Commentary

Determination of Point Mutations or Single-Nucleotide Polymorphisms by Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry: Sample Preparation, Assay Formats, and Instrumentation

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Commentary on Harksen A, Ueland PM, Refsum H, Meyer K. Four Common Mutations of the Cystathionine β-Synthase Gene Detected by Multiplex PCR and Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (*Clin Chem* 1999;45:1157–61)

Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MALDI-TOF MS) has been a powerful tool for the analysis of various biomolecules. Although MALDI-MS has been used as a routine method for studies of peptides and proteins for the last 10 years, ultraviolet (UV) MALDI-MS has recently become a global player in oligonucleotide analysis, and an increasing number of MALDI-MS instruments with hardware and software optimized for genotyping have been marketed. For the detection of point mutations, as reported in our recent paper *(1)*, and in particular for the determination of single-nucleotide polymorphisms (SNPs), MALDI-MS has become a very powerful tool *(2–5)*. In this paper, we briefly summarize recent developments in assay design and sample treatment, matrix composition and preparation, and instrumental innovations that enhance spectra quality and enable automation.

Assay Formats and Design

Mutation detection has been accomplished by DNA sequencing of enzymatic cleavage product or Sanger ladder using MALDI-MS *(6–10)*. However, because of fragmentation and peak broadening as well as low desorption/ionization yield and decreasing detection sensitivity with increasing DNA size, sequencing reading length is typically less than 100 nucleotides (nt) for UV-MALDI-MS. Analysis of PCR products meets with the same limitation *(11)*. Peptide nucleic acids (PNAs) are more stable than DNA and do not fragment under MALDI conditions. The ability of PNAs to hybridize with DNA has been used to construct PNA hybridization probes immobilized on magnetic beads and detected by MALDI-MS *(12, 13)*. Nevertheless, the high variability in thermal stability of these probes makes multiplex analysis difficult.

Photocleavable peptide-DNA conjugates have recently been used as mass markers for indirect detection of oligonucleotide hybridization by UV-MALDI-MS. These probes have some attractive features, including no metastable fragmentation or cation adduct formation, and the hybridization properties could be easily controlled. These properties make them potentially useful for multiplex assay *(14)*.

Primer extension or minisequencing is a convenient assay format for the multiplex detection of known alleles *(15)*. The PROBE *(16)* and the Pin-Point *(17, 18)* are assay formats run on the MALDI-TOF MS platform that allow the simultaneous detection of up to 12 mutations (Fig. 1). Primer extension also enables assessment of allele frequencies by quantitative analysis of pooled DNA samples *(19)*. The PROBE uses a mixture of deoxy- and dideoxynucleotide triphosphates (NTPs), whereas the Pin-Point primers are extended by dideoxy-NTPs only. The PROBE assay entails a bigger mass gap for heterozygous samples and thereby avoids interference from adducts signals. In case the available instrumentation does not provide sufficient performance in terms of resolution, dideoxynucleotides can be mass-tagged to achieve an increased mass difference *(20)*.

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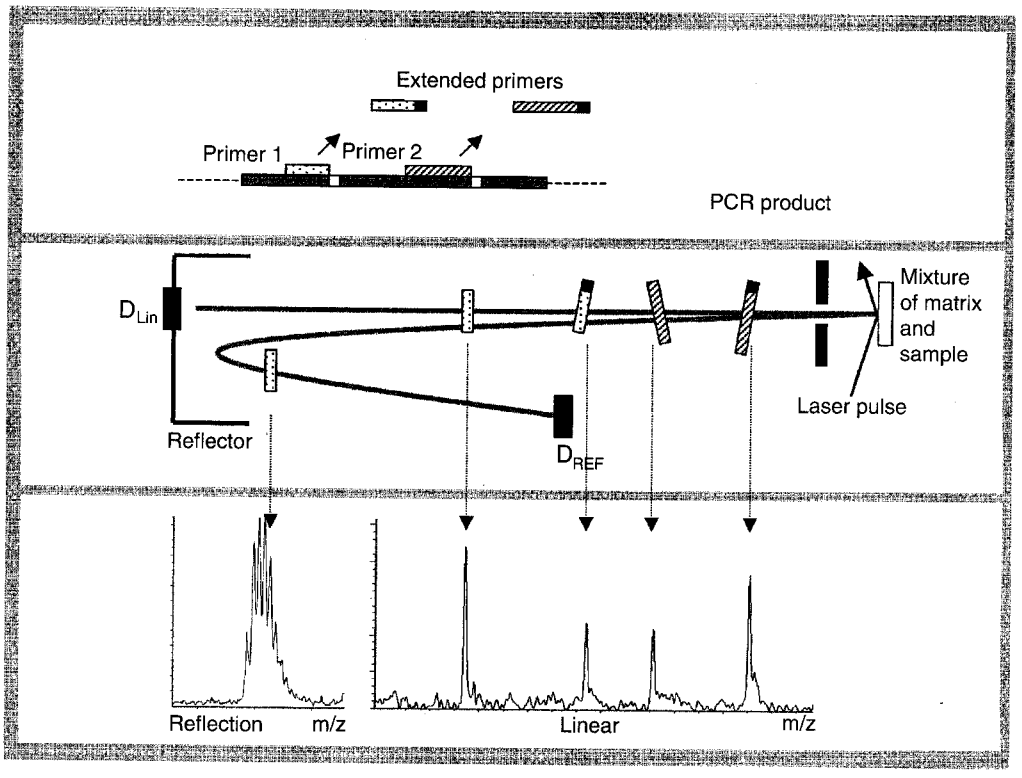

Fig. 1. Multiplex genotyping by minisequencing and MALDI-TOF MS.

Site-specific primers of different lengths anneal to PCR amplicons adjacent to the point mutations (*top*). Primers are extended and the polymerase reaction terminated by incorporation of one dideoxy-NTP. After purification (not shown), the reaction product is mixed with matrix and analyzed by MALDI-TOF MS (*middle*). The desorbed/ionized oligonucleotides are accelerated into the TOF MS at different velocities and registered by detector D_{Lin} or D_{Ref}. The *bottom panel* shows the corresponding mass peaks of unextended and extended primers. The *left trace* (expanded scale) is a signal profile corresponding to one primer obtained by TOF MS in reflector mode, which affords isotopic resolution, whereas the trace to the *right* shows the detection of four oligonucleotides by linear TOF MS.

The PCR amplification required for primer extension has limitations in connection with high-throughput multiplex genotyping: crossover contamination, different yields for different amplicons *(21)*, and time-consuming PCR purification. A new approach, called the Invader assay, requires no initial target amplification *(22)*. The technique involves hybridization of an Invader probe followed by enzymatic cleavage and linear amplification of two allele-specific signal probes. It has the potential to replace the PCR-based minisequencing. The Invader format has recently been adapted to the MALDI-TOF platform *(23)*.

Sample Desalting and Matrices

Sample desalting and the choice of an appropriate matrix are probably the most critical factors in MALDI-MS of DNA. Different purification methods are used that reduce salt adduct formation with the DNA backbone *(24)*. Efficient methods based on reversed-phase separation *(17, 25, 26)* or purification on streptavidin-biotin solid-phase supports *(11, 27)* have been described. We use cation-exchange beads for desalting both DNA samples and matrix *(28)*. The beads are inexpensive and can be added to the samples by robotic workstations, incubation is carried out overnight, and samples can be analyzed the next morning. This method produces samples with insignificant salt adduction to DNA.

Several matrices and additives have been tested with respect to desorption/ionization yield of oligonucleotides, fragmentation, mass accuracy and resolution, alkali adduction, and matrix morphology. 3-Hydroxy-picolinic acid *(29)*; picolinic acid *(30)*, 2,3,4- or 2,4,6-trihydroxyacetophenone *(31, 32)*; 2,5-dihydroxybenzoic acid *(33)*; and 6-aza-2-thiothymine *(34)* are among the commonly used UV matrices. As matrix additives, ammonium salts *(35, 36)* or organic base solutions have been recommended *(37, 38)*. The dried droplet preparation method is widely used *(39)*, but new procedures, such as the fast evaporation method, can improve spectrum quality *(40)*.

Time-of-Flight MS

The Pin-Point assay requires a mass resolution sufficient to distinguish a base A from T, i.e., 9 Da. This performance is obtained in the relevant mass range of up to 10,000 Da with a reflector TOF instrument, which delivers isotope resolution (Fig. 1; bottom panel). Linear instruments have lower mass resolution than reflector analyzers, but demonstrate higher sensitivity because of higher transmission. Since the introduction of time lag focusing (delayed extraction) *(41)*,

linear TOF instruments have become popular because they offer sufficient resolution and high sensitivity at a lower cost. New developments in ion extraction techniques, such as SIDFA-MALDI-MS, may further improve spectrum quality *(42)*. Consequently, the linear instrument may hold its position as the most commonly used mass spectrometers for genetic analyses.

Automation and Miniaturization

Automation and miniaturization of sample processing are paramount to increasing sample throughput. Modern instruments are equipped with fuzzy logic controllers *(43)*, which affords complete automation. Data processing takes place during measurement and generates both spectra and genotyping reports *(44, 45)*. The sample probes furnished with hydrophilic anchors define the positions of matrix droplets and promote homogeneous crystallization, which in turn enhances signal intensity *(46, 47)*.

With lithography, samples arrays (~1-inch square) of 96, 384, or more positions can be defined on silicon chips *(3)*. This miniaturization requires handling of submicroliter volumes. New liquid handling robots are equipped with piezoelectric needles that can dispense volumes of a few nanoliters *(47)*. We use silicon chips together with a nano-dispensing system in our laboratory and obtain high-quality spectra without the time-consuming search for "hot spots". The sample throughput with linear TOF is 96 genotypes in <20 min, depending on the settings of accumulation conditions.

Further Development

High-density MALDI chips can simultaneously act as solid-phase reaction supports, as demonstrated by Tang et al. *(48)* with a PROBE assay. Chip hybridization will further reduce costs and processing times, and MALDI-MS may become a powerful and widely used technology for high-throughput determination of SNPs.

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