Individual multiplex sets may be designed and tested using unlabeled primers with intercalating dye detection for optimization purposes and then converted to dye-labeled primers for routine testing once the multiplex primers have been finalized. Multi-color multiplexes can also be developed with this approach by purchasing dye-labeled primers with spectrally resolvable dyes [3].

In addition, the use of a shorter capillary leads to more rapid run times. High-resolution DNA separations are not necessary during multiplex PCR development and optimization since relative peak intensities are the crucial information being evaluated. Finally, handling double-stranded DNA makes sample preparation easier because no heat denaturation is required, which makes the approach illustrated in Fig. 4A more amenable to automation via robotic setup under room temperature conditions.

Capillary electrophoretic DNA separations permit an evaluation of the primer to product ratio, which is an indication of PCR amplification efficiency. Notice in Fig. 3 that most of the primers are consumed meaning that the PCR reaction has been driven well towards completion. In a qualitative sense, a large amount of remaining primer after PCR amplification appears to indicate that the PCR conditions are not well optimized. Agarose or polyacrylamide gel systems cannot detect and quantify the primers in the same run as the PCR products as well as CE.

As part of our routine multiplex assay development, we typically run the singleplex reactions for each primer pair and compare the results to the multiplex reaction where all of the primers are combined. These tests help determine which, if any, primer pairs produce non-specific products. Finally, we perform “drop out” experiments where one or more of the primer pairs are removed at a time to see which, if any, of the primers cross-react. As noted earlier, careful primer design during the initial stages of multiplex development can reduce or eliminate cross-reactions between primers.

Conclusions

We have demonstrated the use of capillary electrophoresis for both SNP and STR genotyping assay development. These types of assays especially in a multiplex PCR format will continue to play an important role in a number of fields for assessing genetic variation. We illustrated that CE separations with intercalating dye labeling produce comparable results to end-labeled PCR products, but with more rapid separation times and less expensive development costs. We are currently using this CE approach for multiplex design and primer screening of Y chromosome STR markers and well as aiding multiplex SNP assay development using time-of-flight mass spectrometry detection [19]. The combination of good primer design and assay optimization measurement techniques makes multiplex PCR reactions easier to optimize.

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