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Optimization of a multiplex minisequencing protocol for population studies and medical genetics

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ABSTRACT. Several technologically sophisticated high-throughput techniques have been recently developed for the study of human single nucleotide polymorphisms and the diagnosis of point mutations in human diseases. However, there is also a need for simple and inexpensive techniques suitable for clinical services and small research laboratories. Minisequencing meets the latter requirements. It is simple, non-radioactive and can be easily multiplexed by adding oligonucleotide tails of increasing size to the sequencing oligonucleotide primers. To optimize the minisequencing protocol, we designed a test multiplex system capable of typing simultaneously 12 different human autosomal single nucleotide polymorphisms. We discovered that the quality of minisequencing primers and the careful selection of the tail sequences were especially critical for success. This optimized protocol permits rapid genotyping at low cost and can serve as a blueprint for the creation of multiplex minisequencing systems suitable to virtually any typing application in population studies and medical genetics.

Key words: DNA, Polymorphisms, Single nucleotide polymorphisms, Minisequencing, Multiplex

INTRODUCTION

The Human Genome Project has produced a large amount of genetic data, including the identification of more than ten million single nucleotide polymorphisms (SNPs) that are available in public databases (e.g., dbSNP: http://www.ncbi.nlm.nih.gov/SNP and TSC: http://snp.cshl.org). Because of their abundance and widespread genomic distribution, SNPs have become the most useful genome markers in human molecular genetics and are used in gene mapping (Rioux et al., 2001; Ozaki et al., 2002; Kammerer et al., 2005), in population genetics and in evolutionary studies (Underhill et al., 2001; Sanchez et al., 2003; Liljedahl et al., 2003; Shriver et al., 2004) as well as individual identification markers in forensic applications (Syvanen et al., 1993; Just et al., 2004; Inagaki et al., 2004). Moreover, point mutations, which are studied using the same techniques as SNPs, have a fundamental importance in molecular medicine, as causative agents of human diseases (Amir et al., 1999; Lai et al., 2001; Prokunina et al., 2002; see Human Gene Mutation Database at http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html).

Several molecular methodologies have been developed for discovery and genotyping of SNPs, as reviewed by Syvanen (2001) and Kwok and Chen (2003). SNP discovery relies mostly on direct DNA sequencing or on denaturing high-performance liquid chromatography (dHPLC), while SNP genotyping methods rely on methodology for allelic discrimination (Kwok and Chen, 2003). Very advanced techniques have been developed with amazing throughput capabilities. For instance, recently Hinds et al. (2005) used a microarray platform to type simultaneously 1,586,383 SNPs in 71 Americans of European, African, and Asian ancestry!

In spite of these fantastic advances, most clinical services and small research laboratories still make extensive use of the rather inefficient restriction fragment length polymorphism approach to genotype SNPs (Gibbs et al., 1986; Lander and Botstein, 1986; Dietz-Band et al., 1990; Santos et al., 1996; Bazrafshani et al., 2000; Haak et al., 2004) and diagnose diseases. Although restriction enzyme digestion of PCR products is a simple technique, it is not amenable to automation and cannot be used in a multiplex format. Thus, there is a need to develop multiplex techniques that are efficient and yet simple enough to be used in unsophisticated laboratories.

In 1990, Syvanen et al. first proposed the technique of primer extension (minisequencing) for SNP genotyping. The same group later made several alterations on the method, substituting fluorescent labels for radioactivity and introducing several improvements (Syvanen, 1999). In order to set up an efficient and yet simple and low-cost technique for SNP typing, we decided to optimize Syvanen's extension primer technique in a multiplex format. As a model test, we constructed a multiplex system with 12 polymorphic SNPs. We here demonstrate the applicability of this method, which indeed has proven to be easy, uncomplicated and yet highly effective.

MATERIAL AND METHODS

DNA samples

In the development of the minisequencing protocol we used DNA samples from white Brazilian individuals as described previously (Alves-Silva et al., 2000; Carvalho-Silva et al., 2001). DNA samples from 200 unrelated individuals were mixed in a pool to verify allele heterozygosity.

SNP selection and primer design

We chose SNPs from the public database dbSNP (http://www.ncbi.nlm.nih.gov/SNP/). We picked 15 polymorphisms, each one placed at a different chromosome, all with heterozygosity in the range between 0.4 to 0.6 and all located in introns or the 3'- or 5'-untranslated region of mRNAs. All selected primers were checked for spurious matches with other human sequences using Genome BLAST (http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html).

Primers were designed using the primer analysis software *Oligo*, version 4.0 and synthesized commercially. We added a T7 universal tail to one primer of each pair in order to improve multiplex optimization. Detailed information about the primers, the amplified product and the SNP detected by each pair, can be seen in Table 1.

Liquid-phase minisequencing

Syvanen et al. (1990) were the first to propose the principle of minisequencing, initially using a solid phase format. In contrast, we chose a liquid phase and developed a multiplex typing strategy that can, in principle, be used to study any SNP. Figure 1 shows the principle of the method.

PCR

PCR primers for the 15 SNP amplicons and the T7 primer were combined into a single multiplex reaction, generating different PCR product sizes. The best conditions obtained are provided in Table 1. The primer containing the T7 tail was used at a concentration 10 times smaller than that of the non-tailed primer and the T7 primer. This protocol seems to improve the multiplex results, possibly by making the reaction more uniform. All PCR reactions were done in a volume of 12.5 µl with 50 ng genomic DNA, 2.0 mM $MgCl_2$, 1x AmpliTaq Gold buffer, 200 µM dNTPs, 1 unit *Taq* DNA Polymerase (AmpliTaq Gold; Applied Biosystems, Foster City, CA, USA). Amplification was achieved in an M.J. Research Cycler (M.J. Research, Watertown, MA, USA) and consisted of 95°C for 5 min, followed by 34 cycles for 30 s at 95°C, 30 s at 50°C, 1 min at 72°C, and a final extension for 30 min at 72°C. The PCR products were resolved in 6% polyacrylamide gels and silver stained according to Santos et al. (1993).

ExoSap treatment

In order to eliminate the excess of primers and dNTPs, the PCR products were digested by an "ExoSAP" mix consisting of 2 units/µl *E. coli* exonuclease I (Exo I) and 0.2 units/µl shrimp alkaline phosphatase (SAP) and 1x SAP buffer. The enzymes were purchased from the U.S.B. Corporation (Cleveland, OH, USA). Five microliters of PCR product was added to five microliters of ExoSAP mix and incubated at 37°C for 30 min. The enzymes were afterwards inactivated by heating at 80°C for 15 min.

Design of minisequencing primers

Table 2 shows the genotyping primers designed for each SNP. Primers were designed

Table 1. Sequence primers and PCR conditions to amplify flanker SNP regions of 15 autosomic SNPs in multiplex format.

Marker location and dbSNP rs	PCR product size (bp)	dbSNP allele frequency in Caucasian population	Primers to amplify flanker SNP regions	Primer concentration in multiplex reaction
15qrs7322	80	T: 0.594 C: 0.406	F: TTT TGA AGA CTC CCA TTT TA R*: TGG TGA AGA CTT TTG GTA	F: 1.15 µM R: 0.15 µM
7prs2069845	90	A: 0.520 G: 0.550	F: TTC CCA GTC CTC TTT ACA CC R*: CCC TCT CAC CAT CCC TTT AG	F: 0.50 µM R: 0.05 µM
11prs680	98	A: 0.470 G: 0.530	F: TGG CCT GGA CTT GAG TCC CTG A R*: ACG AGC GAC GTG CCC ACC TGT	F: 0.50 µM R: 0.05 µM
8prs285	109	A: 0.410 G: 0.590	F*: CTG CTG CCT GCA AGG GTT TTG CT R: AAC AGA AGA AÇA ACA ACA AAA CCC	F: 0.05 µM R: 0.50 µM
18qrs1801018	113	A: 0.510 G: 0.490	F: AGA GGT GCC GTT GGC CCC CGT TGC R*: TTC ATC ACT ATC TCC CGG TTG TCG	F: 0.50 µM R: 0.05 µM
2prs512535	138	A: 0.450 G: 0.550	F: GGT GGG AAA TGG GCA GTG R*: GGA GGC GGA CGA GGA AAA	F: 0.50 µM R: 0.05 µM
3qrs1520137	155	A: 0.545 G: 0.454	F: AAA AGG CTA CCA GAG AAG AAT A R*: CAC ATT TTT TTC CCT CAT	F: 0.50 µM R: 0.05 µM
9prs944700	172	A: 0.470 G: 0.530	F: TAG ACC GAC CAT AGA GTT CC R*: TTT TTC TCT CCA CTC TCA GG	F: 0.50 µM R: 0.05 µM
10qrs14327	220	A: 0.514 G: 0.486	F: TTA TTT TAT CGT CGA TTT GG R*: CTG CAC AAA TAT CTT TTA AAG A	F: 1.15 µM R: 0.15 µM
13qrs2774030	311	A: 0.674 G: 0.326	F: GCT CCT CTG CCT TCT GCT R*: CCA CCC ACT CCT AAA GTT	F: 1.15 µM R: 0.15 µM
14qrs2069974	337	A: 0.587 G: 0.413	F: TCC TCC AAA ATG TGT CCC R*: TGG CTG TTC TTT GTT CTG	F: 1.15 µM R: 0.15 µM
16prs3093319	352	A: 0.500 G: 0.500	F: CTC AGC AGC CCA GAC CAA R*: GGC TGG TCT CAA TCT CCT	F: 0.50 µM R: 0.05 µM
17qrs3093692	366	A: 0.478 G: 0.522	F: ACC CCC AAA CTA CTC CAG R*: CCT GCT CTG TTG GAC TGA	F: 1.15 µM R: 0.15 µM
5qrs2243057	413	A: 0.565 G: 0.435	F: ATT TCT TTG TGG TTT TTA GG R*: TCA AGT GTG TAA AAT AGC GA	F: 1.15 µM R: 0.15 µM
12rs1480474	576	A: 0.460 G: 0.540	F: CT TTA TCA TGA TTA CAC TCC T R*: CAG TCA ACC ATA ATT TCA GAA T	F: 1.15 µM R: 0.15 µM
T7			TAA TAC GAC TCA CTA TAG GGA GA	1.50 µM

F: foward primer; R: reverse primer ; PCR: polymerase chain reaction; SNP = single nucleotide polymorphism; dbSNP = database SNP.
*Primer with T7 tail: TAA TAC GA TCA CTA TAG GGA GA

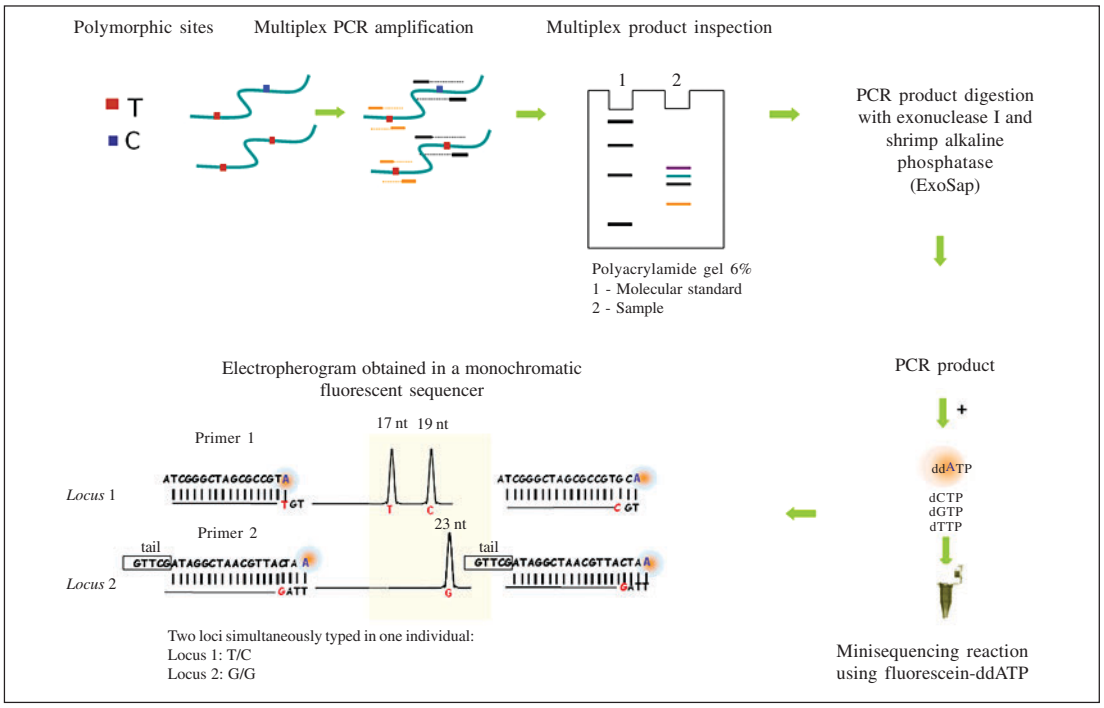

Figure 1. Schematic representation of the multiplex minisequencing protocol. Four distinct major steps can be distinguished. 1) Polymerase chain reaction (PCR): each SNP target-containing region is amplified using primers that flank it. We designed different PCR product sizes for each SNP selected, optimizing them in a multiplex reaction. 2) ExoSap treatment: after inspection of the PCR product in a polyacrylamide gel, the amplicons are treated with exonuclease I (Exo I) and shrimp alkaline phosphatase (Sap) to eliminate primers and dNTPs not used in the reaction. 3) Minisequencing reaction: the technique is based on the annealing of a single primer adjacent to the polymorphic target site. The 3'primer is extended by a DNA polymerase in a cycle sequencing reaction using a fluorescently labeled dideoxynucleotide (ddATP) and the other deoxynucleotides (dNTPs). The DNA polymerase will extend the minisequencing primer until a dideoxynucleotide is incorporated, when it stops. The product size varies according to the primer size and the nucleotide sequence that is adjacent to it. Tails of different sizes are added to each primer to allow the type resolution of several SNPs in the same reaction. 4) The minisequencing products are then visualized using an automatic fluorescent DNA sequencer.

with the 3'-extremity complementary to the last base immediately before the polymorphic residue. In order to distinguish between the sizes of the detection products, the primers were synthesized ranging from 20 to 107 nucleotides. The desired sized of the primers were adjusted by addition of a piece of a "neutral" sequence at the 5' extremity. The polynucleotide sequence of the "neutral" sequences was based on the sequence of pUC18, since this plasmid does not match any human sequences in the NCBI non-redundant database. In the minisequencing multiplex, the distance between the two SNP alleles varied from one to 10 nucleotides, depending on the allele present at the SNP target and the subsequent sequences (Table 2). For each 3-bp DNA fragment size interval, one SNP locus could be detected. All the primers used in minisequencing were purified by HPLC.

Minisequencing reaction and electrophoresis

Multiplex PCR minisequencing was performed in a 12.5-µl volume with 1 µl purified PCR product and 0.01-0.5 µM of the primers (Table 2). The PCR reaction contained 0.5 µM

Table 2. Minisequencing (MS) primers to type 15 autosomic SNPs.

1° Multiplex product	MS marker name	M13 Tail sequence and size	Target-specific sequence	Total primer size (nt)	MS product ddATP*		Primer concentration in multiplex reaction
					Allele A (nt)	Allelle G (nt)	
2prs512535	MS2p	GTA AAA CGA CGG CCA GTG CCA AGC TTG CAT GCC TGC AGG TCG ACT CTA GAG G - 52 nt	GGAAATGGGCAGTGCCTAGAAG	74	75	78	0.10 µM
3qrs1520137	MS3q	GTA AAA CGA CGG CCA GTG CCA AGC TTG CAT GCC TGC AGG TCG ACT - 45 nt	CTCTCACATATTGTAGCACAGAC	68	69	72	0.10 µM
5qrs2243057	MS5q	GTA AAA CGA CGG CCA GTG CCA AGC TTG CAT GCC TGC AGG TCG ACT CTA GAG GAT CCC CGG GTA CCG AGC TCG AAT TCG TAA TCA T - 85 nt	CCCTGCCATTGTTGAGGCTATC	107	108	118	0.50 µM
7prs2069845	MS7p	GTA AAA CGA CGG CCA GTG CCA AGC TTG CAT GCC TGC AGG TCG ACT CTA GAG GAT CCC CGG GTA CCG AGC TCG AAT - 75 nt	CCCAGTCCTCTTTACACCACC	96	97	99	0.01 µM
8prs285	MS8p	GTA AAA CGA CGG CCA GTG CCA AGC TTG CAT GCC TGC AGG TCG ACT CTA GAG GAT CCC CGG GTA CCG AGC TCG AAT TCG T - 79 nt	ACAACAACAAAACCCCACAGCT	101	102	105	0.10 µM
9prs944700	MS9p	GTA AAA CGA CGG CCA GTG CCA AGC TTG CAT GCC TGC AGG T - 40 nt	GAATGCAAGTCCCAGACATCTG	62	63	66	0.10 µM
10qrs14327	MS10q	GTA AAA CGA CG - 11 nt	ATTTTATCGTCGATTTGGTAGTTC	35	36	37	0.50 µM
11prs680	MS11p	GTA AAA CGA CGG CCA GTG CCA AGC TTG CAT GCC TGC AGG TCG ACT CTA GAG GAT CCC CGG GTA CC - 65 nt	GAACCAGCAAAGAGAAAAGAAGG	88	89	94	0.10 µM
12qrs1480474	MS12q		CTATCTAGTATAATTTGAAG	20	21	23	0.5 µM
13qrs2774030	MS13q	GTA AAA CGA CGG CCA GTG CCA AGC TTG CAT GCC T - 34 nt	GAGGGGAGGCCCTTCTTGGT	54	55	60	0.10 µM
14qrs2069974	MS14q	GTA AAA CGA CG -11 nt	GGGTGCCATCCCTTCTCTTT	31	32	33	0.10 µM
15qrs7322	MS15q	GTA AAA CGA CGG CCA GTG C - 19 nt	CTCCCATTTTAAGAACCGTGCA	41	42	43	0.10 µM
16prs3093319	MS16p	GT - 2 nt	AGCCACCAAAAATGAACCTACTT	25	26	27	0.10 µM
17qrs3093692	MS17q	GTA AAA CGA CGG CCA GTG CCA AGC - 24 nt	TGGTGAAAGGGTTCAGGGGCA	45	46	52	0.50 µM
18qrs1801018	MS18q	GTA AAA CGA CGG CCA GTG CCA AGC TTG CAT GCC TGC AGG TCG ACT CTA GAG GAT CCC CGG - 60 nt	ATGGCGCACGCTGGGAGAAC	80	81	86	0.01 µM

ddATP labeled with fluorescein (NEL402-Perkin Elmer Life Sciences, Boston, MA, USA), 0.5 µM unlabeled dCTP, dTTP and dGTP, 3.5 mM $MgCl_2$, 1x Thermo Sequenase buffer, and 1 unit Thermo Sequenase DNA Polymerase (Amersham Biosciences, Uppsala, Sweden). The thermal cycling was performed as "hot start": the enzyme was added 2 min after an initial denaturation. The "cycle sequencing" reaction consisted of 80°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 20 s.

The minisequencing products were applied in a 6% denaturing polyacrylamide gel and resolved in a fluorescent automatic DNA sequencer ALF (Amersham Biosciences). The analyses were done in the software package Allelinks (Amersham Biosciences).

RESULTS

PCR multiplex

It was necessary to experiment with the concentration of each primer to obtain a good multiplex result. The final concentrations are shown in Table 1. A silver-stained polyacrylamide gel of the multiplex products is shown in Figure 2. We were forced to eliminate one SNP (12q) from the multiplex format, because it did not produce visible peaks in it, despite working very well in the simplex format.

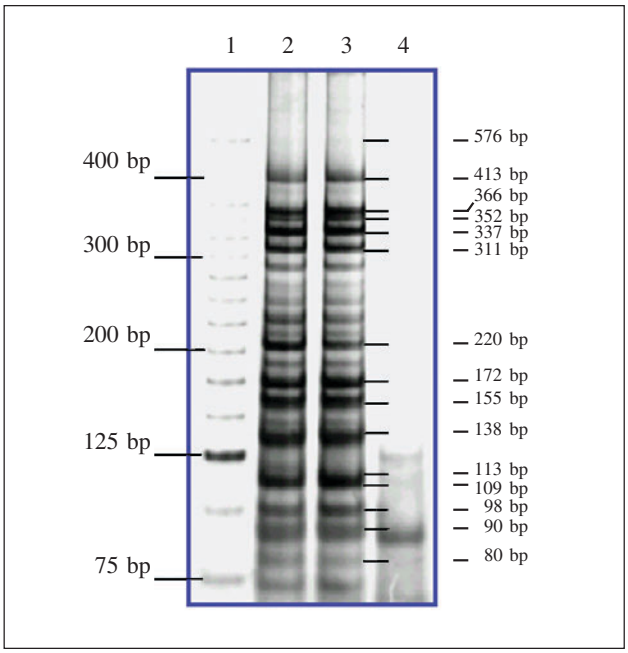

Figure 2. Silver-stained polyacrylamide gel showing the first genomic SNP multiplex. The size of each product is specified in base pairs. *Lane 1*: molecular standards; *lane 2*: individual 1; *lane 3*: individual 2; *lane 4*: blank.

Minisequencing simplex

Before performing the multiplex minisequencing, we checked the quality of each SNP

minisequencing in a simplex reaction. We used a single individual to do this as can be seen in Figure 3A. We had to exclude one SNP (10q) from the multiplex because its product was consistently weak and the background in its vicinity was high (see Figure 3A). Figure 3B shows the simplex minisequencing results using a pool of 200 unrelated Brazilian individuals and permits visualization of both alleles at all selected loci.

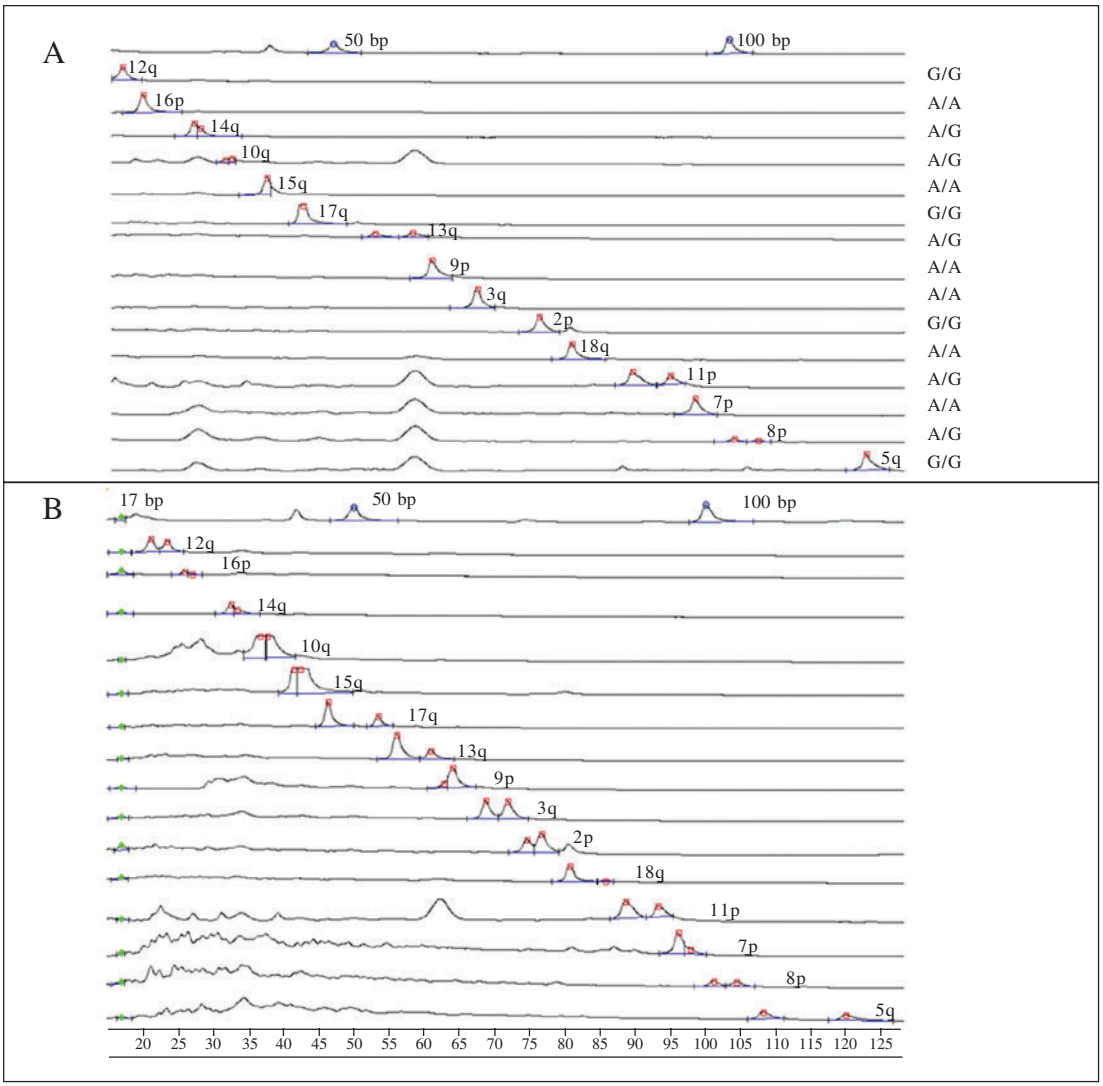

Figure 3. Simplex SNP minisequencing. Denaturing polyacrylamide gel (6%; ALF, Amersham Biosciences), with each lane showing a SNP minisequencing product. The chromosome map position of each SNP is specified. **A.** Typing of a single individual. **B.** Typing of a pool containing DNA from 200 individuals.

Minisequencing multiplex

To obtain good results in the multiplex minisequencing protocol it was necessary to experiment with primer concentrations, so that peak sizes became relatively even (Figure 4).

We were forced to eliminate one SNP (5q) from the multiplex format, because it did not show products despite working very well in the simplex format. Thus, after having to remove three SNPs from the original set of 15, we obtained a reliable multiplex minisequencing protocol that allowed us to type 12 SNPs in a simple, direct and routine fashion.

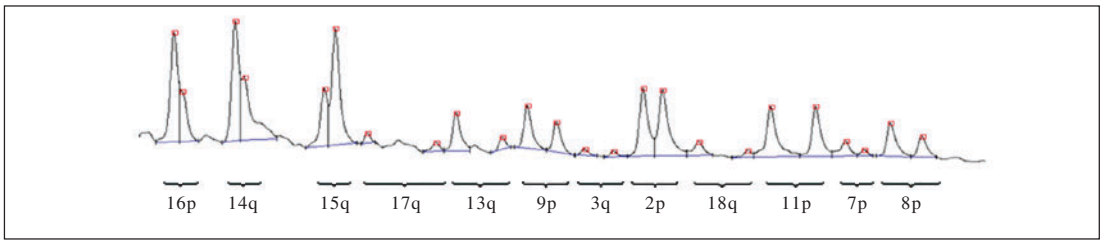

Figure 4. Multiplex minisequencing system with 12 SNP *loci*. The multiplex was used to type a DNA "pool" of 200 individuals.

DISCUSSION

We used SNPs chosen from a public database as a test model to develop a multiplex minisequencing protocol. The protocol has wide applicability in the genotyping of SNPs or in the diagnosis of point mutations in human diseases. During the development of this protocol we observed several important aspects that have to be observed to obtain satisfactory results.

In the first multiplex PCR reaction that will provide the templates for the minisequencing reaction, the most important aspect identified was the optimization of the yields of each PCR product. Weakly amplified products never worked well in the minisequencing protocol. However, strong amplification is no guarantee of success, especially with large amplicons. For instance, the SNPs 5q (413 bp) and 12q (576 bp) both presented a good yield in PCR and strong minisequencing peaks if they were done in simplex format, but not in multiplex. In general, shorter products are easier to adjust to the multiplex format and should be preferred. A uniform tail in the primers, such as the T7 sequence that we utilize, helps with the optimization of the multiplex system, because it will reduce the sequence differences among the primers during the PCR.

During the optimization of the purification step we also tested other protocols. One of them was based on magnetic beads coated with streptavidin (Syvanen et al., 1993). We synthesized biotin-linked primers, which have affinity to the streptavidin beads, allowing the elimination of primers and dNTPs not used in the PCR reaction. Although this is an elegant approach, the enzymatic purification method using ExoSAP was easier, faster and more efficient.

The quality of the primers was observed to be an important factor in minisequencing protocols. The multiplex reaction is done using a mix of different size primers with diverse sequences that could interact and be extended by DNA polymerase generating strong background signals. Besides, each primer has contaminants produced during its synthesis. Initially we used non-purified primers during minisequencing multiplex optimization and obtained high background signals and some weak peaks. When we switched to HPLC-purified primers we observed that the purification step was especially important for primers longer than 60-mers (data not shown).

Some minisequencing products presented stronger signal than others. Because of this we altered each primer concentration in the multiplex until we could obtain relatively even results. We could not establish why the primers presented such different peak heights. We used the Spearman coefficient and Kendall coefficient (SPSS Inc., Chicago, IL, USA) to evaluate the relationship between the peak signal quality in the multiplex and specific primer characteristics such as melting temperature (Tm), CG content or primer size through. However, no significant correlation coefficients were seen (data not shown).

The minisequencing primer tail must be selected with caution. Initially, we used tails formed by GATC repetition but we obtained product sizes different from the predicted ones. Most probably GATC tails can form internal annealing bonds that resist the denaturing conditions used in the gels. The selection of tails based on the sequence of pUC18 solved this problem.

In conclusion we successfully produced a reliable multiplex system for simultaneous typing of 12 SNPs. This can be used as a model for accurate, simple and inexpensive genotyping of single nucleotide polymorphisms or in the diagnosis of point mutations in human diseases.

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