Towards molecular sexing by MALDI-TOF

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Abstract

Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) is an alternative method for rapid and accurate nucleic acid analysis. Here, we demonstrated a potential of MALDI-TOF for robust gender identification of forensic DNA samples. The process is practical and straightforward. PCR product of amelogenin gene amplification was used as the template for primer extension reaction, and the extended product was analyzed with MALDI-TOF. Sex can be determined from the two distinguishable mass spectra patterns generated by male and female samples. This primer extension-coupled MALDI-TOF could analyze as low as 30 pg of genomic DNA with reproducibility.

Keywords: MALDI-TOF, primer extension, amelogenin, forensic science

Introduction

Mass spectrometry (MS) is a useful analytical tool that separates and detects molecules according to their mass per charge (m/z) properties. Last decade saw rapid development of MS as well as adoption in a wide range of applications. The soft ionization, matrix-assisted laser desorption ionization (MALDI) technique was first introduced in 1988 to improve the ability of MS to analyze large organic molecules (1) and is often coupled to time of flight (TOF) mass analyzer. The most distinctive characteristic of MALDI-TOF is that samples must be mixed and co-crystallized with small acidic organic compound called matrix. Major functions of the matrix are to promote the ionization of sample and to minimize sample degradation from the laser radiation (2). After the ionization step, all ions are transferred to TOF

mass analyzer where ions are separated and detected according to their mass. The analysis is rapid (a few seconds per sample) (3), label-free, and has high precision due to mass measurement (4-6). In the area of forensic DNA analysis, MALDI-TOF has been used in the detection of Simple Tandem Repeat (STR) markers for forensic DNA profiling without requiring allelic ladder (3,7). Butler et al. (7) have successfully analyzed three STR fragments by redesigning PCR primers to generate shorter PCR products (55-125bp). The study also suggested that the technique would benefit the analysis of degraded DNA sample (7).

Sex identification by MALDI-TOF analysis of DNA samples has also been established (8). In this study, PCR products from two sex-typing markers, amelogenin (AMEL) (9) and sex-determining region Y (SRY) (10), were analyzed. Sex was identified by the different patterns between male and female in mass spectra. Male samples showed three peaks of AMEL PCR product from X-chromosome (AMELX: 32,754 Da), AMEL PCR product from Y-chromosome (AMELY: 34,608 Da) and SRY PCR product (28,737 Da) in the mass spectra, while female samples showed a single peak of AMELX (32,754 Da) (8). However, the protocol requires pooling of products from four amplification reactions, which must then be purified by DNA purification kits. In addition, due to the large size of AMEL PCR products, salt adducts were not completely removed. This caused error in the mass measurement as well as low peak resolution. Here, we proposed an introduction of a primer extension step in order to improve the quality of the analysis of AMEL by MALDI-TOF. Our study demonstrated that the analysis of AMEL primer extension product generated high peak-resolution mass spectra, which enabled confident gender determination of the sample. The reproducible limit of detection at 30 pg of genomic DNA would also be useful in the analysis of degraded samples.

Objectives

To improve the application of MALDI-TOF for gender identification through DNA analysis.

Research Methodology

DNA Sample preparation

DNA samples were obtained from buccal swabs of male and female volunteers which had been extracted using Wizard® SV Genomic DNA Purification System (Promega). The genomic DNA samples were then quantified with Thermo Scientific NanoDrop™ 1000 Spectrophotometer and stored at -20°C until use.

PCR amplification

AMEL PCR primers were modified from (9). The nucleotide sequences were 5'-CCCTGGGCTCTGTAAAGAATAGTG-3' for forward primer and 5'-ATCAGAGCTTAAACTGGGAAG-3' for reverse primer. A 25-µl reaction was comprised of 1 U of AmpliTaq Gold®DNA polymerase (Applied Biosystems), 1x PCR reaction buffer, 1.5 mM of $MgCl_2$, 0.2 µM each of forward and reverse primers, 0.2 mM of dNTPs, and 10-100 pg of genomic DNA sample. PCR was performed in GeneAmp® PCR system 9700 (Applied Biosystems) with an initial denaturing step of 10 min at 94°C, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, then a final extension at 72°C for 7 min. The PCR products were separated in 5% denaturing polyacrylamide gel electrophoresis and visualized by silver staining. The gels were photographed using GBOX gel documentation (Syngene).

PCR clean up

To remove all excess dNTPs and primers after amplification, the PCR products were subjected to shrimp alkaline phosphatase and *E.coli* exonuclease I (SAP/ExoI) treatment prior to primer extension reaction. Ten-microlitre aliquot of PCR products was incubated with 1 U of SAP (Fermentas) and 10 U of ExoI (NEB) at 37°C for 20 minutes and 85°C for 20 minutes.

Primer extension reaction

The reaction was carried out in a 20-µl reaction volume containing 9 µl of SAP/ExoI treated PCR product, 1 U of Thermo Sequenase™ DNA Polymerase (GE Healthcare), 0.2 µM of AMEL extension primer (Table 1), 10 µM of ddCTP (Amersham Bioscience), 10 µM of dATG, dTTG and dGTP (Promega) mixture and 1 µl of Thermo Sequenase reaction buffer. The thermocycling condition was 50 cycles of 94°C for 20 sec, 48°C for 20 sec, and 60°C for 20 sec, then a final extension at 60°C for 2 min.

MALDI-TOF analysis

Sample desalting was carried out using cation-exchange resin 50 W-X8 (mesh size 200-400 μm, Bio-Rad). The resin was prepared following the protocols from (11) and (12). A 0.2-μl matrix solution (saturated 3-hydroxypicolinic acid (3-HPA) in 50% (v/v) ACN/ ultrapure water and 12 mg/ml dibasic ammonium citrate in ultrapure water at 1:1 ratio) was applied onto a 400-μm SmallAnchor target (MTP AnchorChip™ 384 TF, Bruker Daltonics) (13, 14). After the matrix solution droplet was completely dried, an aliquot of 0.2-μl desalted primer extension product was added on top of the dried matrix. Samples were analyzed with Autoflex™ II TOF/TOF (Bruker Daltonics) with nitrogen laser at 20kV in linear positive mode. All mass spectra were analyzed with Bruker Daltonics flex Analysis software.

Results

Primer design

For primer extension reaction, a new AMEL extension primer was designed to anneal close to the 6-bp deletion on AMELX (Figure 1). In the presence of ddCTP, AMEL primer extension would result in two different sizes of extended products (Table 1): the 5789.845-Da AMELX-specific extended product and the 5172.345-Da AMELY-specific extended product. The extension primer and extended product sequences were used to establish calculated mass in Daltons using the formula from (15).

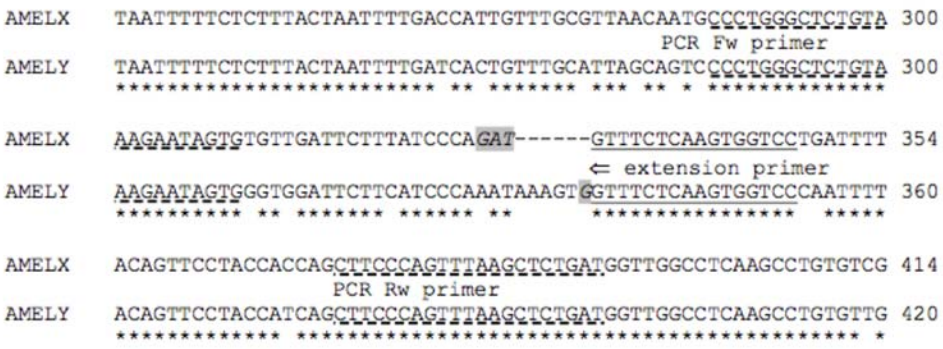

Figure 1 Alignment of AMELX (Genbank no. M55418) and AMELY (Genbank no. M55419) showing location and sequence of AMEL PCR and extension primers. The priming sequences of AMEL primers are underlined with dashes and the extension primer annealing sites are underlined with straight lines. Shaded letters indicate primer extended nucleotides.

Table 1 Sequence, size and mass in Daltons of AMEL extension primer and its products. Italic latters indicate extended nucleotides.

	Sequence from 5' to 3'	Size(bp)	Mass (Da)
AMEL extension primer	GGACCACTTGAGAAAC	16	4899.255
AMELY-specific product	GGACCACTTGAGAAAC*ddC*	17	5172.435
AMELX-specific product	GGACCACTTGAGAAAC*ATddC*	19	5789.845

PCR

During the optimization step, 100 pg of genomic DNA extracted from male and female buccal swab samples was employ in the PCR and the results are shown in Figure 2. Lane 1 which contained AMEL PCR products from male sample showed two bands of 106 and 112 bp, while Lane 2 containing the PCR products from female sample displayed only a band of 106 bp PCR product.

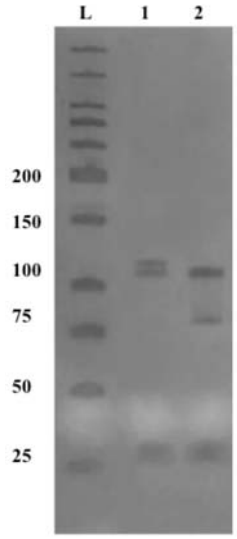

Figure 2 Optimized AMEL PCR results. The electrophoresis was performed in 5% denaturing polyacrylamide gel which was then silver stained. Lane L: low molecular weight ladder (NEB); Lane 1: AMEL PCR products from 100 pg of male DNA sample (106 and 112 bp); Lane 2: AMEL PCR products from 100 pg of female DNA sample (106 bp). The unexpected band in Lane 2 is commonly found in polyacrylamide gel electrophoresis and is known as shadow or ghost bands.

Primer extension reaction with MALDI-TOF analysis

The AMEL primer extension reaction was optimized according to the guidance in (16). It was found that 0.2 μM of extension primer was adequate to generate extended products as well as acting as an internal mass calibration for the MALDI-TOF analysis. Thermo Sequenase™ DNA Polymerase (GE healthcare, Buckinghamshire UK) is a modified DNA polymerase that can incorporate ddNTPs at the same rate as dNTPs (17), thus generating reliable results for primer extension reaction (15, 18). According to the mass spectra results, male samples displayed two peaks of AMELY-specific extended product (5172.435 Da) and AMELX-specific extended product (5789.845 Da; Figure 3A). Female sample was recognized by the presence of AMELX-specific extended product (5789.845 Da; Figure 3B). In this study, the minimum number of laser shots that showed results with acceptable signal-to-noise ratio of 3 (19) is 200.

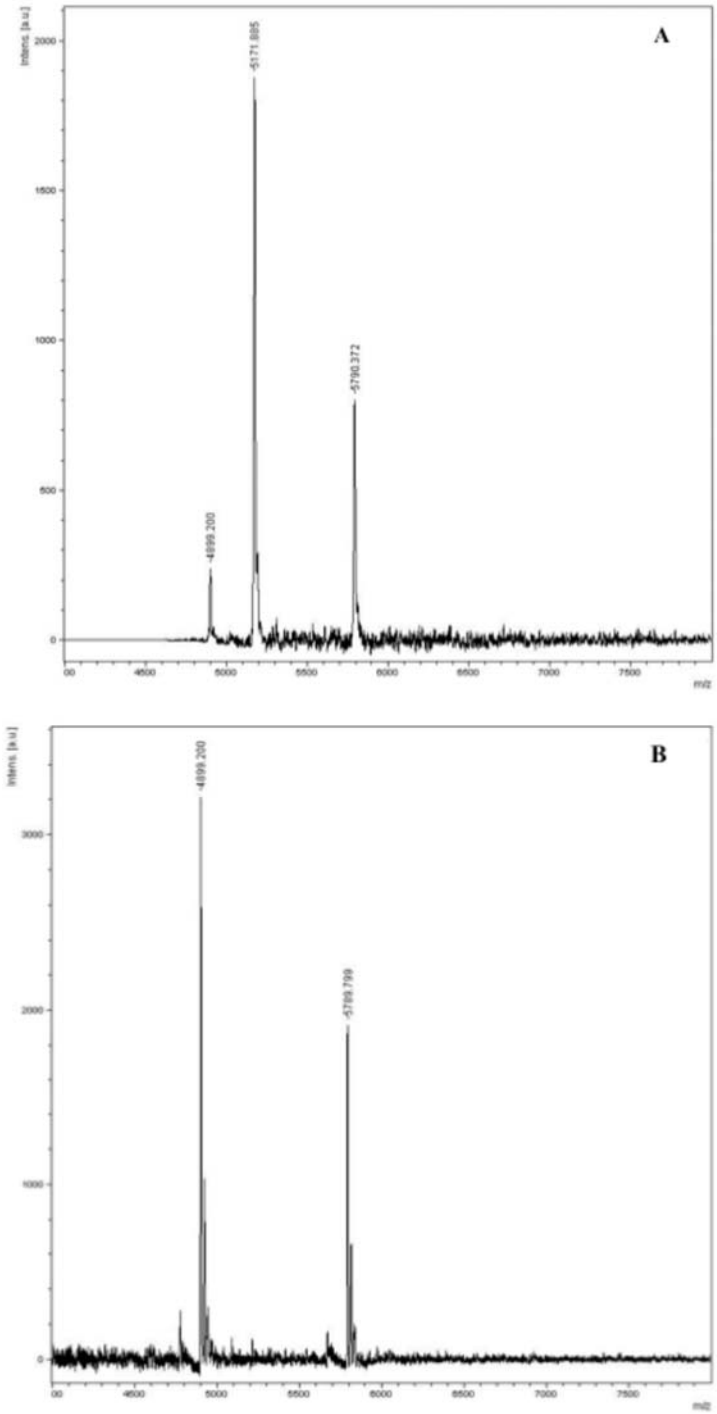

Figure 3 Representative mass spectra obtained from MALDI-TOF analysis of the AMEL primer extension products. A) Male sample showed two extended products of 5172.435 and 5789.845 Da. B) Female sample showed only one extended product of 5789.845 Da. The instrument was operating in linear positive mode with 200 shots of laser at 20kV. Signal-to-noise ratio of each peak must be greater than 3 in order to be recognized in a mass spectrum.

Determination of limit of detection

After the optimization of primer extension reaction, 10, 20, 30, and 50 pg of male DNA sample were tested to verify the limit of detection of the method. As expected, the peak height of the extended products decreased with the amount of template DNA added during PCR (Figure 4). It was also found that the AMELY-specific extended product started to drop out when less than 20 pg of DNA was employed (Figure 4D). Results from the reproducibility study which used 20 and 30 pg of male DNA template in 10 replicated reactions showed that dropout of AMELY-specific extended product was observed in 8 out of 10 replicates in 20 pg of DNA, while none was observed in the 30-pg replicates. Therefore, the results suggested that the reproducible limit of detection of this primer extension-coupled MALDI-TOF technique could be guaranteed at the level of 30 pg of DNA as the starting material of the analysis.

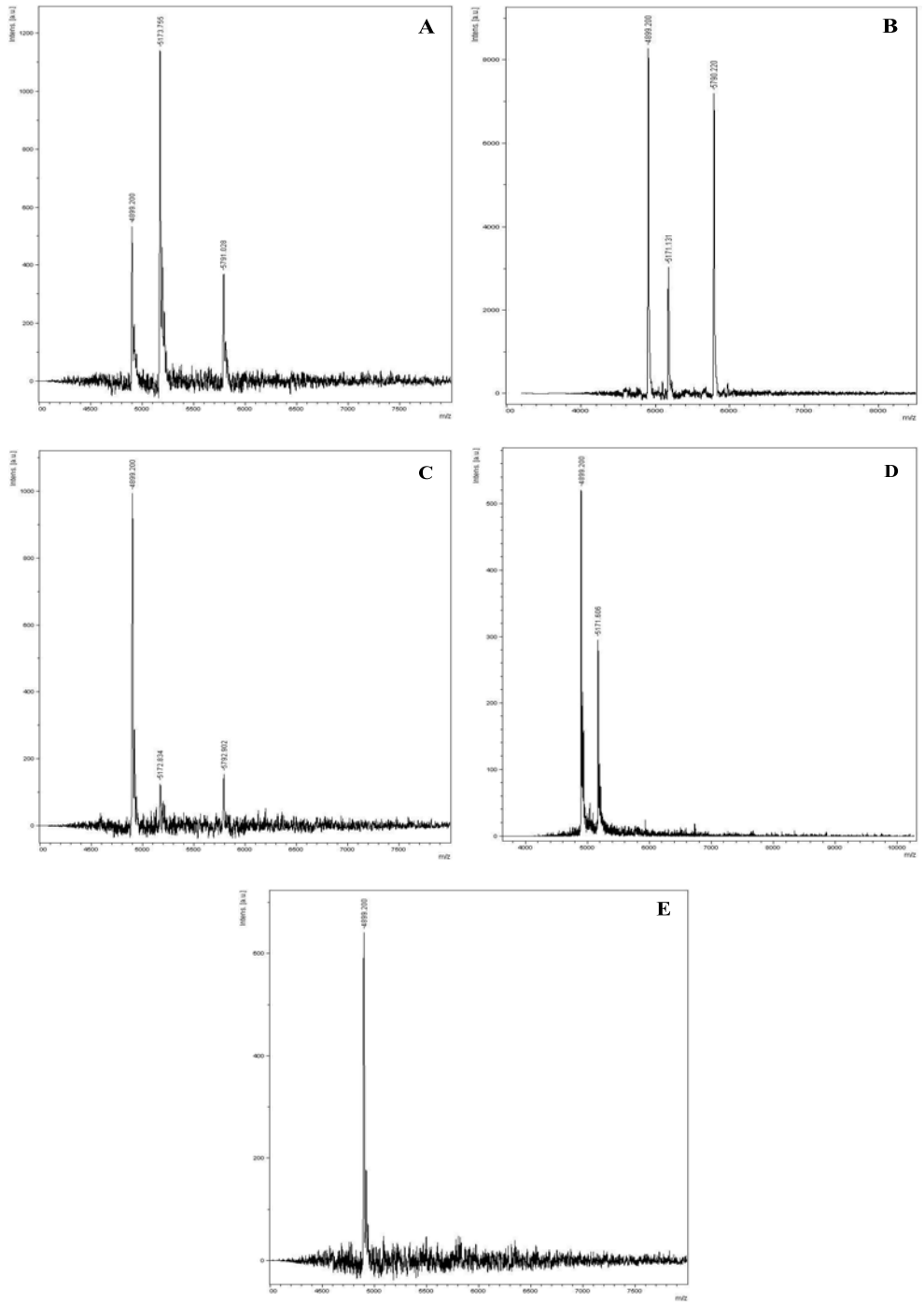

Figure 4 Representative mass spectra obtained from MALDI-TOF analysis of AMEL PCR-primer extension products generated from 50, 30, 20, 10, and 0 pg of male genomic DNA (Figures 4A-E, respectively). The peak of AMEL extension primer (4899.255 Da), which is present in all mass spectra, is used as the internal mass calibration. Peaks of 5172.435 and 5789.845 Da are AMELY- and AMELX-specific extended products, respectively, and were present in the mass spectra of 50, 30, and 20 pg DNA (Figure 4A-C). Dropout of the AMELX peak was observed only in 10 pg DNA (Figure 4D). The instrument was operated in linear positive mode with 200 shots of laser at 20kV. Signal-to-noise ratio of each peak must be greater than 3 in order to be recognized in a mass spectrum.

Discussion

The addition of primer extension step resulted in improvements when compared with the work of Taranenko et al. (8). The limit of detection of the method was showed to be as low as 30 pg and quality of mass spectra showed better resolution. Cation-exchange resin is a cost-effective method to reduce salt from DNA sample prior to MALDI-TOF analysis. We found that samples showed a good signal-to-noise ratio and sharper peaks in mass spectra when desalted with cation-exchange resin. Even though the trace of salt adducts was still observed in mass spectra, it did not affect the mass accuracy measurement. Moreover, it is important that both external and internal mass calibration is performed when analyzing oligonucleotides in order to increase the accuracy of mass measurement (20). In this study, a set of oligonucleotides of known sequences and masses in Daltons were used as external mass calibration and the peak of AMEL extension primer that was present in the mass spectra was used as internal mass calibration. In conclusion, this study provided an alternative method for molecular sexing that is rapid, sensitive, accurate and precise.

Recommendations

We hypothesize that this approach would be advantageous for sex determination of degraded DNA samples.

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