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Nucleic Acids Research, 1998, Vol. 26, No. 11 **2827–2828**

MALDI-TOF mass spectrometric typing of single nucleotide polymorphisms with mass-tagged ddNTPs

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Received January 20, 1998; Revised and Accepted April 5, 1998

ABSTRACT

A matrix-assisted laser desorption/ionization time-of-flight mass spectrometry based method has recently been reported for the typing of single nucleotide polymorphisms using single nucleotide primer extension. This method is limited in some cases by the resolution of the mass determination, as the mass difference between nucleotides can be as little as 9 Da (the difference between A and T). A variation of this method is described here in which a mass-tagged dideoxynucleotide is employed in the primer extension reactions in place of the unmodified dideoxynucleotide. The increased mass difference due to the presence of the mass-tags substantially improves the accuracy and versatility of the procedure.

Single nucleotide polymorphisms (SNPs) are the most common type of genetic variation in the eukaryotic genome. There is about one SNP per kb in the human genome (1). These SNPs have been found to be the causes for a number of heritable diseases such as cystic fibrosis (2) and sickle cell anemia (3) and have been widely used in a variety of applications including the diagnosis of common genetic diseases, forensic identification of individuals and genetic mapping applications (4).

The recent development of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) has made it possible to analyze large macromolecules of biological importance (5). It has shown potential as an alternative to gel-based DNA sequencing (6,7), providing very high speed analyses with no requirement for fluorescence or radioactive labeling. Recently, a new method for the analysis of single nucleotide polymorphisms by MALDI-TOFMS has been described (8). In this method, a single dideoxynucleotide complementary to the base at the polymorphic position in the DNA template is added to the 3′ end of an oligonucleotide primer by polymerase extension. Determination of the mass of the extended primer identifies the added nucleotide, and thus reveals the nature of the base at the polymorphic position in the template. One limitation of this approach stems from the small mass differences between certain nucleotides, rendering it difficult to unambiguously identify the added nucleotide due to a lack of resolution in the mass spectrum. For instance, A/T transversion mutations were found to be the most frequent mutation of the k-ras gene in rat lung tumor and of the p53 gene in rat liver tumors (9,10). However, due to the small mass difference (9 Da) between ddT and ddA, the polymerase extension MALDI-TOFMS procedure described above was unable to distinguish the extension products of an A/T heterozygote (Fig. 1). Oligo(dT) sequences varying in length have been employed as mass-tags in primers to permit multiplexing of the procedure, as described in a recent publication (11).

In this report a new strategy is described in which a mass-tagged dideoxynucleoside triphosphate is employed in the strand extension reaction in place of the unmodified dideoxynucleoside triphosphate (ddNTPs). The increased mass difference due to the presence of dyes greatly facilitates the accurate identification of the added nucleotide, and is particularly useful for typing heterozygous samples.

A 15mer primer (molecular weight 4592.83 Da) and four 44mer synthetic oligonucleotide templates containing A, C, G and T respectively at the variable position six bases from the 5′ terminus were obtained from Integrated DNA Technologies, Inc. [The sequence of the 15mer primer employed was 5′-AAC-GACGGCCAGTAA-3′. The synthetic targets employed were 5′-Biotin TCTCCNTTACTGGCCGTCGTTTTACATGTGTGTT-TGGCCAAATA-3′, where N = A, C, G or T.] The primer was annealed to the template placing its 3′ end directly adjacent to the polymorphic site. The typical primer extension reaction mixture (20 µl) contained 2.0 µM primer, 1.0 µM each template, 6.25 µM ddNTP, 25.0 µM Renaissance® Nucleotide Analogs ddATP (NEN™ Life Science Products, Boston, MA) or 2 µl T Dye terminator, 4 U of AmpliTaq DNA polymerase FS, and 2 µl 10X sequencing buffer from a Dye Terminator Cycle Sequencing Core kit (Perkin-Elmer Corporation, Applied Biosystems Division, Foster City, CA). The primer extension reaction was carried out in a GeneAmp PCR system 9600 thermal cycler (Perkin-Elmer Corporation). The thermal cycling conditions employed were 15 s at 96°C, 60 s at 37°C and 120 s at 72°C for 25 cycles. The thermal cycling is to make sure the primer is completely extended. The extension products were purified using streptavidin-conjugated paramagnetic beads as described (12), and incubated with 2 µl of water at 90°C for 2 min to release the extended strand. An aliquot of 1 µl of the supernatant was mixed with 1 µl of matrix (saturated 3-hydropicolinic acid in a 1:1:2 mixture of water, acetonitrile and 0.1 M ammonium citrate), and was analyzed using a Bruker Reflex II time-of-flight mass spectrometer (Bruker Analytical Systems, Inc. Billerica, MA), equipped with a 337 nm N_2 laser and operated in reflectron, positive-ion mode with an acceleration voltage of 25 kV. Spectra are typically acquired by averaging 50 laser shots.

Figure 1 shows mass spectra obtained in extension reactions using unmodified ddNTPs, from equimolar mixtures of templates containing either A or G at the variable position of the template (corresponding to a A/G heterozygote; Fig. 1a), or A or T (corresponding to an A/T heterozygote; Fig. 1b). The incorporated nucleotides are the complements, ddT or ddC in Figure 1a, and

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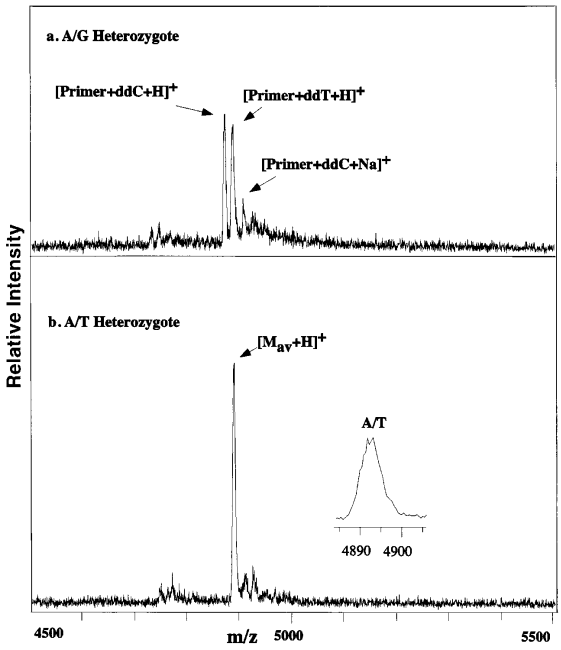

Figure 1. Single nucleotide primer extension assay employing 40 µM unmodified dideoxynucleotides (ddNTPs mixture), 4 µM primer, 25 mM ammonium acetate at pH 9.3, 2 mM magnesium acetate, 0.8 U/µl ThermoSequenase and equimolar mixtures of two synthetic oligonucleotide templates varying in the nature of the base at the polymorphic position: (**a**) 2 µM of template oligos 'A' and 'G'; (**b**) 2 µM of template oligos 'A' and 'T'. M_{av} is the average mass of the two extension products in (b). The extension reaction was performed using the following conditions: 95°C for 10 s, 37°C for 30 s and 72°C for 60 s for 25 cycles.

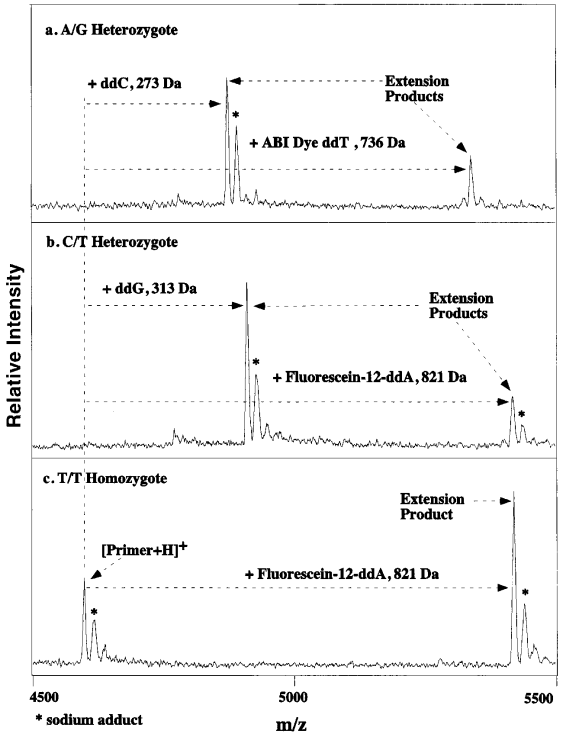

Figure 2. The single nucleotide primer extension assay was performed with AmpliTaq DNA polymerase FS as described in the presence of: (**a**) ddCTP, T Dye terminator, template 'A' and 'G' ; (**b**) ddGTP, Fluorescein-12-ddATP, template 'C' and 'T'; (**c**) Fluorescein-12-ddATP and template 'T'.

ddT or ddA in Figure 1b. Whereas the A/G heterozygote was readily identified due to the mass difference of 15 Da between the incorporated ddT and ddC, the A/T heterozygote was very poorly resolved and unassignable due to the small 9 Da mass difference between ddT and ddA. The presence of sodium or potassium adducts can make it even more difficult to measure peak masses correctly. For example, the sodium adduct of the ddC extension product from the target containing G at the variable position has a mass of 4889.01, only 2 Da less than the mass of 4891.05 corresponding to the ddA extension product from the target containing T at the variable position (data not shown).

Three examples showing how this approach to SNP typing is improved by use of mass-tagged ddNTPs are presented in Figure 2. Figure 2a shows the spectrum obtained from an A/G heterozygote using ddCTP and T Dye Terminator (Taq DyeDeoxy™ Terminator Cycle Sequencing Kit, Applied Biosystems, Inc., Foster City, CA). Incorporation of ddCTP adds 273.19 Da (ddC, $C_9H_{12}N_3O_5P$) to the mass of the primer, whereas addition of T Dye Terminator adds 736.10 Da ($C_{37}H_{31}N_5O_{10}P$). The mass difference between the two alleles is thus 462.91 Da, which is very easily resolved irrespective of the presence of sodium adduct peaks. Similar results are shown in Figure 2b for a C/T heterozygote extended with a mixture of ddGTP and fluorescein-12-ddATP (NEN™ Life Science Products, Boston, MA) with a mass difference in this case of 508.00 Da. Incorporation of ddGTP adds 313.21 Da (ddG, $C_{10}H_{12}N_5O_5P$), whereas addition of fluorescein-12-ddATP adds 821.20 Da ($C_{41}H_{38}N_6O_{11}P$). An example of a homozygote is presented in Figure 2c, in which a single major peak corresponding to the fluorescein-12-ddA extension product is present. The lower signal intensity for the extension products carrying the mass tags compared to those obtained using unmodified ddNTPs (Fig. 2a and b) was due to the higher efficiency of incorporation of unmodified ddNTPs by AmpliTaq DNA polymerase FS. Although fluorescent dyes were used here as mass-tags, they could be replaced by other types of modified molecules. However, this would require that a suitable DNA polymerase capable of efficient and specific incorporation be identified.

In summary, it is shown here that typing of SNPs by polymerase extension and MALDI-TOFMS analysis may be significantly improved by use of mass-tagged dideoxynucleotides as base-specific mass tags.

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