

# Multiplex genotyping of PCR products with MassTag-labeled primers

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## ABSTRACT

**A simple mass spectrometric based assay, the PinPoint assay, has previously been described for typing single nucleotide polymorphisms. The identity of a polymorphism is determined by mass differences of single base extended genotyping primers as determined by MALDI-TOF mass spectrometry. A simple method for multiplexing the assay is described, employing multiple primers with 5' oligo(dT) sequences (MassTags) which serve to mass discriminate the peaks of multiple extended and non-extended primers. The assay is extremely rapid and requires no labeling reagents.**

Single nucleotide polymorphisms are highly informative loci for genetic mapping, clinical and forensic applications (1). We recently reported a simple mass spectrometric method, the PinPoint™ assay, for SNP typing of polymerase chain reaction (PCR) products (2) which is extremely rapid. In the assay, a genotyping primer is extended by a single dideoxynucleotide residue complementary to the base at the polymorphic site and the identity of the base or bases added is determined by the incremental mass of the extended primer. Mass differences are determined by delayed extraction matrix assisted laser desorption ionization mass spectrometry (DE-MALDI-TOF) to an accuracy typically much better than 1 Da (2,3), which is sufficient to unambiguously discriminate between the four naturally occurring deoxynucleotide bases. Here, we report a simple modification supporting multiplexing the PinPoint assay at least 5-fold.

The multiplex PinPoint assay employs primers with a 3' portion of the sequence, typically 12–25 bases, complementary to the target and a 5' portion of 0–20 bases which are not complemen-

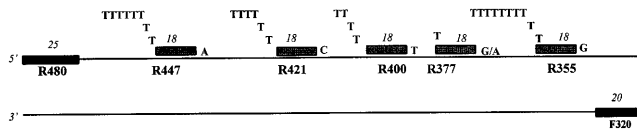
tary to the target, termed a MassTag. Here, the target-complementary sequence length was kept constant at 18 bases (Table 1) and the MassTags were composed entirely of thymidylic acid residues (dT). Since the MassTags are on the 5' end of the primer, they have little effect upon the  $T_m$  of the base-complementary region of the primer to its target. In principle, MassTags can be of any base composition, but dT residues are desirable since they are more resistant to fragmentation during MALDI-TOF than other bases (4). It is convenient to employ MassTags in increments such that the total length of each primer differs from any other primer by two bases. It then becomes virtually assured that all unextended and extended primers will be fully resolved in the mass spectrum.

As an example, a 185 bp PCR product from the *BRCA1* exon 13 locus (2,5) was genotyped at five loci. Four of these loci were homozygous and one locus was a (CT) heterozygote as confirmed by DNA sequencing on an ALF DNA sequencer (Pharmacia). The PCR product (0.4  $\mu$ M) was simultaneously treated with shrimp alkaline phosphatase and exonuclease I to destroy residual dNTPs and PCR primers (2). The PinPoint reaction mixtures contained 25 mM ammonium acetate, pH 9.3, 2 mM magnesium chloride, 10  $\mu$ M of each ddNTP, 1  $\mu$ M each of the genotyping primers, 10% v/v PCR product and 0.32 U/ $\mu$ l ThermoSequenase™ DNA polymerase (Amersham, Arlington Heights, IL). The mixtures were thermal cycled in a Perkin-Elmer 9600 DNA Thermal Cycler at 95°C for 10 s, 37°C for 30 s and 72°C for 60 s for 25 cycles. A 10  $\mu$ l aliquot was desalted by absorption/elution with Poros™ R2, as previously described by Nordhoff *et al.* (6), briefly mixed with cation anion exchange beads (2), mixed with one half volume of 50 g/l 3-hydroxycolonic acid (2) and analyzed on a DE-Voyager Mass Spectrometer Workstation (PerSeptive Biosystems, Framingham, MA).

**Table 1.** Primers used in this study

Primer	Length	+dT MassTag	Mass (Da)	Sequence (5'→3')
F320	20	–	5996.943	AGCCTTCTAACAGCTACCCT
R355	18	10	8599.613	TTTTTTTTTTGTCTCAAGGGCAGAAGA
R377	18	1	5861.77	pTTGCTTTGTTCTGGATTTC
R400	18	4	6594.343	TTTTACAATACACACCTTTTTC
R421	18	6	7393.839	TTTTTTTAAGATATCAGTGTTTGG
R447	18	8	8112.336	TTTTTTTTATAAAGGGGAAGGAAAGA
R480	25	–	7696.042	CATAAAATGTTGGAGCTAGGTCCTT

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**Figure 1.** Model of 5-fold multiplexed PinPoint assay. Each genotyping primer was composed of 18 bases complementary to the template and an oligo(dT) MassTag between 1 and 10 bases. The dideoxy base(s) complementary to the polymorphic site are indicated. The PCR primers were treated with exonuclease I and so did not participate in the multiplex genotyping.

Figure 1 depicts the loci of the 5-fold multiplexed PinPoint assay. Each primer was 18 bases in length with an additional oligo(dT) MassTag of 0–10 bases, and all extended and non-extended primers were resolved. (Primer R377 contained a 5' phosphate for purposes unrelated to this study.) Since the PCR product was treated with exonuclease I, the PCR primers did not carry over to the genotyping assay. In the spectra obtained (Fig. 2), the mass additions all corresponded to the known genotypes within 1 Da. The known heterozygous position following primer R377 produced two clearly mass resolved peaks corresponding to a ddG and ddA addition.

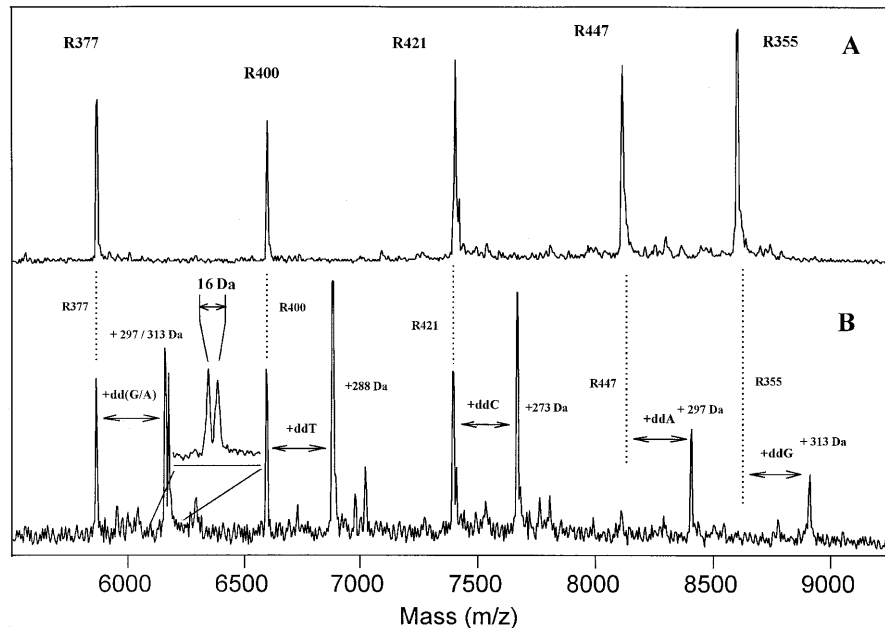
The efficiency of primer extension varied somewhat. Typically, in reaction mixtures containing these five primers, virtually all of primer R355 was extended, but only a portion of the other primers. The presence of at least one unextended primer peak in the spectrum of extended primers is desirable. Since the masses of all the unextended primers are known, the presence of at least one unextended primer peak serves to calibrate the masses of all the other peaks and so simplifies accurate determination of mass differences. Although it is not generally necessary, to assure the presence of one or more unextended primers in the reaction mixture a stop solution containing unextended primers and 10 mM EDTA can be added following thermal cycling.

The 5' dT-MassTags do not interfere with primer extension, even for primers that nearly abut one another on a target sequence. For example, there was a distance of only 4 bases between the 3' end of primer R377 and 5' end of the target-complementary portion of primer R355; yet the dT<sub>10</sub> MassTag did not interfere with primer extension of either primer. MassTags up to 20 bases have been tested with no evidence of adverse effects upon primer annealing or extension.

More than five primers can be simultaneously typed, with the major limitation appearing to be the detection limit for the longest length genotyping primer. Currently, the maximum length of extended primer that can be robustly detected, at the concentrations normally formed, is ~40 bases. The throughput is quite high, since mass spectra can be acquired at the rate of ~1–2/min in an automated data acquisition mode. For high throughput genotyping, relatively simple liquid handling devices are currently under development for automated sample desalting and spotting on the sample plates. Such an integrated system will be capable of preparing and acquiring spectra for many thousands of genotyping samples per day.

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**Figure 2.** MALDI-TOF of primer extension assay. The upper spectrum represents the reaction mixture before the single base extensions of the five primers annealed to BRCA1 PCR product and the lower spectrum is of the reaction mixture following the primer extension reaction. The polymorphic site following primer R377 is a (CT) heterozygote.