Research Report

Multiplex Automated Primer Extension Analysis: Simultaneous Genotyping of Several Polymorphisms

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ABSTRACT

Accurate and fast genotyping of single nucleotide polymorphisms (SNPs) is of significant scientific importance for linkage and association studies. We report here an automated fluorescent method we call multiplex automated primer extension analysis (MAPA) that can accurately genotype multiple known SNPs simultaneously. This is achieved by substantially improving a commercially available protocol (SNaPshot™). This protocol relies on the extension of a primer that ends one nucleotide 5' of a given SNP with fluorescent dideoxy-NTPs (minisequencing), followed by analysis on an ABI PRISM® 377 Semi-Automated DNA Sequencer. Our modification works by multiplexing the initial reaction that produces the DNA template for primer extension and/or multiplexing several primers (corresponding to several SNPs) in the same primer extension reaction. Then, we run each multiplexed reaction on a single gel lane. We demonstrate that MAPA can be used to genotype up to four SNPs simultaneously, even in compound heterozygote samples, with complete accuracy (based on concordance with sequencing results). We also show that primer design, unlike the DNA template purification method, can significantly affect genotyping accuracy, and we suggest useful guidelines for quick optimization.

INTRODUCTION

Recent data indicate that more than 1.4 million single nucleotide polymorphisms (SNPs) exist on the human genomic sequence (16). This translates to a frequency of one polymorphism every 1.9 kb in the human genome (16). Accurate identification of multiple SNPs can thus benefit many fields that depend on this extreme diversity, such as population genetics, molecular and genetic epidemiology, medical diagnostics, forensics, pharmacogenomics, and pharmacogenetics.

Many methods have been developed that genotype DNA samples for known SNPs. DNA sequencing and RFLP are accurate techniques (2,12), but they are also laborious and expensive. Thus, other higher-throughput and lower-cost methods have emerged for the accurate genotyping of multiple SNPs (4). Most of these techniques depend on the recognition of heteroduplex DNA or primer extension analysis. The first kind of methods includes molecular beacon signaling, denaturing HPLC, mismatch cleavage detection, oligonucleotide array hybridization, fluorescent monitoring of PCR products, and electronic dot blot assay (3,8,10,11, 15,17,22). The second kind of assays includes allele-specific primer extension on microarrays, real-time pyrophosphate DNA sequencing, detection of allele-specific alkylated primer extension products by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS), detection of differentially sized extension products with MALDI-time of flight (TOF) MS,

allele-specific PCR with universal energy transfer-labeled primers, and solid-phase minisequencing (1,7,9,13, 18,20). Most of these techniques suffer from various problems such as the use of multi-step sample processing, the need for real-time monitoring or laborious optimization, the use of expensive probes and/or microarrays, or the difficulty of multiplexing.

We present here a modified version of a novel commercially available semi-automated fluorescent primer extension technique, *SNaPshot*™ (Applied Biosystems, Foster City, CA, USA), which accomplishes accurate genotyping of multiple SNPs simultaneously in the same reaction and gel lane, with minimum optimization. We call our version multiplex automated primer extension analysis (MAPA). Multiplexing primer extension reaction is not a novel idea (6,14,19,21), yet MAPA combines multiplexed semi-automated detection, proven chemistry (fluorescent primer extension with DNA polymerase), easy optimization, and the availability of reaction kits and analysis software (Applied Biosystems) together with the option of multiplexing the template-generating PCR. In comparison, Tully et al. (21) utilized expensive biotinylated primers and do not demonstrate accurate detection (1:1 ratios) of heterozygous SNPs. Pastinen et al. (14) had to load each of the four reactions (G, A, T, and C) in a separate gel lane, resulting in loss of throughput. Shumaker et al. (19) present two different solid phase-based methods that both extend the SNP-specific primers at room temperature with Sequenase

Table 1. Genotyping Concordance between MAPA and DNA Sequencing for Various Primer Combinations

	Combination 1 (18F and 25F)				Combination 2 (17R and 22F)				Combination 3 (17R and 25F)				Combination 4 (18F and 22F)			
SNPs	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Samples																
A	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
B	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+

Note: All combinations include primers 28F and 32F. For further details regarding the identity of each primer used, refer to Figure 1. Key: SNPs: “1”, C682G polymorphism; “2”, G888A; “3”, G927; and “4”, G1008C (cf., Figure 1); Concordance symbols: “+”, positive genotyping concordance between MAPA and DNA sequencing; “-”, negative (no) genotyping concordance between MAPA and DNA sequencing. MAPA template was purified by extraction from an agarose gel after electrophoresis.

(USB, Cleveland, OH, USA). The latter technique may result in unequal primer annealing and/or ddNTP incorporation, and thus inaccurate genotyping, because of secondary structure (especially if one wants to use many multiplexed templates and interrogate many SNPs). Lindblad-Toh et al. (6) multiplexed the template-generating PCR and geno-

typed many SNPs per reaction and gel lane with fluorescent semi-automated primer extension. However, they used two rounds of template-generating PCR amplifications—the second round (with expensive biotinylated primers) to eliminate the unincorporated PCR primers (a labor-intensive approach)—and they applied extensive primer de-

sign, including the design of a “neutral” random sequence that was added to the 5’ end of the SNP-specific primers (6). We will show that both the latter modification and the time-consuming template purification are unnecessary. We will also demonstrate that primer design, unlike the DNA template purification method, can significantly affect the genotyping accuracy of MAPA (and presumably other minisequencing methods), and we will suggest guidelines for quick optimization.

MATERIALS AND METHODS

PCR Amplification of the DNA Template Used for Primer Extension

A DNA template comprising 725 bp of the putative promoter and exon 1 of the SRD5A2 genomic sequence (5) was prepared by PCR. The PCR buffer was supplemented with 1.5 mM MgCl₂, 1 U *Taq* DNA polymerase (all from Invitrogen, Carlsbad, CA, USA), and 200 μM each of the four dNTPs (PanVera, Madison, WI, USA). Amplification primers (0.25 μg each, synthesized by Invitrogen) were as follows: 57F (forward) 5’-GACCGACGGCACAGAGGGTGTG-3’ and 4R (reverse) 5’-TGC-ACTGGGCGCCCGCAAGGG-3’.

Genomic DNA (50–150 ng) ob-

Figure 1. Diagram of all the MAPA primers and primer extension products. Either a single 725-bp PCR fragment or two (330 and 420 bp) PCR fragments were used as templates for primer extension with fluorescent ddNTPs. Four positions (1–4)—three corresponding to three SNPs (C682G, G888A, and G1008C) and a wild-type control position (G927)—were interrogated in each sample. The numbers indicate each SNP’s position in the published SRD5A2 genomic DNA sequence (5). Four primers, each corresponding to each of the four positions, were multiplexed in every reaction. Two alternative primers were used for positions 1 and 2. Each primer is labeled according to its length (in nucleotides) and orientation (F: forward, or R: reverse). All possible primer extension products are shown, by the (fluorescent) color of all potentially incorporated ddNTPs (e.g., blue labeled-ddG or green labeled-ddA for the G888A SNP). The figure is not drawn to scale.

Molecular Diagnostic Techniques

tained from samples that had been previously screened for polymorphisms (by fluorescent automated sequencing of the respective PCR products) was utilized in 50- μ L reactions, including 5% DMSO (Fisher Scientific, Pittsburgh, PA, USA). The PCR was conducted in a Robocycler[®] PCR machine (Stratagene, La Jolla, CA, USA) as follows: 3 min at 95°C for 1 cycle (step 1); 1 min at 95°C, 30 s at 62°C, and 2 min at 72°C for 40 cycles (step 2); and 5 min at 72°C for 1 cycle (step 3).

Purification of the PCR DNA Template

All PCR products were purified by either using a QIAquick[™] PCR Purification kit (Qiagen, Valencia, CA, USA) or by excising the appropriate fragment from an agarose gel after agarose gel electrophoresis, followed by QIAquick gel extraction.

MAPA

SNaPshot Ready Reaction Mix (5 μ L), containing AmpliTaq[®] DNA Polymerase FS, four fluorescently (dRhodamine) labeled ddNTPs (ddATP, ddCTP, ddGTP, and ddTTP) and reaction buffer, was mixed with 0.10–0.25 pmol purified PCR DNA template, in a 10- μ L final reaction volume. Combinations of the following six primers (synthesized by Invitrogen), using four primers (1.5 ng each) in every tube, were included in each reaction (Figure 1): C682G-18F: 5'-GTCTGGCGCTCCATAAAG-3', C682G-17R: 5'-GCGCGGCCCCCGCAACC-3', G888A-22F: 5'-GCCGGCGGCTACCCGCCTGCCA-3', G888A-25F: 5'-GAAGCCGGCGGCTACCCGCCTGCCA-3', G927-28F: 5'-CTGGTTCTGCAGGAGCTGCCTTCCTTC-3', and G1008C-32F: 5'-CACCTGGGAGTACTTCTGGGGCTCTTCTGC-3'.

The primers were extended in a TouchDown PCR machine (Hybaid, Middlesex, UK) as follows: 30 s at 96°C for 1 cycle (step 1) and 10 s at 96°C, 5 s at 50°C, and 30 s at 60°C for 25–35 cycles (step 2).

After primer extension, each reaction was treated with 1 U calf intestine alkaline phosphatase (CIAP) (Invitro-

gen) for 1 h at 37°C, followed by a 15-min incubation at 72°C for CIAP inactivation.

Electrophoresis/Data Analysis

All samples were run on an ABI PRISM[®] 377 DNA Sequencer (Applied Biosystems), following the manufacturer's recommendations, on a 5% Long Ranger[™] 6 M urea polyacrylamide gel (BMA, Rockland, ME, USA). GS Run 36E-2400/ or 36E-1200 Control Modules were used for the run. To generate a Matrix File that corrects

for spectral overlap of fluorescent dyes, dRhodamine Matrix Standards (Applied Biosystems) were run.

After the run, samples were analyzed using GeneScan[®] 3.1 software (Applied Biosystems).

Alternative (Multiplex PCR) MAPA Protocol

A multiplex PCR producing two PCR templates (330 and 420 bp) for MAPA, designed to amplify two different parts of the human SRD5A2 genomic sequence (5) that include all four

Figure 2. MAPA analysis of a compound heterozygote sample: effect of primer design. Positions 1, 2, 3, and 4 correspond to the three SNPs and one invariant (control) position shown in Figure 1. Different primer combinations for positions 1 and 2 were used (together with primers 28F and 32F for positions 3 and 4, respectively): (A) 17R and 25F. (B) 18F and 25F. (C) 18F and 22F. Color decoding: blue, ddG; black, ddC; green, ddA; and red, ddT (see also Figure 1). MAPA template was purified by extraction from an agarose gel after electrophoresis.

previously interrogated positions (Figure 1), was prepared as follows: 0.05 µg each of primers 57F and 4R (see above) and 16F and 54R (5'-GCAGCGGCCACCGGCG-3' and 5'-CTGAACCTGCATCGCGCCGTGTTCC-3', respectively) were used.

The multiplex PCR was run in a Robocycler as follows: 3 min at 95°C for 1 cycle (step 1); 1 min at 95°C, 30 s at 62°C, and 1 min at 72°C for 40 cycles (step 2); and 5 min at 72°C for 1 cycle (step 3). All other materials and methods were as described above. The PCR templates were then purified by QIAquick PCR purification.

MAPA primer extension reactions were performed as above, with primers 17R, 25F, 28F, and 32F, and a 25-cycle primer extension. CIAP treatment, electrophoresis, and analysis were done exactly as described above.

RESULTS

Simultaneous Accurate Genotyping of Several SNPs with MAPA

The recently developed ABI PRISM *SNaPshot* ddNTP Primer Extension kit allows the accurate automated genotyping of previously known SNPs by fluo-

rescent primer extension analysis of single oligonucleotides (singleplex reactions) (www.appliedbiosystems.com/310/pdf/SNaPshotprotocol.pdf). We have modified this technique to allow the simultaneous genotyping of multiple SNPs in the same tube and gel lane (MAPA). We tested this method by genotyping several genomic DNA samples that were previously shown to contain three SNPs within 725 bp of genomic DNA sequence by semi-automated fluorescent DNA sequencing analysis on an ABI PRISM377 DNA Sequencer (data not shown).

We PCR amplified the 725-bp target DNA sequence containing the putative promoter and exon 1 of the human type II steroid 5 α -reductase (SRD5A2) gene (5) from different genomic DNA samples. Then, we purified the PCR products to reduce the amount of the unused primers, primer byproducts (e.g., primer dimers), and dNTPs that would interfere with the subsequent primer extension. The purified template was then mixed with four differentially sized oligonucleotides, each ending one nucleotide 5' of either each of the three SNP positions or a fourth control (wild-type) position (Figure 1) and the *SNaPshot* ready reaction mixture. The reaction mixture includes AmpliTaq DNA

polymerase FS and all four ddNTPs (ddGTP, ddCTP, ddTTP, and ddATP, each labeled with a different fluorescent dye). The primers were then extended in a PCR machine for 25–35 cycles, but only by a single nucleotide. All the potential primer extension products are shown in Figure 1. The primer extension products were digested with CIAP and were subsequently run in a single polyacrylamide gel lane on a semi-automated ABI PRISM377 DNA Sequencer, where they were separated by size. The GeneScan 3.1 software was then used to analyze each multiplex reaction.

The use of either the 25- or 35-cycle profiles produced similar results in our hands (data not shown). Figure 2A shows the MAPA genotyping results of a compound heterozygote sample run in a single polyacrylamide gel lane. The shorter primers run faster and each incorporated dideoxynucleotide corresponds to a different color (Figures 1 and 2), which allows for fast genotyping. The genotypes inferred from Figure 2A for this genomic DNA sample are: CG heterozygote for the C682G SNP, GA heterozygote for the G888A, GG homozygote for the G927, and GC heterozygote for the G1008C (Figures 1 and 2A). These genotypes are identical to those obtained by automated sequencing (sequencing data not shown). We have genotyped more than 30 samples and observed that two out of the four combinations of primers produced 100% concordance between MAPA and sequencing, while the other two primer combinations produced less than 100% concordance (Table 1 and data not shown). The five samples selected for inclusion on Table 1 represent all possible genotype combinations.

Figure 2A also demonstrates that all four primers produce similar intensity of fluorescence after extension (all heterozygote signals exhibit roughly half the G927 homozygote signal) when multiplexed. In addition, since the background signal is extremely low (Figure 2A), there is facile distinction between homozygote (e.g., Figure 2A, position 3) and heterozygote (e.g., Figure 2A, position 1, 2, or 4) peaks.

For the running conditions on the ABI PRISM 377 Sequencer, Applied Biosystems recommends two alterna-

Figure 3. Effect of agarose gel DNA template purification on MAPA analysis. Primers used were 17R, 25F, 28F, and 32F, on the same sample as Figure 2. PCR template purified by (A) agarose gel followed by QIAquick gel extraction or (B) QIAquick PCR purification only. For color coding, see Figure 2.

Molecular Diagnostic Techniques

tive control modules (*SNaPshot* protocol; www.appliedbiosystems.com/310/pdf/SNaPshotprotocol.pdf): GS Run 36E-1200 (2-h run) or GS Run 36E-2400 (2.5-h run). Both of these running modules work well in singleplex reactions (data not shown), but, when multiplexing, we observed much better peak separation and lower background with the longer, denser scanning mode run (36E-2400) (data not shown). This may be a more significant issue the more SNPs and more compound heterozygotes one is genotyping because the number of (real and extraneous) peaks increases accordingly.

Effect of Primer Design and Method of Purification on Genotyping Accuracy

To test the effect of primer design on the accuracy of the MAPA genotyping, we designed primers of different length and orientation for two of the SNPs studied (C682G and G888A) and examined the genotyping accuracy for each of the four resulting combinations of primers. We utilized highly polymorphic genomic DNA samples for that purpose. Table 1 shows that two out of the four combinations of primers produced 100% accurate genotypes (based on concordance with sequencing) for both the three SNPs and the wild-type control position, while the other two combinations did not. Most of the genotyping problems, though, involved a single polymorphism (the C682G polymorphism; Table 1). Representative individual problems were selected for inclusion in Figure 2, using a compound heterozygote sample: panels 2A and 2B differ only by the use of different C682G polymorphism primers (17R and 18F, respectively). The use of the 17R (reverse) primer results in a 1:1 ratio of G to C for this heterozygote sample (Figure 2A, position 1), while use of the 18F (forward) primer results in an approximately 4:1 ratio of G to C (Figure 2B, position 1). Thus, primer design can affect the accuracy of genotyping (a 4:1 ratio may be mistaken for homozygosity, especially if the peak height is low). Furthermore, the use of the shorter (22-nucleotide) G888A polymorphism primer 22F instead of the longer (25-nucleotide)

primer 25F, in combination with the 18-nucleotide C682G polymorphism primer 18F, results in all four primers migrating at about the same gel spot (positions 1 and 2, Figure 2C versus 2B). This co-localization of peaks significantly increases the difficulty of interpreting the genotypes.

To evaluate the effect of the PCR template purification on the genotyping accuracy, we performed all MAPA reactions using template purified by either QIAquick column purification (as recommended by the *SNaPshot* protocol) or agarose gel extraction followed by QIAquick purification. Figure 3 demonstrates that the agarose gel purification step (Figure 3A) reduces the background (compare Figures 3A and 3B) and thus makes genotyping interpretation easier. Accuracy, though, is not significantly affected.

An Alternative MAPA Protocol May Increase Genotyping Throughput

Given the fact that SNPs occur at a frequency of approximately one SNP per 1.9 kb in the human genomic sequence (16), it is unlikely that one can interrogate several SNPs in a single PCR fragment. Thus, multiplexing the PCR template generation step would greatly increase the technique's usefulness. In addition, the inclusion of the agarose gel purification step reduces the throughput of MAPA. Examination of Figure 3B reveals two major back-

ground bands around the A49T peak that are presumed to correspond to extension products from the primers of the template-generating PCR. Thus, minimizing the amount of primers used in the template-generating PCR may lead to low enough MAPA background that makes gel purification unnecessary. Therefore, an alternative MAPA protocol was tested, which accordingly uses 0.05 μ g (or five times less than previously) each of two sets of primers, in a multiplex PCR designed to produce two distinct PCR templates that include all four SNP positions previously interrogated. The first PCR template (420 bp; generated by primers 57F and 54R) is expected to amplify part of the *SRD5A2* gene promoter that includes the C682G SNP (5) (Figure 1). The second PCR template (330 bp; generated by primers 16F and 4R) is expected to amplify exon 1 of the same gene that includes the G888A, G927, and G1008C positions (5) (Figure 1). The multiplex PCR was run as before, except that the polymerization time was reduced to 1 min. Only the two expected PCR fragments (330 and 420 bp) and no primers or primer dimers were observed in an agarose gel, after amplification of 10 genomic DNA samples (data not shown). Following QIAquick PCR purification, MAPA analysis was performed as described before, using primers 17R, 25F, 28F, and 32F. Analysis of the same sample displayed in Figure 3 shows that this alternative

Figure 4. An alternative MAPA protocol results in accurate genotyping in the absence of the laborious gel purification. MAPA analysis was performed as described before, except that (i) a multiplex template-generating PCR was used to amplify two fragments (330 and 420 bp) containing the four desired positions (1–4; Figure 1), using 0.05 μ g each of primers 57F, 54R, 16F, and 4R, and (ii) QIAquick PCR purification was used to purify the template for MAPA. Primers used for MAPA were the same as in Figure 3, and the same sample is shown. For further explanation, see the Materials and Methods section.

multiplex PCR MAPA protocol produces faster genotyping (since we omitted the gel electrophoresis step) without any sacrifice in genotyping accuracy (compare Figure 3A and Figure 4; also notice the reduction of the background from Figure 3B to Figure 4). Analysis of the rest of the samples produced similar results (data not shown).

DISCUSSION

Accurate genotyping of known SNPs is of major importance in genetic and molecular medicine, pharmacogenetics, and many other fields. Given the near completion of the human genome project and the high prevalence of SNPs in the human genome (16), it is desirable to genotype as many SNPs in a single reaction as possible. We present here a semi-automated fluorescent method (MAPA) that can accurately and easily genotype multiple SNPs simultaneously. This is achieved by modifying a commercially available protocol (*SNaPshot*) that relies on the extension of a primer that is designed to end one nucleotide 5' of a given SNP, with fluorescent ddNTPs, followed by semi-automated analysis on an ABI PRISM 377 Sequencer. Our modification includes the incorporation of several primers (corresponding to several SNPs) in the same reaction and loading the primer extension products on a single gel lane. (Applied Biosystems claims a loss of fluorescent intensity when multiplexing; see *SNaPshot* protocol). In our hands, the multiplexed technique works with 100% genotyping accuracy for all the SNPs examined in more than 30 samples (based on concordance with sequencing results; Table 1 and data not shown). In addition, an alternative MAPA protocol that incorporates a multiplex PCR step and eliminates the need for agarose gel purification results in similarly high genotyping accuracy. This modification is expected to increase the throughput of MAPA (by avoiding the laborious gel purification) and its usefulness (by allowing the simultaneous genotyping of SNPs taken from more than one PCR template in the same reaction).

However, there is a practical limit to the number of SNPs one can accurately

genotype with MAPA. This limit is dictated by the range of applicable primer lengths (16–50 nucleotides) and the minimum spacing in the primer length that allows unambiguous separation (in general, 2–4 nucleotides; but also notice the inadequate separation between the 18- and 22-nucleotide primers in Figure 2C, positions 1 and 2). Thus, MAPA cannot probably be used to efficiently genotype more than 10–12 SNPs per sample and gel lane. Throughput can be increased, though, by two additional modifications (6): pooling together complementary SNP primers (e.g., a 22-mer primer pair specific for a C/T SNP with another 22-mer primer pair specific for an A/G SNP) and loading samples consecutively on the same gel (e.g., at 0, 0.5, and 1 h for each 2.5-h run) (6). We have shown the technique's usefulness for up to four SNPs and with no need for optimization of the primer extension conditions.

MAPA can be used to genotype DNA samples with great reproducibility (compare Figures 2A and 3A) and accuracy (Table 1). Plausible reasons for this include the very low fluorescence background, the almost equal apparent incorporation of ddNTPs in heterozygote samples (evident by the close to 1:1 ratio observed between the two peaks for each SNP position; Figure 2A), and the almost equal apparent annealing of all multiplexed primers (evident by comparison of the sum of the peak heights for each position; Figure 2). Some optimization, however, may be necessary to achieve accurate genotyping. Indeed, two out of the four primer combinations tried (regarding two of the SNPs) yielded ambiguous genotyping results, favoring one allele over the other (even though mostly for one of the SNPs (Table 1 and Figure 2). This problem can be easily overcome by the use of alternative primers for the problematic SNPs. When designing primers for MAPA, it may be useful to keep in mind the results of Figure 2: primer orientation appears to affect the accuracy of heterozygous genotyping more than primer length (perhaps because of the formation of strand-specific secondary structure). A minisequencing primer design rule of thumb could thus be to try one primer per SNP with (sequenced) positive controls and then

Molecular Diagnostic Techniques

design alternative primers, in the reverse orientation, for SNPs that resulted in ambiguous and/or erroneous genotyping. The genotyping accuracy of homozygous samples, though, is not affected by the choice of primers (data not shown). Also, our results prove that extensive primer design to keep the melting temperature of the minisequencing primers similar [as done by Lindblad-Toh et al. (6)] is not necessary to achieve accurate genotyping (our primers had very different melting temperatures). This may become an issue though with large numbers of SNPs.

In contrast to primer design, the choice of PCR template purification method appears to play only a minor role in the genotyping accuracy of MAPA. In general, QIAquick column purification (as recommended by the *SNaPshot* protocol) may purify the PCR template adequately, but genotyping becomes easier and more accurate when the primer concentration of the template-generating PCR is reduced to a minimum. The more SNPs one needs to multiplex in each reaction, though, the more likely it is that the background will affect genotyping accuracy (especially of heterozygous samples), and, thus, the need for the laborious agarose gel purification step probably increases.

In conclusion, we present here a modified version of a novel commercially available technique (*SNaPshot*) that we call MAPA. This multiplexed technique allows the simultaneous and accurate genotyping of multiple known SNPs (even compound heterozygotes) with minimum optimization by semi-automated analysis of fluorescent primer extension products on an ABI PRISM 377 Sequencer. The combined effect of multiplexed semi-automated detection, proven chemistry (fluorescent sequencing/primer extension), available reaction kits and software (Applied Biosystems), and easy optimization together with the option of multiplexing the template-generating PCR creates a method that rivals most other available genotyping techniques for known SNPs.

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