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Reliable genotyping of short tandem repeat loci without an allelic ladder using time-of-flight mass spectrometry

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Abstract DNA separations which traditionally have been performed by slab gel or capillary electrophoresis, may now be conducted via time-of-flight mass spectrometry (TOF-MS). The advantages of using a mass spectrometry approach for short tandem repeat (STR) characterization include a dramatic increase in both the speed of analysis and the accuracy of mass measurements. We report here typing of the STR loci TH01, TPOX, and CSF1PO as well as the sex-typing marker amelogenin using TOF-MS. Allelic ladders, which are typically used with electrophoretic separation systems to correct for mobility differences of DNA fragments under various conditions, are not needed for accurate genotyping with TOF-MS. A mass precision of 0.1% RSD, which corresponds to approximately 0.1 nucleotide, was routinely observed. Mass accuracies were better than a fraction of a single nucleotide when a daily mass calibration was used. STR microvariants, such as the TH01 allele 9.3, could be detected and resolved from alleles which differ by as little as a single base. In addition, the smaller PCR product sizes (55–125 bp) examined in this study have the potential advantage of being more successful when amplifying forensic samples with degraded DNA.

Key words Short tandem repeats (STRs) · Allelic ladders · Mass spectrometry · DNA fragment analysis · Microvariants

Introduction

Short tandem repeat (STR) loci are useful DNA markers for human identity testing and genetic mapping (Edwards et al. 1991; Fregeau and Fourney 1993; Kimpton et al. 1993). Dozens of tetranucleotide STRs have been examined by the forensic DNA community and validated for use in paternity testing and forensic identification (Hammond et al. 1994; Kimpton et al. 1996; Sprecher et al. 1996). The polymorphic variation in allele length has been previously detected by slab gel electrophoresis with silver staining (Sprecher et al. 1996) or multiple color fluorescent detection (Fregeau and Fourney 1993; Kimpton et al. 1993). More recently, capillary electrophoresis (Butler et al. 1994; Wang et al. 1995; Mansfield et al. 1996) has been used to resolve and type STR alleles. We introduce here the application of time-of-flight mass spectrometry to STR typing (see also Ross and Belgrader 1997).

Mass spectrometry offers unprecedented analysis times – on the order of seconds per sample – with excellent accuracy in measuring DNA fragment size. Substantial improvements have been made in recent years with the development of an effective ionization procedure, known as matrix-assisted laser desorption ionization (MALDI) and the discovery of new matrices, particularly 3-hydroxypicolinic acid (Wu et al. 1993). In MALDI, DNA samples are mixed with an organic matrix and allowed to co-crystallize on a sample plate. Multiple samples are typically spotted in an array on the sample plate and then sequentially analyzed. After the sample plate is placed in the mass spectrometer, which is under vacuum, a pulse of laser energy liberates a small portion of the DNA sample. While the generated ions travel to the detector in a matter of microseconds, multiple spectra are averaged for signal processing, which extends the measurement time to a few seconds. The DNA size is calculated by the time-of-flight to the detector in comparison to mass standards. A daily calibration (or alternatively when instrument conditions are varied) is usually sufficient to maintain a high degree of mass accuracy. We demonstrate here that the STR results from time-of-flight mass spectrometry analysis may be obtained more accurately than gel electrophoresis and orders of magnitude faster. In addition, STR alleles may be reliably typed without comparison to allelic ladders.

Materials and methods

Human genomic DNA samples representing several ethnic groups (African American, European, and Oriental) were purchased from

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Table 1 PCR product sizes and primer sequences used in this study

Locus Name (GenBank accession)	Known alleles*	PCR product size ranges	Primer sequences for this study	Locus reference
Amelogenin (M55418, M55419)	X, Y	106, 112 bp	5′-**B**-CCCTGGGCTCTGTAAAGAATAGTG-3′ 5′-ATCAGAGCTTAAACTGGGAAGCTG-3′	Sullivan et al. 1993
TH01 (D00269)	3,5,6,7,8,8.3,9,9.3, 10,10.3,11,12,13.3	55–98 bp	5′-CCTGTTCCTCCCTTATTTCCC-3′ 5′-**B**-GGGAACACAGACTCCATGGTG-3′	Edwards et al. 1991
TPOX (M68651)	6,7,8,9,10,11,12,13,14	69–101 bp	5′-**B**-CTTAGGGAACCCTCACTGAATG-3′ 5′-GTCCTTGTCAGCGTTTATTTGC-3′	Anker et al. 1992
CSF1PO (X14720)	6,7,8,9,10,11,12,13, 14,15	89–125 bp	5′-ACAGTAACTGCCTTCATAGATAG-3′ 5′-**B**-GTGTCAGACCCTGTTCTAAGTA-3′	Hammond et al. 1994

*: as reported in the literature; see STR Fact Sheets in STRBase (http://ibm4.carb.nist.gov:8800/dna/home.htm)
B: biotinylated strand

Bios Laboratories (New Haven, Conn.). K562 cell line DNA (Promega Corporation, Madison, Wis.) was used as a control in our experiments since the genotypes have been reported for this cell line with the STR loci examined here (GenePrint STR manual Promega 1995). Allelic ladders were reamplified using the PCR conditions and primers listed below from a 1:1000 dilution of AmpFlSTR Green I (CSF1PO, TPOX, TH01, amelogenin) allelic ladders (PE Applied Biosystems, Foster City, Calif.).

Primers were designed for each STR locus using Gene Runner software (Hastings Software, Inc., Hastings, N.Y.) and sequence information from GenBank (http://www.ncbi.nlm.nih.gov). To improve the sensitivity and resolution in the mass spectrometer, primers were placed close to the repeat region to make the PCR product size ranges under 120 bp in size when possible (Table 1). In the case of amelogenin, previously published primers were used. Primers were purchased from Biosource/Keystone (Menlo Park, Calif.) or synthesized in-house. One primer in each locus-specific set was biotinylated at the 5′ end.

Each PCR reaction contained 20 pmol of both forward and reverse primers, 1 U Taq polymerase (Promega Corporation, Madison, Wis.), 1X STR buffer (Promega) and typically 1–10 ng human genomic DNA. PCR was performed in 20 µl reaction volumes using a MJ Research DNA Engine (MJ Research, Watertown, Mass.). The PCR thermal cycling conditions were 94 °C for 2 min, 35 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and 72 °C for 5 min.

The final incubation at 72 °C favors non-templated nucleotide addition (Clark 1988; Kimpton et al. 1993). Following PCR, a 1 µl aliquot of the PCR product was typically checked on a 2% agarose gel to verify amplification success. A purification procedure involving solid-phase capture and release from streptavidin-coated magnetic beads was then utilized (Monforte et al. 1997) to remove salts which interfere with the mass spectrometry process (Shaler et al. 1996). In the final step, samples were evaporated to dryness using a speed vac and then reconstituted in 0.5 µl of matrix and spotted on the sample plate. The matrix typically used for STR analysis was 3-hydroxypicolinic acid (3-HPA; Lancaster Synthesis, Windham, N.H.) in 25 mM ammonium citrate (Sigma) and 25% acetonitrile. A GeneTrace-designed and built linear time-of-flight mass spectrometer was used as previously described (Wu et al. 1994).

Results and discussion

Mass calibration and precision

Data points in mass spectrometry are collected in spectral channels that must be converted from a time value to a mass value. This mass calibration is normally done at the beginning of each day or whenever conditions are changed for the mass spectrometer. We typically use two oligonucleotides, a 15mer (4507.0 Da) and a 36mer (10998.2 Da) for mass calibration. Over the course of multiple analyses under the same conditions, the calibration drift is neglible. Reanalysis of the standards, even after several hours, results in a shift of only a few Daltons. While the use of these mass standards was not ideal for calibration (since all of the examined STR alleles fell outside the mass range defined by 15mer and 36mer), reliable results were still obtained. It should be pointed out that a two-point method of calibration is typically used in time-of-flight mass spectrometry and works very well (Whittal et al. 1997). Future work will examine improvements in mass determination using internal standards as well as external standards over various size ranges.

Sample purity can play an important role in mass accuracy, resolution and precision. In particular, sodium and potassium ions in a sample can interact with the DNA molecules. A salty sample will be evidenced by adducts which are multiples of 23 Da or 39 Da (or a combination of the two) larger in size than the DNA molecule of interest. In addition, photo adducts from the matrix, which are approximately 139 Da in the case of 3-HPA, may be seen as a result of the MALDI process. These adducts can reduce the spectral resolution and mass accuracy, especially with larger sized DNA molecules. If a sample is not well purified, peak masses may be shifted slightly higher due to unresolved adducts (Shaler et al. 1996). We carefully purified our DNA samples and removed contaminating salts using a solid-phase capture and release method (Monforte et al. 1997). This procedure has been fully automated on a robotic workstation and will be described in a future communication. Using our robotic workstation in combination with a single high-throughput mass spectrometer, we have been able to purify and analyze over 2,000 samples in a single day.

To demonstrate the excellent reproducibility of mass spectrometry, we collected 15 mass spectra of a TPOX allelic ladder. A histogram plot of the obtained masses for alleles 8, 9, 10, and 11 show that all alleles were easily segregated and distinguishable (Fig. 1). Statistical analysis of the data found that the standard deviation about the mean for each allele ranged from 20 to 27 Da or approximately 0.1% relative standard deviation (RSD). For each TPOX allele, all of the data fell within four 30 Da bins, which corresponds to 120 Da or approximately 0.4 nucleotides since each nucleotide is near 300 Da in mass (C = 289.2, T = 304.2, A = 313.2, and G = 329.2 Da). The mass between alleles is equal to the repeat unit, which in

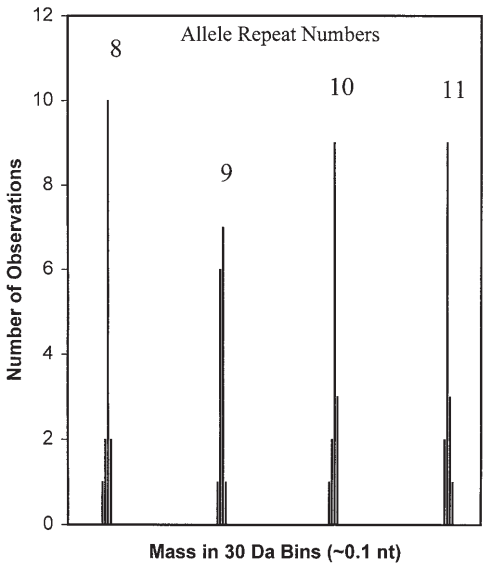

Fig. 1 Histogram showing mass precision in sizing TPOX alleles. The allele masses from 15 consecutive measurements of a TPOX allelic ladder were placed into mass bins of 30 Daltons (Da), which corresponds to approximately 0.1 nucleotide (nt). The standard deviation about each allele ranged from 20–27 Da

the case of TPOX is 1260 Da (AATG). Thus in Fig. 1, each allele differs by forty-two 30-Da bins and are easily distinguishable. Comparison to a similar histogram of data generated from fluorescent scanning of a polyacrylamide gel (Kimpton et al. 1993, Fig. 2) clearly shows the higher precision with mass spectrometry.

Mass accuracy compared to expected STR allele sizes

The GenBank sequence for each STR locus was downloaded into our Gene Runner software, and the number of repeats was counted. The mass of this reported GenBank sequence was then calculated, again using the Gene Runner software. The expected masses for other reported alleles in each STR system were obtained by adding or subtracting the mass of the repeat sequence. An additional mass of 313.2 Da was added to each allele to account for the nontemplate addition of adenine (Kimpton et al. 1993).

The two different strands of DNA can be examined in the mass spectrometer. For example, with the short tandem repeat TH01, if the bottom strand from GenBank is used, the expected repeat mass is 1259.8 Da for an AATG repeat. Alternatively, if the top strand is examined, the expected repeat mass is 1210.8 Da for a TCAT repeat. An examination of mass differences between alleles in a TH01 allelic ladder showed that the sequence content of the repeat could be differentiated (Table 2). The upper strand was discernible from the lower strand due to the different sequence contents of the repeats on the two strands. On the bottom strand with an AATG repeat, the mass difference observed between the 9.3 and 10 alleles was 315 Da, or the deletion of an adenine (expected mass = 313.2 Da), as previously reported (Puers et al. 1993). Alternatively, on the top strand with a TCAT repeat, the mass difference between the two alleles was 306 Da, or the deletion of a thymine (expected mass = 304.2 Da). Thus, even the STR repeat structure and nucleotide content of microvariants can be seen using our mass spectrometry technique.

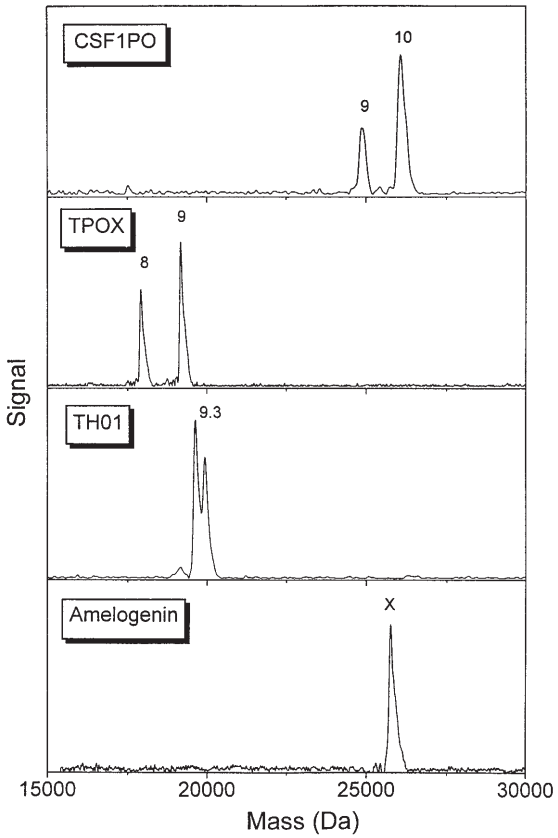

Fig. 2 Mass spectra from several STR loci and the sex-typing marker amelogenin using a K562 DNA template. The numbers above the peaks represent the allele calls based upon the observed mass. The expected types for K562 were obtained for all measured loci: CSF1PO (9, 10), TPOX (8, 9), TH01 (9.3, 9.3) and amelogenin (X, X). The TH01 peak is split because it is not fully adenylated

When STR samples are analyzed using mass spectrometry, peaks that come within 100 Daltons of an expected allele mass are assigned to a particular allele. Since the measured standard deviation was approximately 30 Da, a mass window of plus or minus 100 Da from the expected allele mass should define 99.9% (3.3 standard deviations) of all measurements for a particular allele. This 100 Da mass window represents a fraction of a single nucleotide and has applied well to the STR data we have taken thus far from over a dozen different DNA templates obtained from Bios Laboratory. A mass window of ± 100 Da should also allow the flexibility to correctly determine PCR-amplified alleles that include other nucleotides besides adenine resulting from non-templated nucleotide addition (Clark 1988). We have correctly typed the standard K562 DNA template at each of the loci examined (Fig. 2). When a mass calibration has been performed at the instrument conditions used to analyze the sample, peak masses have fallen within 100 Da of expected allele masses. As a fur-

Table 2 Mass differences between TH01 alleles using upper strand (TCAT repeat) or lower strand (AATG repeat)

	Upper Strand			Lower Strand	
	Expected (Da)	Observed (Da)		Expected (Da)	Observed (Da)
Allele 5–6	1211	1210	Allele 5–6	1260	1259
Allele 6–7	1211	1211	Allele 6–7	1260	1262
Allele 7–8	1211	1215	Allele 7–8	1260	1269
Allele 8–9	1211	1215	Allele 8–9	1260	1267
Allele 9–9.3	907	915	Allele 9–9.3	947	948
Allele 9.3–10	304	306	Allele 9.3–10	313	315
Allele 9–10	1211	1221	Allele 9–10	1260	1263
Repeat = TCAT = 1210.8 Da = –CAT = 906.6 Da			Repeat = AATG = 1259.8 Da = –ATG = 946.6 Da		

ther verification that the correct type was assigned to a sample, the mass difference between heterozygous alleles may be used to confirm the repeat sequence and number of repeats.

Advantages of smaller PCR products and resolution of STR alleles

By designing primers that are near the repeat region, smaller PCR products are amplified than are normally used with standard gel or capillary electrophoresis studies (see Table 1). The primary purpose in using smaller PCR products is that they may be detected with higher sensitivity and resolution in the mass spectrometer than larger amplicons. There are several advantages to using smaller PCR products besides an improvement in the mass sensitivity and resolution.

PCR efficiency typically improves with smaller amplicon size and shorter cycle times may be used. In a forensic context, smaller PCR product size ranges have the potential to handle highly degraded DNA samples. For example, in a recent validation study, the Forensic Science Service found that amelogenin and TH01, the two loci with the smallest allele sizes, were the last to drop out when extremely degraded DNA samples were used (Sparkes et al. 1996). Larger PCR products are typically examined with gel electrophoresis because the optimum resolution for separation of DNA fragments is in the size range of 100–350 bp. By examining smaller PCR products, we improve the detection of STR alleles in the mass spectrometer with added benefits to the molecular biology of the PCR reaction and to potential forensic situations with degraded DNA.

An excellent test for resolution with STR typing is the separation of the HUMTH01 allele 10, which contains 10 repeats of AATG and allele 9.3, a common variant of allele 10 with an adenine deletion in the seventh repeat (Puers et al. 1993). Using the mass spectrometry technique described here, a HUMTH01 allelic ladder containing alleles 9.3 and 10 can be fully resolved (Fig. 3). In addition, the loss of the adenine can be detected because a mass difference between the two alleles of 315 Da was observed, which is near the expected value for adenine (313.2 Da).

Comparison of allele sizing with and without allelic ladders

With the development of each new STR system, an allelic ladder is typically prepared from a mixture of known alleles to ensure reliable typing (Edwards et al. 1991; DNA recommendations 1994; Smith 1995). While multiple color fluorescent detection systems permit DNA fragment sizing using internal standards added to each sample (Mayrand et al. 1992), an allelic ladder is typically run for accurate determination of STR alleles (Edwards et al. 1991; Smith 1995; Kline et al. 1997). The variability of DNA mobilities under different electrophoretic conditions make an allelic ladder important to reliable genotyping of STR markers (Kline et al. 1997). However, the construction of an allelic ladder can be a time-consuming process. Also, whether an allelic ladder is prepared in a DNA typing laboratory or purchased from a commercial source, it increases the overall cost of typing each sample. Eliminating the need for allelic ladders to perform reliable STR genotyping would result in lower sample costs and lower costs for research and development of new STR loci.

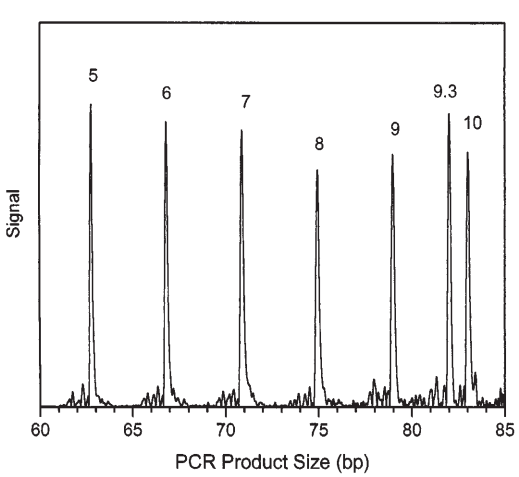

Fig. 3 TH01 allelic ladder demonstrating single-base resolution of alleles 9.3 and 10. The allele names, corresponding to the number of repeats, are included over each peak. The mass difference between alleles 9.3 and 10 was 315 Da, which corresponds to the difference of a single A (expected mass: 313.2 Da)

The high degree of accuracy for sizing STR alleles using mass spectrometry permits reliable typing of STR loci without the use of an allelic ladder. For example, in four different spectra of a heterozygote TPOX sample with alleles 8 and 11, the measured mass deviated from the expected mass by 41, 39, 29 and 38 Da for allele 8, and 16, 12, 28, and 53 Da for allele 11. On the other hand, the mass deviation for these same sample alleles from an allelic ladder, run just previous to their analysis, was 3, 1, 9, and 0 Da for allele 8 and 37, 9, 7, and 26 Da for allele 11. As might be expected, there was a slight improvement in mass accuracy when using an allelic ladder although the advantages are not significant. A mass difference of over 630 Da (half of a 1260 Da repeat) from the expected value would be required to incorrectly assign an adjacent allele with a tetranucleotide repeat. As part of another study, we reliably genotyped several thousand samples containing dinucleotide repeat loci as determined through obtaining comparable results by conventional gel electrophoresis methods (Butler et al., manuscript in preparation). With all samples tested to date, the correct alleles were accurately typed using the simple mass calibration procedure described here without need of an allelic ladder. In addition, further improvements in mass accuracy may be possible with mass calibrants that more closely reflect the mass range of measured STR alleles.

In conclusion time-of-flight mass spectrometry lends itself well to high-throughput STR typing with only a few seconds required for analysis of each sample. With mass spectrometry, the actual DNA oligomer resulting from PCR is detected intact rather than a fluorescent or radioactive label, as in gel electrophoresis. In addition, the high accuracy of mass spectrometry permits a measured peak mass to be correlated back to an STR allele without the use of an allelic ladder. Finally, the smaller PCR products (55–125 bp) described here for TH01, TPOX, and CSF1PO improve the sensitivity and resolution in the mass spectrometer as well as benefiting the molecular biology of the PCR reaction and potential forensic situations with degraded DNA.

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