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SNP genotyping using alkali cleavage of RNA/DNA chimeras and MALDI time-of-flight mass spectrometry

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ABSTRACT

Single nucleotide polymorphisms (SNPs) are now widely used for many DNA analysis applications such as linkage disequilibrium mapping, pharmacogenomics and traceability. Many methods for SNP genotyping exist with diverse strategies for allele-distinction. Mass spectrometers are used most commonly in conjunction with primer extension procedures with allele-specific termination. Here we present a novel concept for allele-preparation for SNP genotyping. Primer extension is carried out with an extension primer positioned immediately upstream of the SNP that is to be genotyped, a complete set of four ribonucleotides and a ribonucleotide incorporating DNA polymerase. The allele-extension products are then treated with alkali, which results in the cleavage immediately after the first added ribonucleotide. In addition, to obtain fragments easily detectable by mass spectrometry, we have included a ribonucleotide in the primer usually at the fourth nucleotide from the 3′ terminus. The method was tested on four SNPs each with a different combination of nucleotides. The advantage over other mass spectrometry-based SNP genotyping assays is that this one only requires a PCR, a primer extension reaction with a universal extension mix and an inexpensive facile cleavage reaction, which makes it overall very cost effective and easy in handling.

INTRODUCTION

Single nucleotide polymorphisms (SNPs) are thought to be the ideal markers for the dissection of complex traits in association studies and linkage disequilibrium mapping (1). Furthermore, SNP analysis will quite likely become a powerful tool for pharmacogenetics (2) and traceability in the agro-alimentary sector.

Many methods for SNP genotyping with detection by DNA microarrays, electrophoresis, mass spectrometry and plate-readers have been introduced (3). Particularly for mass spectrometry-based SNP assays, matrix-assisted laser desorption/ionization mass spectrometry (MALDI) is the method of choice due to its reliability, accuracy and speed (4). DNA analysis by mass spectrometry was reviewed extensively and for a general overview we refer the reader to publications cited therein (5,6). In order to analyze a SNP with a mass spectrometer, allele-specific products have to be prepared. Primer extension is the most commonly used format in conjunction with mass spectrometric detection. A primer is annealed upstream of a SNP and a combination of dNTP and/or ddNTP is used to synthesize an allele-specific product with the aid of a DNA polymerase. Two formats are used. One is multiple-nucleotide primer extension, such as in the Mass Extend assay [(7); www.sequenom.com]. The other is single-base primer extension, such as the GOOD assay (8). For the GOOD assay, single-base primer extension suffices because by including a cleavage step into its protocol the product mass is moved into a mass range where resolution of the mass spectrometer allows distinguishing even the smallest mass difference between the two alleles of a SNP (the smallest difference is 9 Da between thymine and adenine). However, the additional processing and enzymatic cleavage step requires the use of a 5′phosphodiesterase, which is a fairly expensive and somewhat temperamental enzyme (cleavage of cytidine is less efficient). Recently a method for the removal of primers via alkali induced RNA cleavage of chimeric primers was presented (9).

Here we present a novel concept for allele-preparation for SNP genotyping combined with MALDI mass spectrometry for detection. Rather than extending a primer immediately upstream of the polymorphism to be genotyped with deoxynucleotides (dNTP) and dideoxynucleotides (ddNTP), four

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ribonucleotides (NTP) are used in conjunction with a modified thermostable DNA polymerase that readily incorporates NTPs. Ribonucleotide incorporating DNA polymerases that manage to incorporate a limited percentage of ribonucleotides have been described in the literature (10). However, complete transcription by this mechanism is novel. Further, we have included a ribose sugar at the fourth nucleotide from the 3′ end of the primer. After the ribonucleotide primer extension reaction, cleavage is carried out using alkali. This results in the cleavage 3′ of the first added ribonucleotide and 3′ of the ribonucleotide in the primer leaving a product that carries the allele-information (Figure 1). We have applied this allele-distinction procedure with two conditioning procedures: the first uses a regular DNA backbone and ion exchange desalting for conditioning, while the second one uses phosphorothioate bridges in the primer that are charge-neutralized by alkylation and analyzed without desalting as is done in the GOOD assay. The method has been tested on four SNPs each with different combinations of nucleotides. Owing to the 1 Da mass difference between C and U, distinction between the two alleles of a Y SNP is achieved by replacing either cytidine by 5-methyl-cytidine or uridine by 5-methyl-uridine. This separates the two alleles by an easily measurable mass difference.

MATERIALS AND METHODS

Sixteen DNA samples from the CEPH collection (www.cephb.fr) were used for this work. They were selected based on representing all possible genotypes with the four SNPs used here. All samples were genotyped for these four SNPs by other methods for reference. Primers for PCR were synthesized by Biotez (Berlin, Germany) or MWG (Ebersberg, Germany), extension primers were from Eurogentec (Liège, Belgium) and PAGE purified. Sequences of the primers used in the four systems are listed in Table 1. SNPs with different nucleotide combinations were selected. Deoxynucleoside triphosphates, dNTPs, (N = A, C, G, T) and ribonucleoside triphosphates, NTPs, (N = A, C, C^{Me}, G, U, U^{Me}) were purchased from Roche Diagnostics (Mannheim, Germany) and Trilink Biotechnologies (San Diego). Platinum™ *Taq* HiFi DNA polymerase high fidelity was obtained from Invitrogen (Karlsruhe, Germany). General chemical reagents and α-cyano-4-hydroxycinnamic acid were purchased from Aldrich (Steinheim, Germany). G46E CS6R DNA Polymerase was provided by Roche Molecular Systems (Alameda). This Designer DNA Polymerase is a chimeric DNA polymerase comprising the 5′-nuclease domain of *Thermus* sp. Z05 DNA polymerase and the 3′-nuclease and 5′–3′ DNA polymerase domains of *Thermotoga maritima* (*Tma*) DNA polymerase. The 5′-nuclease activity is eliminated in this enzyme by the introduced G46E mutation. The wild-type *Tma* proof reading activity has been eliminated by mutation of 'Motif I' of the 3′–5′ exonuclease domain. Finally, the enzyme contains an E678G mutation that eliminates the selectivity of the wild-type enzyme against incorporation of C′-2-substituted nucleotides and which facilitates efficient incorporation of ribonucleoside triphosphates ['R' designation (11), Research samples of G46E CS6R DNA Polymerase may be obtained from Dr Thomas Myers (thomas.myers@roche.com), Director, Program in Core Research, Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, CA 95401, USA]. Ion exchange resin AG 50W-X8 H^+ was purchased from Bio-Rad (Marmes la Coquette, France). Thermocycling procedures were carried out in Eppendorf Gradient Thermocyclers (Eppendorf, Germany).

PCR amplifications were in 5 µl volumes with 10 ng of genomic DNA, 3.75 pmol of each primer, 0.5 U of DNA polymerase (Platinium HIFI *Taq* DNA polymerase), 1 mM $MgSO_4$, 0.1 mM dNTP (each), 0.5 µl of 10× buffer [600 mM Tris–SO_4, pH 8.9, and 180 mM $(NH_4)_2SO_4$]. Thermal cycling for PCR is carried out with the following profile: 4 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 60°C and 30 s at 72°C. This is concluded with 4 min at 72°C for final extension.

The cycled extension reaction follows without isolation of the PCR product or degradation of residual PCR primers or dNTPs. For a final volume of 10 µl, 0.25 µl of G46E CS6R DNA polymerase (4 U/µl), 1 µl of NTP (4 mM each), 0.5 µl of 200 mM Tris–HCl, pH 8.3, 0.5 µl of 500 mM NH_4Cl, 0.5 µl of 50 mM $MgCl_2$, 0.25 µl of extension primer (40 pmol/µl) and 2 µl of water are added to the amplification reactions. The composition of the chosen NTP mix is a function of the SNP that is to be genotyped. In the case of a Y SNP, the two alleles are not resolvable by mass as C and U are different in mass by only 1 Da. Accordingly, by using a mix of $AC^{Me}GU$ triphosphate or $ACGU^{Me}$ triphosphate, Y SNPs are readily assigned. The thermal cycling profile for the extension reaction is 2 min at 95°C followed by 40 cycles of 15 s at 95°C, 2 min at 65°C and 20 s at 72°C with a final extension of 4 min at 72°C.

For alkali cleavage, 1 µl of 3.3 M KOH or 3.3 M NaOH is added for a final concentration of 0.3 M and incubated at 70°C for 1.5 h. Concentrated NH_4OH (40 µl of 25 M) was also evaluated. For this, the incubation had to be carried out at 55°C overnight and the solvent removed in the SpeedVac at the end.

Following alkali treatment, two different methods for sample conditioning were used. The first was desalting the samples, which was done in the case of the phosphate backbone fragments. The second was alkylation in the case of phosphorothioate backbone fragments. Desalting is carried out by the addition of cation exchange resin charged with H^+. Four microliters of water were added for a final volume of 15 µl. One-third of the total reaction volume is added in resin and incubated for 20 min at room temperature under agitation. Thereafter, the sample was centrifuged for 30 s at 1000 r.p.m. 134 × *g* to sediment the resin. Five microliters of the supernatant was mixed with 7.5 µl of 60% acetonitrile and 2.5 µl of saturated α-cyano-4-hydroxycinnamic acid in acetone. One microliter was transferred to a nickel-coated aluminium MALDI target with 384 positions and dried at room temperature. In the case of cleavage with NH_4OH, the residual was dissolved in 10 µl of water and mixed with the matrix without treatment with ion exchange resin.

For the alkylation of phosphorothioate backbone products 1 µl of 3.3 M HCl was added to neutralize the alkali used for cleavage. Alkylation was achieved by adding 28 µl of a mixture of 12 µl of acetonitrile, 0.5 µl of 2 M triethylamine (TEA, pH 7.5 with CO_2), 2.5 µl 2 M Tris–HCl, pH 8.0, and 6.0 µl of methyliodide. After incubation at 40°C for 30 min, 10 µl of water were added. A biphasic system formed upon standing. Five microliters were sampled from the upper layer and

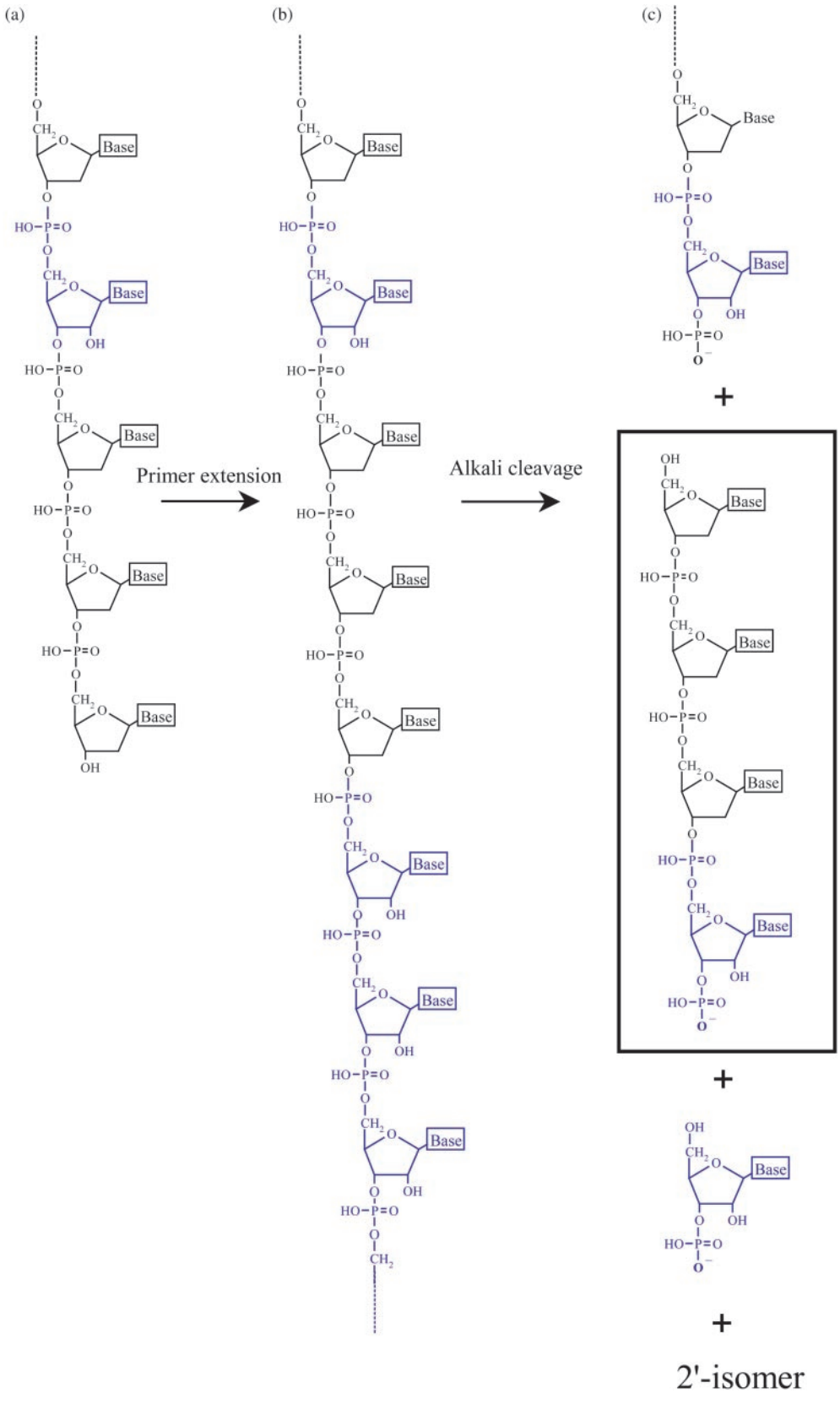

Figure 1. Principle of the ribonucleotide primer extension for SNP genotyping: (**a**) shows the primer prior to extension with a ribose sugar at the fourth nucleotide from the 3′ end; (**b**) extension of the primer with ribonucleotides; (**c**) cleavage of the extended product by alkali results in the cleavage inside the primer and after the first added nucleotide generating a mixture of 2′-HPO_4–3′OH and 2′OH–3′-HPO_4 products of identical mass.

Table 1. Sequences of the primers used for the four tested SNPs

Name		Forward PCR primer	Reverse PCR primer	Extension primer
Cav3-t+543c	Y	5′-TAAACCAGCACGCGGTTCCC	5′-CGTGTACGGTTCCAGCAAACAA	5′-TTCACTTGGCCAAATG**U**AAG 5′-TTCACTTGGCCAAATG**U**A^A^G
FANCA 10162	W	5′-GAGGGCTGTACCCTCCTACC	5′-GAGAGGCAGTCCCCATGATA	5′-CCGCTCTTTTCAGA**G**GCA 5′-CCGCTCTTTTCAGA**G**G^C^A
FANCA 10191	S	5′-TGCCTGTAATCCCAGCTACC	5′-AAGGCCTGGAGATAAGCAGC	5′-GGCCTGAGCATTGGT**C**CTT 5′-GGCCTGAGCATTGGT**C**C^T^T
KIAA 0604_18550	R	5′-CCTGCTCCCTCCTGAGTATG	5′-GGTCCTTGCTCTGCCACTTA	5′-CGCAGAGTGGCCT**G**TCC 5′-CGCAGAGTGGCCT**G**T^C^C

The symbol '^' indicates a phosphorothioate bridge and is combined with a final conditioning by alkylation. Bolded nucleotide in extension primer designates position of ribose sugar in primer.

added to 7.5 µl of 60% acetonitrile and 2.5 µl of a saturated solution of α-cyano-4-hydroxy-cinnamic acid in acetone. One microliter was transferred onto the MALDI target and dried.

The target is introduced into the MALDI mass spectrometer (Bruker, Reflex III or Bruker Autoflex, Bremen, Germany) for analysis. Analysis is carried out in negative ion mode with an acceleration voltage of +18–20 kV using pulsed ion extraction with a delay of 200 ns. Mass spectra were recorded in linear mode with external calibration.

RESULTS

The principle of the SNP genotyping procedure developed here is illustrated in Figure 1. A PCR is used to prepare a template of sufficient amount and reduced complexity for the following extension step. It was not necessary to remove residual primers and dNTPs from the PCR prior to the extension reaction. Extension products with deoxynucleotides were never observed under these extension conditions. Further, if residual PCR primers were extended, these products would be outside the observed mass ranges.

A primer is annealed immediately upstream of the SNP that is to be genotyped. Primer extension is carried out with a reaction mix containing four ribonucleotides and a thermostable, ribonucleotide incorporating chimeric DNA polymerase. This DNA polymerase was derived from *Thermus* sp. Z05 and *T.maritima* DNA polymerases. It has no proof reading activity and no 5′-nuclease activity. The primer extension requires transcription of the SNP sequence and incorporation of at least one additional ribonucleotide. The product is chimeric DNA/RNA where the DNA part is the primer and the RNA part is neosynthetic.

Allele-specific products for the mass spectrometric analysis are generated by alkali cleavage. This results in the cleavage 3′ of the first added ribonucleotide. Under these conditioning and analysis methods, a residual 2′ or 3′ phosphate group remains at the 3′ terminal nucleotide. From the mass we cannot distinguish 2′-HPO_4 + 3′-OH and 2′-OH + 3′-HPO_4. Quite likely products are a mixture of these two species. Products corresponding to a termination of the extension reaction after addition of the first ribonucleotide, which would manifest themselves by the lack of a terminal phosphate group, were never observed. Resolution of the MALDI mass spectrometer is best in the low mass range and not sufficient in the mass range where 20mers lie to distinguish between two allele-products generated by the outlined procedure. By including a ribonucleotide at the fourth position from the 3′ end of the extension primers, the mass of the products is shifted into the mass range of 1000–1500 Da where baseline resolution of products separated by as little as 5 Da is possible. The application of this procedure of SNP genotyping is shown in Figure 2. Different conditions for cleavage were investigated (NaOH, KOH, NH_4OH). A problem arising from the substantial amount of Na^+ or K^+ added is the difficulty to remove it again prior to mass spectrometric analysis. A limited degree of bleed through was observed (Figure 2). However, as adducts appear at defined positions relative to the real allele-masses in mass spectra possible confusion of an allele-peak and an adduct peak can be avoided by the choice of the alkali. We carried out this genotyping procedure with SNPs with different base composition (Figures 3 and 4). No cases of premature termination, preferential incorporation of bases or easier cleavage due to base context was observed. In no instances were the 3-base products of cleaved non-extended primers observed. This might be due to efficient primer extension or low efficiency of ionization of 3mers with two phosphate groups compared with 4mers with three interbase phosphate groups and a terminal phosphate group. Under the cycled extension conditions used one would assume that the extension primer was completely consumed.

Moving from deoxyribonucleotides to ribonucleotides results in an only 1 Da mass difference for a Y SNP due to the mass difference between cytidine and uridine rather than cytidine and thymidine. To resolve the two alleles of a Y SNP, we explored the possibility of replacing either cytidine by 5-methyl-cytidine or uridine by 5-methyl-uridine. Both 5-methyl-cytidine triphosphate and 5-methyl-uridine triphosphate are incorporated with nearly the same efficiency as cytidine triphosphate and uridine triphosphate by G46E CS6R DNA polymerase (Figure 4).

In the past, two major strategies for sample conditioning were developed in order to master the mass spectrometers inherent sensitivity to impurities. Owing to the negative charges on the sugar phosphate backbone, adducts of the phosphate groups with Na^+ and K^+ are observed when samples are not freed of these components. The most common method for desalting reactions is by the addition of an ion exchange resin. Alternatively, the negative backbone charges can be removed chemically by an alkylation reaction as is done in the GOOD assay (8). This requires the replacement of phosphate groups of the backbone by phosphorothioate groups. As shown here, a negative ion mode variant of the GOOD assay can be used where a single negative charge is introduced with the terminal nucleotide in analogy to what was shown by Sauer and Gut

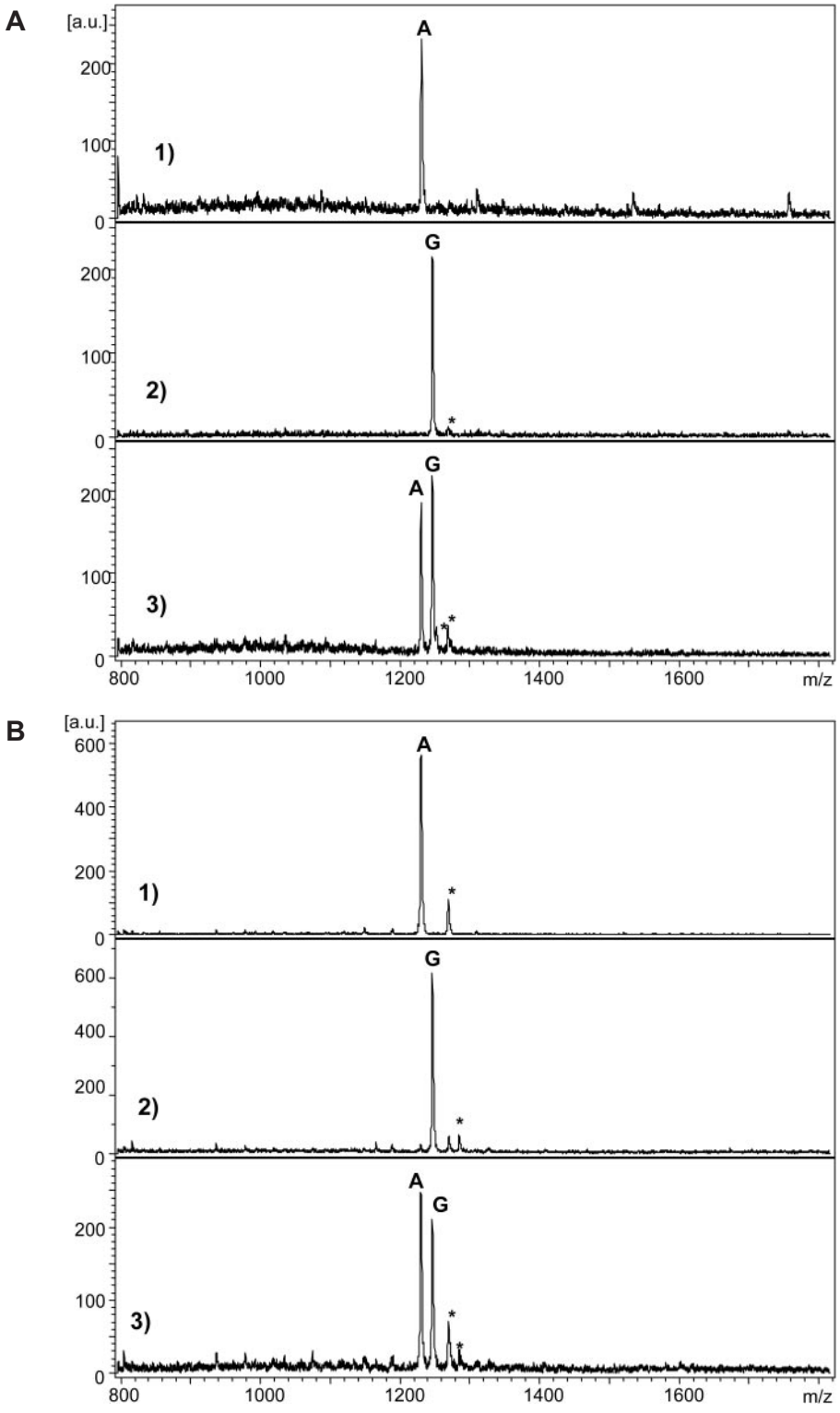

Figure 2. Spectra of the SNP KIAA 0604_18550 (A/G): (**a**) shows cleavage with NaOH. Na^+ adduct peaks (asterisk) are observed at +22 Da from the real allele-peaks; (**b**) shows cleavage with KOH. K^+ adduct peaks (asterisk) are observed at +38 Da from the real allele-peaks. In addition +22 Da Na^+ adducts are detected due to ubiquitous Na^+. (1) Mass spectrum of a homozygous DNA sample for the first allele, (2) mass spectrum of a homozygous DNA sample for the second allele, and (3) mass spectrum of a heterozygous sample.

(12). Both methods for sample conditioning were implemented here. Genotyping results with desalting are shown in Figures 2 and 3a, while Figures 3b and 4 show results using an extension primer containing phosphorothioates and alkylation (no desalting). Phosphorothioate is susceptible to oxidation under harsh alkali conditions. The conditions for the cleavage reaction were selected to cause minimal oxidation. However, peaks with −30 Da ($-SCH_3$ + OH) relative to the

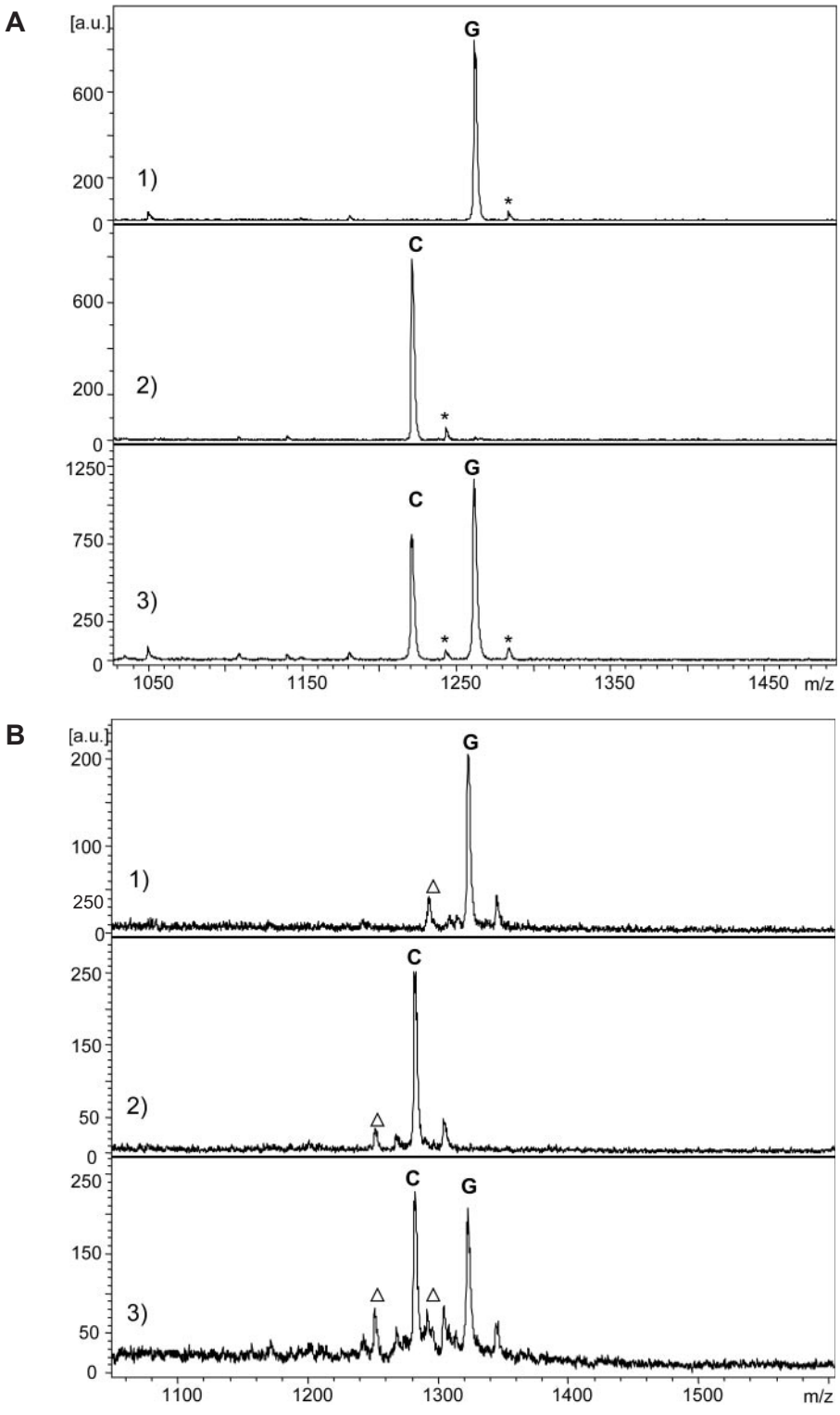

Figure 3. Spectra of the SNP FANCA 10191: (**a**) shows the protocol using desalting with ion exchange resin, Na^+ adducts are labeled with (asterisk); (**b**) shows the protocol with alkylation and no desalting. Peaks associated with exchanges of S by O, which then cannot be alkylated, are labeled with open triangle. Homozygous samples for each of the two alleles and a heterozygous sample are shown (1–3).

allele-peaks were observed (Figures 3b and 4). Again these peaks appeared at defined masses and are thus easy to identify.

The four SNPs shown here were selected from SNPs that had been genotyped previously at the CNG by the GOOD assay (Table 1). Repetition of the genotyping of all four SNPs under the optimized conditions provided in Materials and Methods always resulted in the correct genotypes relative to the control genotyping. The DNA samples of the CEPH collection were selected to be able to verify genotypes also based on the transmission of alleles from one generation to the

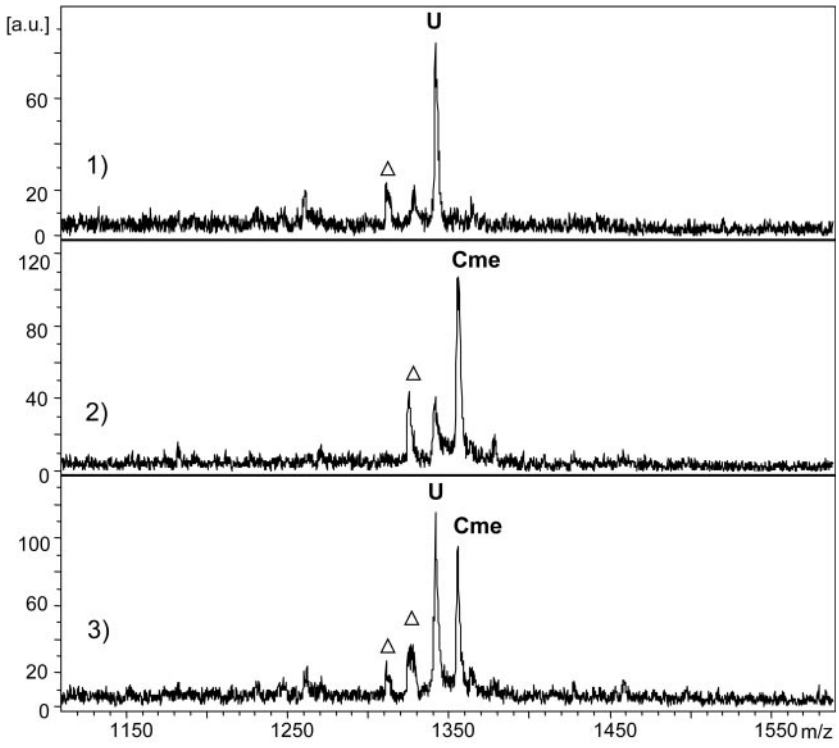

Figure 4. Mass spectra of the SNP Cav3-t+543c: conditioning is carried out according to the GOOD assay where the sample is subjected to alkylation, which neutralizes the negative charges on the phosphate backbone. An example for U and C^{Me} is shown for DNA samples homozygous for either of the alleles as well as a heterozygous DNA sample (1–3). Peaks associated with exchanges of S by O, which then cannot be alkylated, are labeled with open triangle.

Table 2. Sequences and masses of the allele-products

Name	Allele 1	Mass allele 1/Da	Allele 2	Mass allele 2/Da
Cav3-t+543c	AAG**C**	1277.9	AAG**U**^{Me}	1292.9
	AAG**C**^{Me}	1291.9	AAG**U**	1278.9
	A^A^G**C**	1338.0	A^A^G**U**^{Me}	1353.0
	A^A^G**C**^{Me}	1352.0	A^A^G**U**	1339.0
FANCA 10162	GCA**A**	1277.9	GCA**U**	1254.8
	G^C^A**A**	1338.0	G^C^A**U**	1315.0
FANCA 10191	CTT**C**	1219.8	CTT**G**	1259.8
	C^T^T**C**	1279.9	C^T^T**G**	1320.0
KIAA 0604_18550	TCC**A**	1228.8	TCC**G**	1244.8
	T^C^C**A**	1289.0	T^C^C**G**	1305.0

The symbol '^' indicates a phosphorothioate bridge. After alkylation with methyliodide, each phosphorothioate bridge results in a change of +30 Da relative to a regular phosphate bridge (mass difference between OH and SCH_3). Bolded nucleotide designates added ribonucleotide.

next. Masses of the products are listed in Table 2. The deviation of calculated and measured mass was within 1 Da throughout.

DISCUSSION

Here we describe a novel principle for allele-discrimination. A cycled primer extension reaction is carried out using four ribonucleotides and a ribonucleotide incorporating thermostable DNA polymerase. In contrast to an RNA polymerase, this polymerase is used with a sequence specific primer rather than a transcription start site. This allows positioning primers freely. After the extension reaction, the product is cleaved with alkali, which results in an extension primer product that only retains the first added nucleotide and effectively thus constitutes a single-base extension product. As primers are positioned immediately next to the position of the SNP that is to be genotyped, this added base carries the allele-information. Products were analyzed by MALDI mass spectrometry. Ribonucleotides were also included in the extension primers at the fourth base from the 3′ end. This allows removal of a large part of the extension primer during the alkali cleavage and shifts the allele-products into a more favorable detection range of the mass spectrometer. The allele-distinction method was combined with two versions of sample conditioning, desalting with ion exchange resin and alkylation as is used in the GOOD assay. Both methods were equally effective and allowed unambiguous genotyping. Phosphorothioates in the primers used in the alkylation version are susceptible to oxidation under harsh alkali conditions. However, the conditions chosen here for the alkali cleavage of the ribonucleotides was sufficiently mild to only slightly compromise the phosphorothioates. Alkali cleavage can be carried out with NaOH or KOH. As traces of Na^+ and K^+ tend to survive the desalting procedure it is desirable to be able to chose where adducts are detected. Na^+ adducts are 22 Da larger than their mother molecule, while K^+ adducts are 38 Da larger. When genotyping, for example, a T/A SNP (which is U/A for the allele-products, e.g. FANCA 10162) where the mass difference between the two alleles is 24 Da, a potential Na^+ contamination is undesirable as it can compromise allele-calling. The same applies for a C/G (FANCA 10191) where the mass difference is 40 Da. Here a K^+ adduct contamination is

undesirable. The alkali for cleavage can be chosen as a function of the SNP that is being genotyped. However, the desalting procedure is less efficient for K^+ than Na^+. Figure 2 shows genotyping of the SNP KIAA 0604_18550 cleaved with NaOH and KOH. We also tested NH_4OH for cleavage. However, in order to achieve efficient cleavage, the high concentrations and long incubations required seemed impractical even though NH_4^+ is a substrate that is very compatible with mass spectrometry. An advantage of the transition from DNA to RNA is that the mass difference of T/A SNP increases from 9 to 24 Da due to U replacing T. On the other hand this is a disadvantage for C/T SNPs that is remedied with the inclusion of C^{Me} or U^{Me} to replace cytidine or uridine, respectively. Accordingly, sufficient mass resolution is achieved to distinguish all base combinations of SNPs.

The presented procedure constitutes another addition to the ever-growing arsenal of SNP genotyping methods. Its main advantage over other SNP genotyping methods using mass spectrometric detection lies in the cost and ease of operation. As the G46E CS6R DNA polymerase efficiently incorporates NTPs, an intermediate enzymatic clean-up step with, for example, shrimp alkaline phosphatase (and ExoI) can be omitted. Using alkali to cleave both immediately following the allele-specific base and in the primer is very cost effective. Further, ribonucleotides are orders of magnitude less expensive than the ddNTPs that are commonly used in primer extension assays.

Clearly, the mechanism of primer directed transcription described here could also be of use for applications other than genotyping.

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Conflict of interest statement. None declared.

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