*Anal. Chem.* **2008,** *80,* 2342−2350

*Articles*

# Novel Mass Tags for Single Nucleotide Polymorphism Detection

**Klara R. Birikh,† Vladimir A. Korshun,† Pablo L Bernad,‡ Andrei D. Malakhov,† Natalie Milner,‡ Safraz Khan,‡ Edwin M. Southern,§ and Mikhail S. Shchepinov*,‡**

*Shemyakin-Ovchinikov Institute of Bioorganic Chemistry, RAS, Miklukho-Maklaya 16/10, 117997, Moscow, Russia, Tridend Technologies, a division of Oxford Gene Technology Ltd., Sandy Lane, Yarnton, Oxford OX5 1PF, UK, and Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK*

**A new method suitable for single nucleotide polymorphism detection and other applications based on oligonucleotide probe extension has been developed. The method is based on mass spectrometry and utilizes a single surface for affinity purification of extended probes and matrix-independent desorption/ionization of the cleavable labels. A new family of sulfur-linked laser-cleavable trityl labels with vastly improved flying abilities is implemented in this study. Corresponding reagents compatible with automated oligonucleotide synthesis are presented. Utility of this method for SNP genotyping is demonstrated.**

Mass spectrometry-based methods in molecular biology attract substantial interest and represent a very fast developing field. Admittedly, so far biological applications of mass spectrometry have been mostly limited to proteomic studies,[1] although there are some examples of its penetration into the molecular biology of DNA.[2,3]

Two different principles have been used for single nucleotide polymorphism (SNP) detection by mass spectrometry—one was based on the detection of the oligonucleotide probes themselves,[4] and the other relied on detection of cleavable labels.[5] The disadvantage of flying the entire oligonucleotides is that they have a much broader spectrum due to a large number of isotopic variants, and a lower sensitivity, because oligonucleotides do not desorb very well. On the other hand, this method does not require any labels and gives a more direct indication of the SNP nucleotide. Mass labels attract the attention of molecular biologists due to their extremely sharp spectra, which allows one to simultaneously detect labels that are only a few daltons apart in mass. This opens a prospective of a large degree of multiplexing, as opposed to fluorescent labels, which spectra are rather broad. This is especially important in the field of SNP genotyping.[6,7]

Trityl derivatives easily ionize, desorb, and can be efficiently detected by MS techniques (in other words, "fly" well) due to the stability of corresponding carbocations. Importantly, ionization is a result of a laser irradiation and does not require the assistance of matrix, although the presence of the latter does not adversely affect the flying. This has been put to a practical use by employing trityl mass tags (MT) as labels in combinatorial chemistry[8] in combination with (MA)LDI-TOF detection. That study used mass labels comprising the standard dimetoxytrityl protecting group with variable additional masses attached. In the present study, we demonstrate the use of a much improved version of the trityl mass tags for DNA labeling and SNP genotyping.

As opposed to the previously described method for SNP genotyping with cleavable mass labels,[5] the present protocol omits minicolumn purification of the reaction mixtures and a separate step of label cleavage by irradiation. Superb matrix-independent flying abilities of the trityl-based labels made it possible to use the surface of the mass spectrometry target as an affinity substrate in the purification protocol followed by label cleavage by laser upon desorption/ionization, altogether largely reducing sample handling.

## EXPERIMENTAL SECTION

**Procedures for Model Compound 11 and Modifying Phosphoramidites 7 and 15. 5′-*O*-{4,4′-Dimethoxy-4″-[2-(*N*-succinimidyloxycarbonyl)ethyl]trityl}-3′-*O*-(diisopropylamino-2-cyanethoxyphosphinyl)thymidine (7).** Thymidine **5** (727 mg, 3.0 mmol) was coevaporated with dry pyridine (2 × 20 mL), dissolved in pyridine (30 mL), half-evaporated, and cooled on an ice bath. 3-{4-[Chlorobis(4-methoxyphenyl)methyl]phenyl} propionic acid, *N*-oxysuccinimide ester (**6**; 1.42 g, 2.8 mmol) was added in one portion with stirring, and the stirring was continued for 4 h. The mixture was warmed to room temperature, diluted with $CHCl_3$ (150 mL), washed with water (2 × 100 mL), dried over $Na_2SO_4$, and evaporated, and the residue was purified by column chromatography (elution with 20−35% EtOAc in toluene, then 15−30% acetone in EtOAc−toluene (1:2), and, finally, with

* Corresponding author. E-mail: misha@tridend.com. Phone: +44-7766058079. Fax: +7-(495)-330-67-38.
† Shemyakin-Ovchinikov Institute of Bioorganic Chemistry.
‡ Tridend Technologies.
§ University of Oxford.

(1) Lane, C. S. *Cell Mol. Life Sci*. **2005**, *62*, 848−69.
(2) Hofstadler, S. A.; Sannes-Lowery, K. A.; Hannis, J. C. *Mass Spectrom. Rev*. **2005**, *24*, 265−85.
(3) Tost, J.; Gut, I. G. *J. Mass Spectrom*. **2006**, *41*, 981−95.
(4) Storm, N.; Darnhofer-Patel, B.; van den Boom, D.; Rodi, C. P. *Methods Mol. Biol*. **2003**, *212*, 241−62.
(5) Kokoris, M.; Dix, K.; Moynihan, K.; Mathis, J.; Erwin, B.; Grass, P.; Hines, B.; Duesterhoeft, A. *Mol. Diagn*. **2000**, *5*, 329−40.
(6) Marnellos, G. *Curr. Opin. Drug Discovery Dev*. **2003**, *6*, 317−21.
(7) Shi, M. M. *Am J. Pharmacogenomics* **2002**, *2*, 197−205.
(8) Shchepinov, M. S.; Chalk, R.; Southern, E. M. *Tetrahedron* **2000**, *56*, 2713−2724.



10.1021/ac071291y CCC: $40.75 
Published on Web 10/31/2007

acetone−toluene (1:1)) to give 5′-*O*-{4,4′-dimethoxy-4″-[2-(*N*-succinimidyloxycarbonyl)ethyl]trityl}thymidine. Yield 1.35 g (68%), white amorphous solid. NMR (DMSO-$d_6$): 11.3 (s, 1H, N*H*); 7.50 (s, 1H, *H*-6); 7.32−7.20 (m, 8H, Ar*H*); 6.88 (m, 4H, Ar*H*); 6.20 (apparent t, 1H, $J$ = 6.7 Hz, H-1′); 5.30 (d, 1H, $J$ = 4.4 Hz, O*H*); 4.31 (m, 1H, *H*-3′); 3.88 (m, 1H, *H*-4′); 3.74 (s, 6H, OC$H_3$); 3.25−3.13 (m, 2H, *H*-5′); 3.03−2.89 (m, 4H, ArC$H_2$C$H_2$); 2.81 (s, 4H, COC$H_2$C$H_2$CO); 2.3−2.1 (m, 2H, *H*-2′); 1.45 (s, 3H, CC$H_3$). To a solution of 5′-*O*-{4,4′-dimethoxy-4″-[2-(*N*-succinimidyloxycarbonyl)ethyl]trityl}thymidine (250 mg, 0.35 mmol) in dry DCM (10 mL) and DIEA (85 $\mu$L, 0.49 mmol), 2-cyanoethyl diisopropylchlorophosphoramidite (54 $\mu$L, 0.49 mmol) was added dropwise. After 2 h, the reaction mixture was diluted with $CHCl_3$ (50 mL), washed with washed with water (2 × 50 mL), dried over $Na_2SO_4$, and evaporated, and the residue was dissolved in DCM (2 mL) and precipitated in hexane (200 mL). The solid was filtered off and dried in vacuo. Yield 310 mg (97%), white amorphous solid. NMR (DMSO-$d_6$): 8.96 (s, 1H, N*H*); 7.47 (m, 1H, *H*-6); 7.39 (m, 2H, Ar*H*); 7.33 (m, 4H, Ar*H*); 7.24 (m, 2H, Ar*H*); 6.89 (m, 4H, Ar*H*); 6.26 (m, 1H, $J$ = 6.7 Hz, H-1′); 4.64 (m, 1H, *H*-3′); 4.12 (m, 0.5H, *H*-4′); 4.07 (m, 0.5H, *H*-4′); 3.82−3.50 (m, 10H, OC$H_3$, NC*H*, POC$H_2$); 3.39−3.24 (m, 2H, *H*-5′); 3.05−2.90 (m, 4H, ArC$H_2$C$H_2$); 2.78 (s, 4H, COC$H_2$C$H_2$CO); 2.66 (m, 1H, C$H_2$CN); 2.54 (m, 1H, C$H_2$CN); 2.48−2.33 (m, 2H, *H*-2′); 1.54−0.88 (m, 12H, C(C$H_3$)$_2$). $^{31}$P NMR (MeCN-$d_3$): 149.31, 148.28.

**Model Compound 11.** 4-(***N***-Succinimidyloxycarbonyl)-4′-methoxytritanol (447 mg, 1.0 mmol) in DCM (20 mL) was treated with butylamine (1.1 mmol); the solution was stirred for 1 h, then washed with 5% citric acid (20 mL), dried over $Na_2SO_4$, and evaporated to dryness. The residue was dissolved in 10% trifluoroacetic acid in DCM (5 mL), and 6-hydroxyhexanethiol (1.0 mmol) was added. The mixture was stirred at room temperature overnight, diluted with DCM (100 mL), washed with 5% NaHCO3 (2 × 50 mL), dried over $Na_2SO_4$, and evaporated to dryness. The residue was dissolved in dry pyridine (5 mL) and 4-(pentylaminocarbonyl)-4′-methoxytrityl chloride (1 mmol) (the amide was prepared from 4-(***N***-succinimidyloxycarbonyl)-4′-methoxytritanol and pentylamine and then converted to trityl chloride with AcCl) was added, and the mixture was stirred overnight. The mixture was poured into water (100 mL) and extracted with EtOAc (50 mL). The organic layer was washed with water (2 × 40 mL) and 5% citric acid (50 mL), dried over $Na_2SO_4$, and evaporated and coevapotated twice with toluene (30 mL). The resulting crude **11** was not purified. An analytical sample of **11** was isolated using preparative TLC (3% of EtOAc in toluene).

**1-*S*-{4,4′,4″-Trimethoxy-3-[5-(*N*-succinimidyloxycarbonyl)pentyl]trityl}-4-*O*-(diisopropylamino-2-cyanethoxyphosphinyl)-4-hydroxypentanethiol (15).** To a solution of 3-[5-(***N***-succinimidyloxycarbonyl)pentyl]-4,4′,4″-trimethoxytritanol (13) (1.50 g, 2.68 mmol) in AcOH (15 mL), 4-hydroxypenthanethiol (355 mg, 2.95 mmol) was added and the mixture was stirred for 2 h. The mixture was poured in water (300 mL) and extracted with EtOAc (150 mL). The organic layer was washed with water (2 × 100 mL), dried over $Na_2SO_4$, and evaporated to dryness. The residue was purified by column chromatography (30% EtOAc in toluene) to give the intermediate 1-*S*-{4,4′,4″-trimethoxy-3-[5-(*N*-succinimidyloxycarbonyl)pentyl]trityl}-4-hydroxypentanethiol. Yield 1.52 g (85%), colorless oil. $^1$H NMR (DMSO-$d_6$): 7.19 (d, 4H, $J$ = 8.8 Hz, H-2′,6′,2″,6″); 7.07 (d, 1H, $^4J$ = 2.3 Hz, H-2); 7.02 (dd, 1H, $J$ = 8.5 Hz, $^4J$ = 2.6 Hz, H-6); 6.85 (m, 5H, H-5,3′,5′,3″,5″); 4.25 (d, 1H, $J$ = 4.8 Hz, O*H*); 3.76 (s, 3H, OC$H_3$); 3.73 (s, 6H, OC$H_3$); 3.43 (m, 1H, C*H*OH); 2.80 (s, 4H, COC$H_2$C$H_2$CO); 2.60 (t, 2H, $J$ = 7.3 Hz, COC$H_2$); 2.47 (t, 2H, $J$ = 7.5 Hz, ArC$H_2$); 2.08 (m, 2H, SC$H_2$); 1.60 (m, 2H), 1.47 (m, 2H), 1.38 (m, 1H), 1.30 (m, 3H), 1.23 (m, 2H) (COCH$_2$C$H_2$C$H_2$C$H_2$, SCH$_2$C$H_2$C$H_2$); 0.94 (d, 3H, $J$ = 6.2 Hz, CHC$H_3$). This was phosphitylated similar to the nucleoside derivative to give the desired amidite **15** in 86% yield, colorless oil. $^1$H NMR (DMSO-$d_6$): 7.29 (m, 4H, H-2′,6′,2″,6″); 7.16−7.11 (m, 2H, H-2,6); 6.87−6.81 (m, 5H, H-5,3′,5′,3″,5″); 3.87−3.53 (m, 14H, OC$H_3$, POC*H*, POC$H_2$, NC*H*); 2.78 (s, 4H, COC$H_2$C$H_2$CO); 2.64−2.52 (m, 6H, ArC$H_2$, COC$H_2$, C$H_2$CN); 2.16 (m, 2H, SC$H_2$); 1.69 (m, 2H), 1.56−1.32 (m, 8H) (COCH$_2$C$H_2$C$H_2$C$H_2$, SCH$_2$C$H_2$C$H_2$C$H_2$); 1.19−1.10 (m, 15H, CHC$H_3$). $^{31}$P NMR (MeCN-$d_3$): 147.95, 147.08.

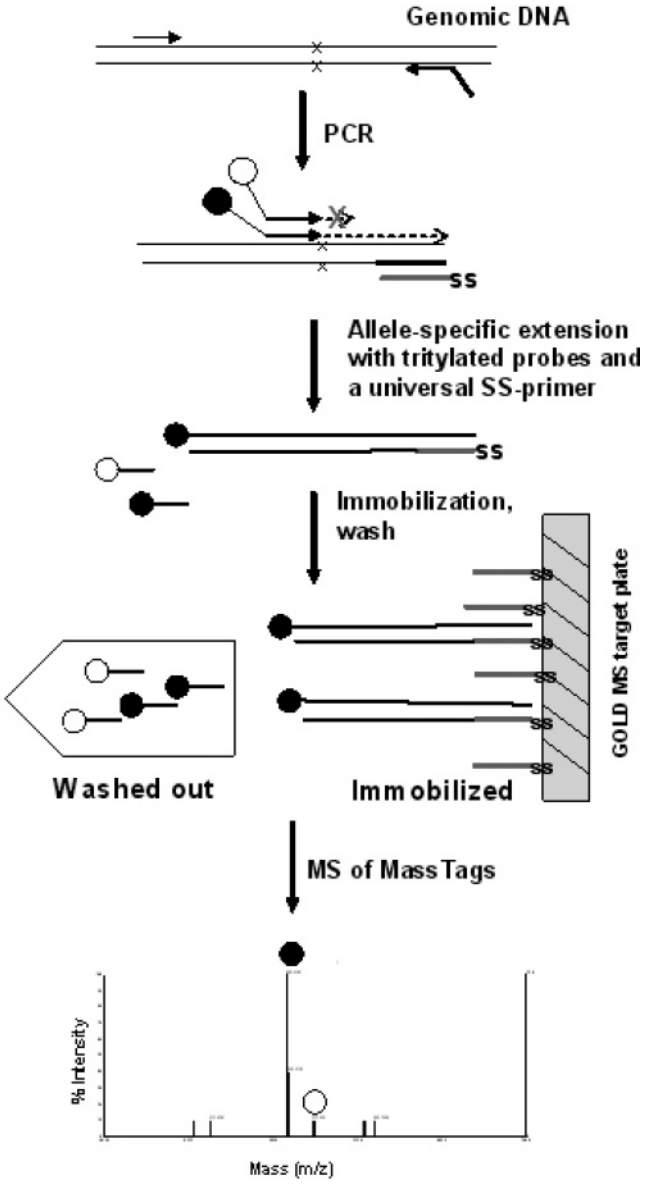


**Figure 1.** Schematic representation of the SNP detection method. Mass tags are depicted as circles; the disulfide group is indicated as SS.

**Oligonucleotide Synthesis.** Oligonucleotides were made in an Applied Biosystems 392 DNA/RNA synthesizer using standard phosphoramidite chemistry.

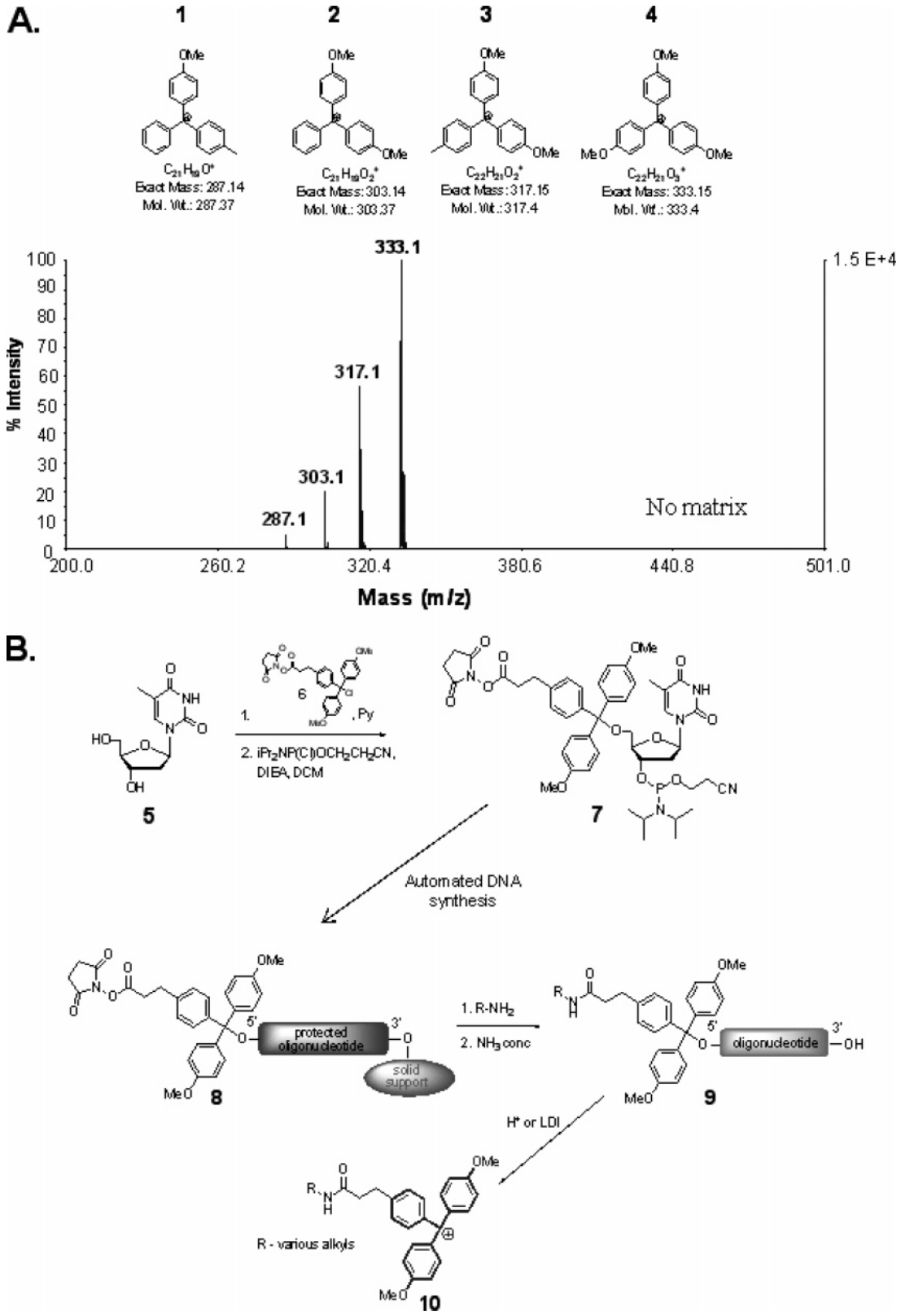


**Figure 2.** Trityls as mass tags for nucleic acids. (A) Trityl cations derived from methoxy/methyl-substituted tritanols and mass spectrum of an equimolar mixture of the tritanols; (B) synthesis of phosphoramidite of dimethoxytrityl MT and its use for oligonucleotide labeling and detection.

A disulfide group was introduced into oligonucleotides using dithiol phosphoramidite from Glen Research (Catalog No. 10-1937-xx). Branching phosphoramidite reagent ("trebler") was purchased from Glen Research (Catalog No. 10-1922-xx). Incorporation of these phosphoramidites was carried out under conditions recommended by the manufacturer.

Incorporation of the modifying phosphoramidites **7** and **15** at the 5′-end of the oligonucleotides was carried out through a

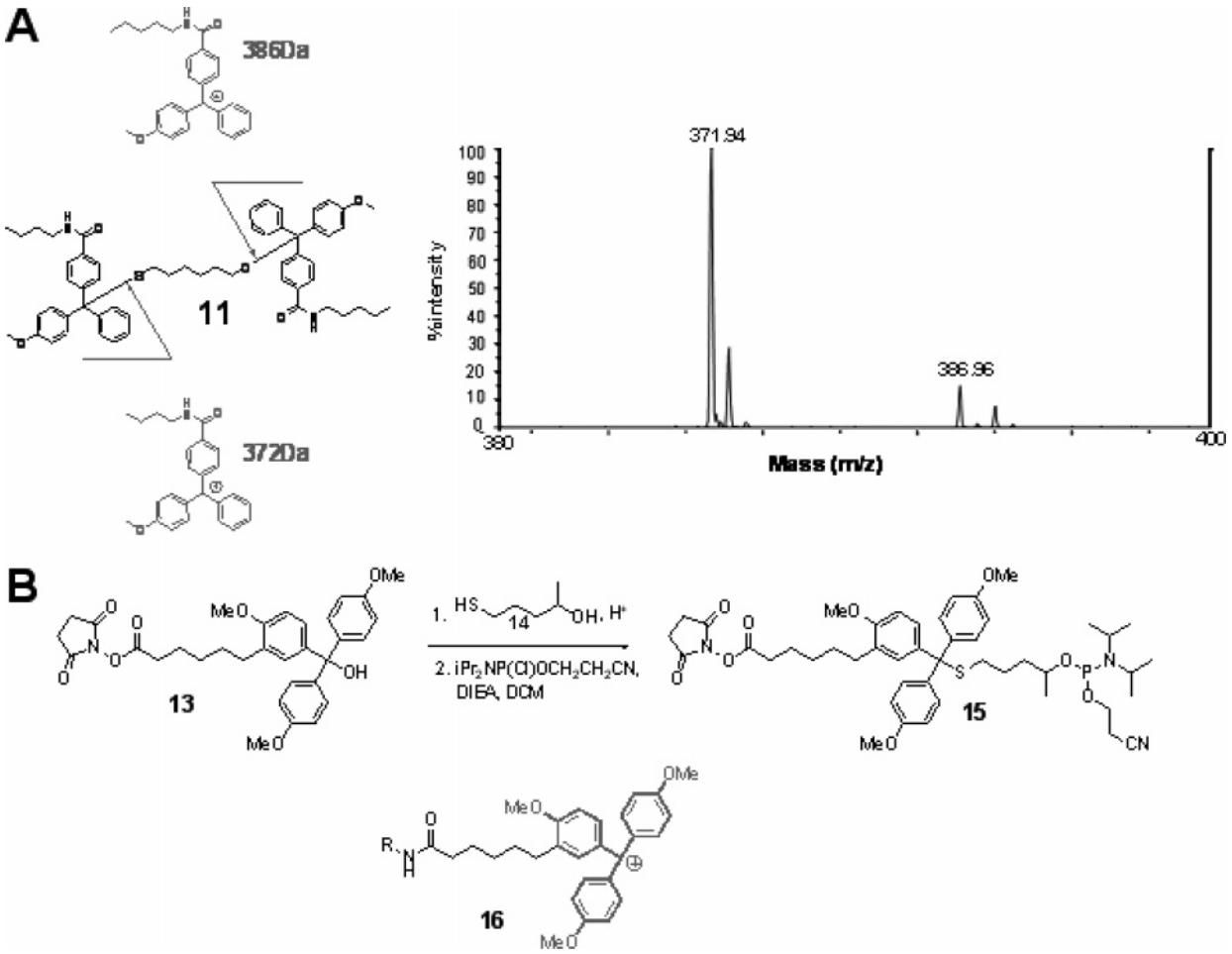


**Figure 3.** Attachment of trityl labels through a sulfide bond. (A) Model ditrityl compound (**11**) and its LDI mass spectrum (the corresponding trityl cations and their masses are depicted for convenience in mass spectrum interpretation; (B) synthesis of trimethoxytrityl MT phosphoramidite.

standard synthetic cycle of automated oligonucleotide synthesis, apart from the oxidation step, in which the concentration of Iodine was lowered 5-fold as compared to the standard conditions.

**PCR and Extension Reactions.** These were carried out using TopoTaq polymerase (Fidelity Systems) under the conditions recommended by the manufacturer. The template PCR was carried out in 20-$\mu$L reaction mixture with 10 ng of genomic DNA, with primers US and DS-tail (0.1 mM each); the thermo cycle was as follows: 45 s 94 °C, 1 min 60 °C, and 30 s 72 °C (30−40 cycles). For the following extension reaction with probe A/C and primer Tail-SS, the template PCR reaction mixture comprised 1/10 of the extension reaction volume; thermo cycle for the extension reaction was 30 s 90 °C, 1 min 55 °C, and 30 s 72 °C (6 cycles). Thermocycling was carried out using PCR amplificator Mastercycler (Eppendorf).

**Gold Affinity Purification.** After the extension, magnesium concentration in the extension mixture was adjusted to 0.1 M using 1 M $MgCl_2$, and then 4 $\mu$L of the extension mixture was spotted on a circular spot of a disposable gold-plated MS target plate (ABI), left at room temperature to dry for ~30 min. Then the plate was rinsed with water, placed in a container with 20−30 mL of washing buffer (3 M guanidine thiocianate, 10 mM Tris-HCl pH 9.0, 0.1 mM $HSCH_2CH_2SO_3Na$, 50% THF), incubated for 10 min at room temperature with gentle shaking, rinsed thoroughly with 50% EtOH, and taken for MS. All solvents used for washings were HPLC grade; salts were Molecular Biology grade.

**(MA)LDI-TOF Mass Spectra.** These were obtained using a Voyager Elite Biospectrometry Research Station (PerSeptive Biosystems, Vestec Mass Spectrometry Products) in a positive ion mode. Laser power was set on maximum. Signal from three to five shots was accumulated for each spectrum.

## RESULTS AND DISCUSSION

**Outline of the Mass Tag-Based SNP Genotyping Method.** Schematic representation of the SNP detection protocol developed in this study is depicted on Figure 1. The first step of the procedure, which is common in most SNP detection methods, is PCR amplification of the fragment, containing the SNP site.

The second step involves a well-developed and widely used principle of allel-specific primer extension in which alleles are discriminated by the inhibited extension of a 3′-mismatched probe. For each allele variant, a probe labeled with a certain trityl is introduced into the reaction. Thereby, each nucleotide in the variable position is assigned to a MassTag (the trityl, which mass is unique in the reaction). As a result of the extension reaction, probes that match the SNP variant are extended and the corresponding trityls get incorporated into a relatively long double-stranded DNA; whereas all mismatched probes along with an excess of matching probes remain in the form of single-stranded oligonucleotides.

The last and crucial step is to detect only the extended probes. To this end, the nonextended probes have to be removed from

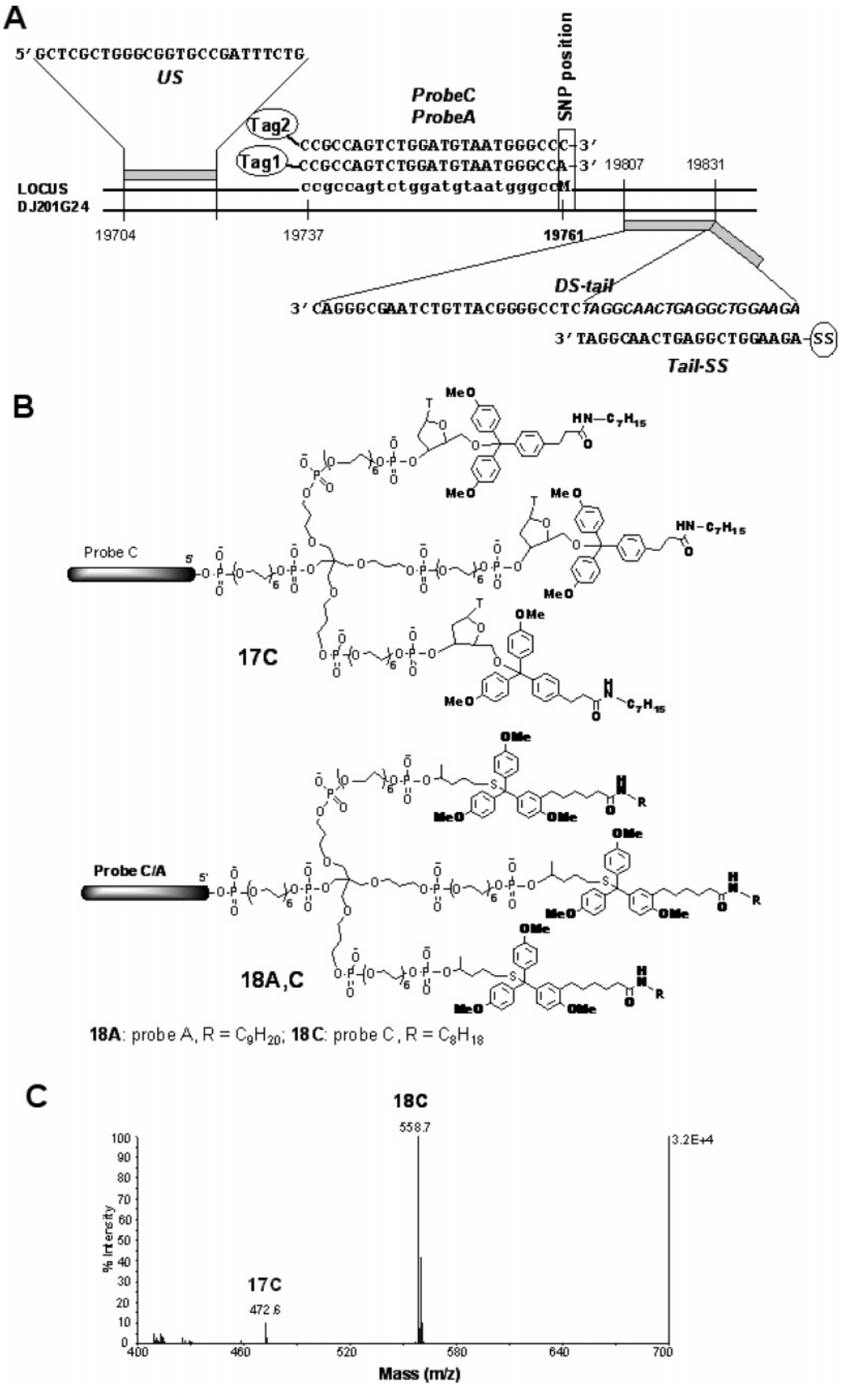


**Figure 4.** Primers and probes for SNP 19761 of MHCIII gene. (A) Sequence information (gray bars indicate the position of the primers on the genomic DNA, corresponding sequences are given aside, names of the oligonucleotides are in italic type, SS—a disulfide-containing group attached at the 5′-end of the oligonucleotide, Tag1 and tag2—mass tags); (B**)** overall structure of the trityl-labeled probes (structures that are cleaved off and detected in the mass spectrometer are depicted in boldface type), and (C) mass spectrum of an equimolar mixture of conjugates **17C** and **18C**.

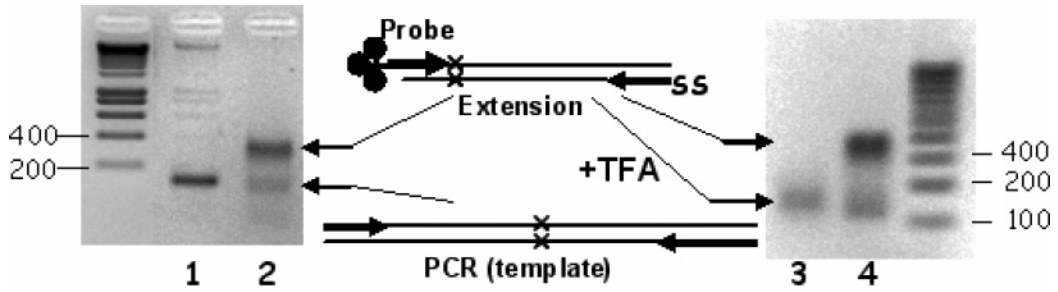


**Figure 5.** Gel electrophoresis analysis of the extension reaction with a trityl-labeled probe and disulfide-labeled primer**.** Lanes: 1, PCR of the SNP-containing fragment (~150 nt); 2, 4, extension reaction; 3, extension reaction treated with TFA.

the mixture, because bearing cleavable labels they would produce the same kind of signal in the mass spectrometer as the extended probes and bury the specific signal. In order to provide means for such a separation, a disulfide group was incorporated at the 5′-end of the reverse primer in the extension reaction. In order to be able to use a universal SS-modified primer for any set of primers and probes, we introduced a dangling segment (tail) at the 5′-end of the reverse PCR primer, which provided an annealing site for a universal primer during the extension reaction (the universal primer is depicted on Figure 1 as a gray line with SS at one terminus). As a result, double-stranded DNA molecules bearing trityl labels would also bear a disulfide group on the opposite terminus. Therefore, these molecules would be able to bind to gold, whereas nonextended probes would not possess such affinity. Crude reaction mixtures (with some extra $MgCl_2$ added) were spotted onto disposable gold-coated MS target plate (ABI) and left to dry at room temperature for 30 min. Usually, salt-containing solutions dry into uneven white patches; however, our samples dried into a transparent film evenly covering the surface of the sample circle of the plate. We attribute it to the presence of some detergent in the reaction buffer for the TopoTaq polymerase used in these experiments. The plate was then washed from unbound material, rinsed with 50% ethanol, and analyzed in a (MA)LDI mass spectrometer without any matrix added. On the mass spectrum, each allele present in the analyzed DNA sample should express itself as a peak of the corresponding mass tag.

**Mass Tags for DNA Labeling.** A series of compounds has been synthesized to define the physicochemical parameters that correlate the structural features of the triarylmethyl group with its MS performance (Korshun et al., manuscript in preparation). It has been found that the single most important factor influencing the stability of triarylmethyl carbocations is the number and position of electron-donating or cation-stabilizing groups. For instance, additional methoxy groups in ortho or para position of the trityl's phenyl rings stabilize the corresponding cation. Some of the investigated trityl cations are shown in Figure 2A along with the mass spectrum of the equimolar mixture of corresponding tritanols. One can see that in LDI conditions tritanols display only trityl cation signals, $[M - OH]^+$, rather than molecular peaks $[M + H]^+$. This is the case for other trityl derivatives as well, e.g., ethers.[8]

Well "flying" trityl cations **3** and **4** (Figure 2A) were elucidated as potential MT. Trityl core is responsible for the flying abilities of the trityl MT. The latter must contain also a "mass variation site" and must be covalently but reversibly bound to an analyte. To obtain a mass diversity, we used the derivatization of an activated carboxyl with various amines.[8] The central carbon atom of the trityl is an obvious "connecting point" to an analyte (in our case oligonucleotide).

Trityl MTs based on **3** were developed and used for oligonucleotide labeling (Figure 2B). Thymidine **5** was 5′-O-tritylated with NHS-containing dimethoxy trityl chloride **6** (Korshun et al., manuscript in preparation) and phosphitylated further to **7** (Figure 2B). The labeling reagent **7** contains a phosphoramidite tool for attaching it to an oligonucleotide during automated DNA synthesis to give **8** and NHS functionality for postsynthetic attachment of an added mass through reaction with an amine. Thus, one phosphoramidite reagent can be used to create a variety of mass tags due to different amines attached (conjugates **9**). With the reagent **7**, the trityl block gets attached to the oligonucleotide at the 5′-end through the central oxygen and can be cleaved off under acidic conditions or by laser irradiation releasing trityl MT **10** (Figure 2B), easily detectable by mass spectrometry. In this arrangement, ionization and flying properties of the mass tag directly correlate with its lability. Therefore, a synthetic scheme similar to **7** could not be accomplished for a better flying trityl cation **4**, because the corresponding ether proved to be too unstable.

It has been known that carbon−sulfur bonds in trityl thioethers show a higher stability than the corresponding trityl ethers. However, it has not been known how efficient the trityl cation release from trityl thioether in the course of LDI is.

In order to compare release of trityl cations from esters versus thioesters under LDI conditions, we synthesized a model compound **11** (Figure 3A). This compound comprises equimolecular composition of two monomethoxytrityl-MTs, one of which is connected through sulfur, while the other one is symmetrically connected through oxygen. The mass spectrum of this compound revealed a surprising result—a more chemically stable thioether turned out to be easier to brake by laser irradiation than ether. In other words, the trityl attached through sulfur gave a higher signal in LDI mass spectrometry (Figure 3A). This combination of properties of trityl thioesters appears to be favorable for designing MTs, because it might allow using labels based on trimethoxy trityl (**4**) by attaching them to an analyte through sulfur. This should increase their sensitivity in LDI and at the same time provide enough stability in chemical and biochemical manipulations.

These results prompted us to synthesize phosphoramidite for oligonucleotide labeling with a trimethoxytrityl-based MT (Figure 3B). Activated ether **13** (Korshun et al. , manuscript in prepara-

tion) reacted with **14** under acidic conditions[9] and phosphitylated to give **15**. This phosphoramidite can be used for DNA labeling similar to **7** in Figure 2B. The resulting oligonucleotides can be decoded in LDI by the mass of the corresponding trityl cation **16**.

**Genetic Model System for SNP Genotyping.** As a model system, we used a polymorphic site within the MHCIII gene (LOCUS DJ201G24; M(19761)= C or A). Primers for amplification of the SNP-containing fragment and allele-specific probes labeled with mass tags are presented on Figure 4A.

**Probes for SNP Detection.** Probe C labeled with two different trityls has been synthesized using phosphoramidite reagents **7** and **15**. To further increase sensitivity, three trityl labels were attached to each DNA molecule during automated synthesis using branching reagent Trebler (Glen Research).[10] PEG-hexamer linkers (Glen Research) were introduced between the oligonucleotide and the Trebler as well as between the Trebler and trityl labels (Figure 4B). These linkers were employed to prevent interference of bulky hydrophobic trityl residues with oligonucleotide hybridization and extension. After the synthesis, added masses were introduced by reacting amines with the NHS functionality of the trityl blocks. Thus, masses of the flying ions of the labels were 472 Da for **17C**-based MT and 558 Da for **18C**. After ammonolysis, fractions containing three labels were purified by HPLC. An equimolar mixture of oligonucleotides **17C** and **18C** was prepared and analyzed by MS without matrix.

As expected, probe **18C** labeled with trimethoxytrityl-based reagent **15** produced substantially higher signal then the probe labeled with dimethoxytrityl based reagent **7** (Figure 4C).

**Extension of Tritylated Probes.** Both labels described in the previous section proved to be stable under PCR conditions in a buffer with pH 9.0. Considering higher sensitivity of the label based on reagent **15**, this label was chosen for further experiments.

An extension reaction with labeled probe C (**18C** in Figure 4B) is demonstrated in Figure 5. The PCR reaction with primers US and DS-tail (Figure 5, lane 1) was diluted 10-fold and used as a source of template for the extension reaction with **18C** and primer Tail-SS. Six PCR cycles were carried out to produce double-stranded extension product (calculated length 115 bp). The reaction was analyzed by gel electrophoresis in 2% agarose gel. The major product of the extension reaction demonstrated a much lower mobility than the corresponding unmodified double-stranded DNA (Figure 5 lanes 2 and 4, compare to PCR template lane 1, and length markers). Control reactions that lacked either probe C or Tail-SS did not give any prominent products (data not shown). We attributed the changed mobility of the extension product to the presence of branched linkers and labels. In order to confirm the presence of trityl labels in the extension product, the reaction mixture was treated with TFA (1:10 v/v of 10% TFA was added to the reaction, incubated for 30 min at room temperature, and DNA precipitated by EtOH). Indeed, removal of the trityls from the extension product caused a reversal change in its mobility (lane 3), making it more similar to the corresponding double-stranded DNA. Somewhat increased mobility of this detritylated product

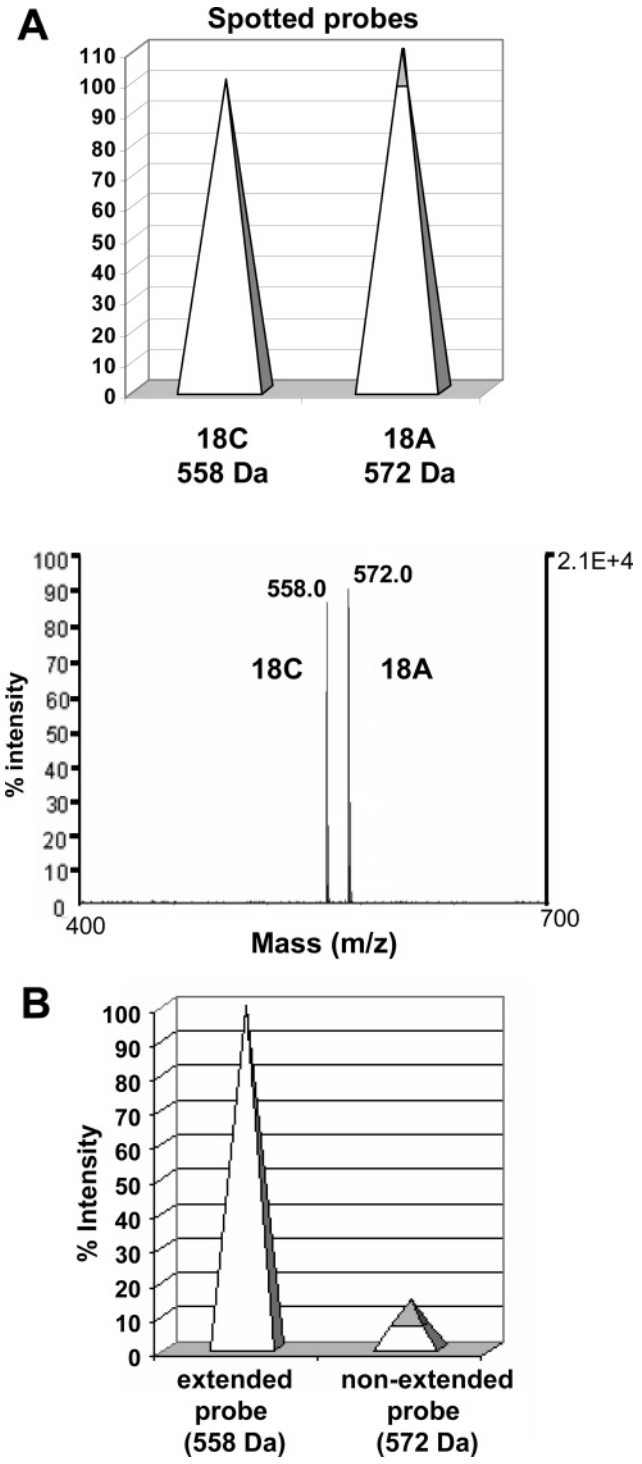


**Figure 6.** Detection of labeled and extended probes by MS**.** (A) Detection of equimolar mixture of labeled probes by MS. An equimolar mixture (0.1 $\mu$M) of probes **18A** and **18C** in 50% MeOH in water was spotted on the MS target and dried. Mass spectra were taken from four different locations on two different spots; a representative spectrum is shown. Intensity of **18C** was always taken for 100%, and relative intensity (see the diagram) and standard deviation (gray tip of the diagram pyramid) of the second probe **18A** was calculated. (B) Nonspecific binding of the tritylated probe to gold. A nonextended probe (0.5 $\mu$M) was added to the extension mixture after the reaction. The mixture was loaded on gold, washed, and analyzed by MS. Signals from the extended probe in each experiment were taken for 100%. The data were averaged from 12 spectra obtained in three different experiments. Gray tip of the pyramid represents standard deviation.

(9) Filippi, J. J.; Fernandez, X.; Lizzani-Cuvelier, L.; Loieseau, A. M. *Tetrahedron Lett.* **2002**, *43*, 6267.

(10) Shchepinov, M. S.; Udalova, I. A.; Bridgman, A. J.; Southern, E. M. *Nucleic Acids Res.* **1997**, *25*, 4447−54.

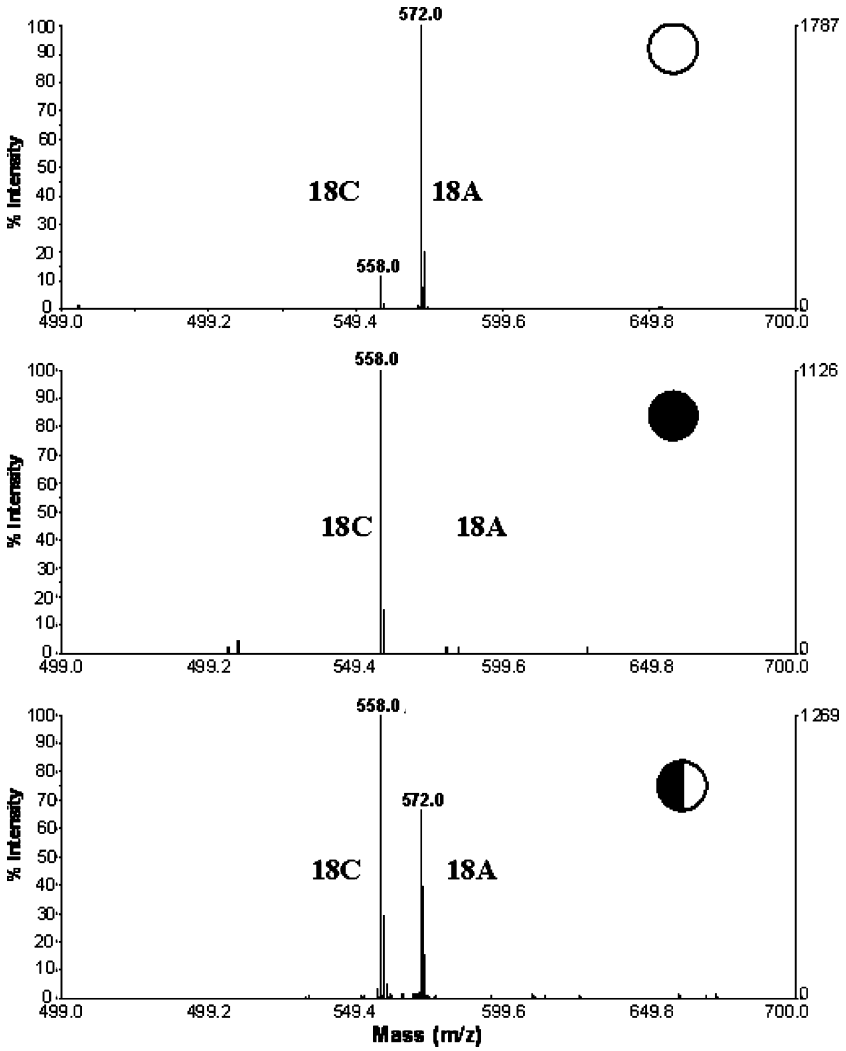


**Figure 7.** SNP genotyping. Representative spectra of the samples obtained from homozygous DNA (C at position 19761), top spectrum; homozygous DNA (A at position 19761), middle spectrum; and heterozygous DNA (both alleles present) , bottom spectrum.

can be attributed to the linkers, which remain attached to the DNA after the trityl removal.

Thus, successful incorporation of the trityl-labeled probe along with disulfide labeled primer into the extension product has been demonstrated.

A second probe, probe A, was also synthesized and labeled with the same type of trityl, but with a different added mass (**18A** in Figure 4B, flying ion mass 572 Da).

When an equimolar mixture of the probes **18A** and **18C** was spotted on a gold MS target plate, it gave approximately the same relative MS signal intensities for both probes (Figure 6A), whereas absolute values of the signal intensity varied in orders of magnitude. To further address the issue of reproducibility of relative signal intensities in a mixture of multiple probes, we prepared a mixture of probes composed of the same oligonucleotide labeled with five different MTs and monitored signal intensities in different locations on the target plate. Relative intensities were plotted as percent of the lightest probe intensity. Standard deviations of these values for all probes were within 10% (see Supporting Information). This confirmed an important notion that relative intensity of MT signals is area independent.

**Gold Surface Affinity Purification and Detection of the Extended Probes.** According to the principle of SNP genotyping protocol depicted in Figure 1, signals from the probes corresponding to genotypes, which are absent from the DNA sample, should not appear on the spectrum. False positive signals, however, might appear either as a result of extension through a 3′-mismatch or due to incomplete washout of the nonextended probes from the surface. In order to distinguish these two backgrounds and develop optimal washing conditions, we carried out a number of experiments where only one probe was taken to the extension reaction, while the second probe (at the same concentration) was added after the reaction, hence remained totally single-stranded. The washing conditions developed in this study provided fairly low nonspecific signal in this setup (within 15% of the main peak intensity, Figure 6B).

**Genotyping.** The background related to the extension through a 3′-mismatch is known to largely depend on the probe structure/SNP environment. Development of efficient allele-specific probes has been extensively studied yet remains mainly empirical. The structure of the probes used in this study (probe A and probe C) has been designed on the basis of experiments with unmodified DNA probes analyzed by conventional methods (data not shown).

To demonstrate the utility of the method depicted in Figure 1, DNA samples with all three known variants at the investigated SNP position were tested using this protocol —a heterozygous

DNA (A and C) and two homozygous—one with A and one with C nucleotide at the position of interest. Human genomic DNAs representing these genotypes were kindly provided by Dr. I.A. Udalova of Wellcome Trust Center for Human Genetics (University of Oxford, Oxford, UK).

As expected, homozygous DNAs produced only one major peak corresponding to the A or C probe, whereas the heterozygous DNA showed both peaks (Figure 7). Slight difference in intensity of the signals from probe C and probe A was consistent. It could not be attributed to the mismatch extension, because in that case we would have also seen such nonspecific extension of the probe C in the homozygous sample (top spectrum). Apparently, this difference in intensities stems from somewhat higher extension efficiency of probe C (probably due to G−C pair at the 3′-end rather then A−T).

## CONCLUSIONS

These experiments demonstrated the utility of the suggested SNP genotyping method, which allows use of multiple mass labels. A new generation of trityl-based mass tags for matrix-independent laser desorption/ionization has been successfully used to implement this method. A more efficient release of trityl cation from trityl thioether as compared to ether in the course of laser-assisted desorption/ionization has been demonstrated.


## ACKNOWLEDGMENT

We thank Dr. Susan Wheeler and Mrs. Kaajal Patel from Oxford Gene Technology Ltd. for their helpful advice in mass-spectrometry, and Dr. Irina Udalova from Wellcome Trust Centre for Human Genetics, Oxford Universuty, for her help in selecting the genetic model.


## SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.


Received for review June 19, 2007. Accepted September 14, 2007.

AC071291Y