USE OF MATRIX-ASSISTED LASER DESORPTION/ IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY FOR MULTIPLEX GENOTYPING

Klaus Meyer[1] and Per Magne Ueland

Bevital AS, c/o Section for Pharmacology, University of Bergen, Bergen, Norway

1. Abstract

After completion of the human genome project, the focus of geneticists has shifted to elucidation of gene function and genetic diversity to understand the mechanisms of complex diseases or variation of patient response in drug treatment. In the past decade, many different genotyping techniques have been described for the detection of single-nucleotide polymorphisms (SNPs) and other common polymorphic variants. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is among the most

[1]Corresponding author: Klaus Meyer, e-mail: klaus.meyer@farm.uib.no

0065-2423/11 $35.00
DOI: 10.1016/S0065-2423(11)53001-X

powerful and widely used genotyping technologies. The method offers great flexibility in assay design and enables highly accurate genotyping at high sample throughput. Different strategies for allele discrimination and quantification have been combined with MALDI (hybridization, ligation, cleavage, and primer extension). Approaches based on primer extension have become the most popular applications. This combination enables rapid and reliable multiplexing of SNPs and other common variants, and makes MALDI-TOF-MS well suited for large-scale studies in fine-mapping and verification of genome-wide scans. In contrast to standard genotyping, more demanding approaches have enabled genotyping of DNA pools, molecular haplotyping or the detection of free circulating DNA for prenatal or cancer diagnostics. In addition, MALDI can also be used in novel applications as DNA methylation analysis, expression profiling, and resequencing. This review gives an introduction to multiplex genotyping by MALDI-MS and will focus on the latest developments of this technology.

2. Introduction

Understanding the basis of human genetic variations is a major task in medical research. Various diseases have been related to changes in gene sequences caused by different types of polymorphisms and mutations, which can alter the structure and function of a protein. Among these variants, attention has been focused in the recent years on single-nucleotide polymorphisms (SNPs). These biallelic single-base changes have a frequency of at least 1% in a given population and are found in the human genome at a frequency of about one in 1000 base [1]. Public databases as the general catalog of genetic polymorphism maintained by NCBI [2,3] actually consist of over 143 million reported SNPs, of which 19 million reference SNP clusters have been validated (dbSNP build 132; http://www.ncbi.nlm.nih.gov/SNP/snp_summary.cgi). Only a small portion of SNPs changes the coding sequence of a protein or affects gene regulation and thus represents a potential genetic risk factor. The majority of SNPs does not change the amino acid sequence and can serve as markers for disease causing genes in genome-wide association studies. The international HapMap [4] has to date registered more than 3.1 million so-called tag-SNPs, which tag common haplotype blocks and help to reduce the number of genotypes screened in genome-wide association studies. The number of registered tag-SNPs will further increase with completion of the 1000 Genomes Projects (www.1000genomes.org), which is aimed to achieve a nearly complete catalog of common human genetic variants. Due to their biallelic nature, SNPs are easy to determine and their interpretation can be highly automated.

Various methods for genotyping of SNPs have been developed in the past few years and validated with respect to accuracy and sensitivity, flexibility of the assay design, throughput, and costs per genotype [5–8]. Methods based on

mass spectrometry provide very reliable platforms for genotyping [9]. Among these, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) belongs to the most powerful techniques for high-throughput multiplexing of SNPs [7,10–16] and has also been used for the detection of other common variations, such as restriction fragment length polymorphisms (RFLPs) [17], short tandem repeats (STRs) [18,19], insertions and deletions [20], and copy number variations (CNVs) [21]. The ability of sensitive and (semi-) quantitative allele determination has enabled pooled DNA analysis [22,23] and noninvasive prenatal fetal diagnosis [24].

3. Genotyping Using Mass Spectrometry

A characteristic feature of mass spectrometry is that the detection is based on an intrinsic physical property (mass to charge ratio), which contributes to highly accurate data. Traditional methods use indirect methods like fluorescence or radioactive reporter tags, which have to be labeled to the target molecule. While the number of different tags is limited and thus only supports low-level multiplex applications per reaction, MS provides multiple data points per experiment and enables high-level multiplex genotyping. In addition, latest mass spectrometers provide sensitive analysis with limits down to the femtomole and attomole range [25,26].

The breakthrough of MS for the analysis of large biomolecules was introduced by the development of new gentle ionization methods, which prevent the decay of the molecules during the ionization process. Nowadays, two ionization methods are commonly used for biomolecules: electrospray ionization (ESI) [27] and MALDI [28]. ESI is a much softer ionization method than MALDI and allows the characterization of very large biomolecules of up to 10^8 Da [29]. In combination with ion trap, QTOF or FT-ICR MS, the technique has been successfully used for the analysis of nucleotides, especially intact PCR products, plasmid, or phage DNA [9]. However, MALDI has become the technique of choice for of MS-based multiplex genotyping [30]. The method is less dependent on highly purified samples than ESI, and the direct combination of MALDI with TOF analyzers enables robust and rapid genotyping with throughputs of several thousand samples per day [31].

4. MALDI-TOF-MS

4.1. Principle

The MALDI process can be separated into three fundamental steps of analyte/matrix preparation, desorption of the upper matrix monolayers, and ionization of the analyte molecules. Successful MALDI involves incorporation

of analyte molecules between the matrix molecules, either in the bulk or within the crystal surface, and separation of the analyte molecules from each other. There is no single MALDI matrix or protocol that is suited to all analytical questions, and a proper choice of both is crucial for the analytical outcome [32–34]. In addition, various factors as the analyte-to-matrix ratio, type of solvent and additives, amount of salts, and characteristic of the target surface influence the crystalline morphology and, thus, the quality of mass spectra.

During the MALDI analyses, a short UV- or IR- laser pulse of a few nanoseconds irradiates the crystals and leads to desorption/ionization of the matrix and analyte molecules into the gas phase (Fig. 1). The wavelength of the laser should be close to the absorption maximum of the matrix molecules. The processes of material desorption and ionization of the matrix and analyte molecules are intertwined and take place on a micrometer geometrical and nanosecond time scale. While the mechanisms of desorption have been partly understood by comparison of experimental data with simulations [35,36], no unifying theory exists that explains the formation of the gas-phase ions [37–39]. This might be caused by the large variety of factors including type of analyte molecules, matrices, preparation, and experimental conditions, which influence the process of ionization.

Various analyzers as orthogonal TOF, FT-ICR, QIT, QTOF, QIT-TOF [29], or Orbitrap [40] have been coupled to MALDI, but axial TOF (including reflectron TOF, TOF/TOF) has become the most common type for MALDI spectrometers. Axial TOF analyzers, as illustrated in Fig. 1, are ideally suited to MALDI sources, as only a very short laser pulse is needed and the ions fly along a flight tube, hit a detector, and are registered by a digital oscilloscope [41]. Ions are typically accelerated by 20 kV into the flight tube, and as all ions have the same kinetic energy $E = zeU = 1/2mv^2$, they travel with different velocities v and arrive the detector at time $t = L(m/2E)^{1/2}$ where L is the length of the flight tube.

For MALDI-TOF-MS instruments, there are two primary sources of error related to the flight time. The first is caused by the initial velocity distribution of MALDI ions and is compensated in current TOF instruments mainly by pulsed-ion extraction (also called delayed extraction) instead of using a static acceleration field [42]. The second source of errors is introduced by energy dispersion due to nonflat sample morphology and is compensated by a one- or two-stage reflectron analyzer. Here, ions with higher energy travel a longer way through the reflector than ions with lower energy and the ion packets can be refocused on a second detector. Modern research-grade instruments are equipped with both delayed extraction and reflectron TOF-MS [43].

4.2. Analysis of Nucleic Acids by MALDI-TOF-MS

Even though modern MALDI-TOF instruments are well suited for high-level multiplexing applications in genomics, determination of nucleic acids by MALDI is generally difficult when compared to peptides. Assay

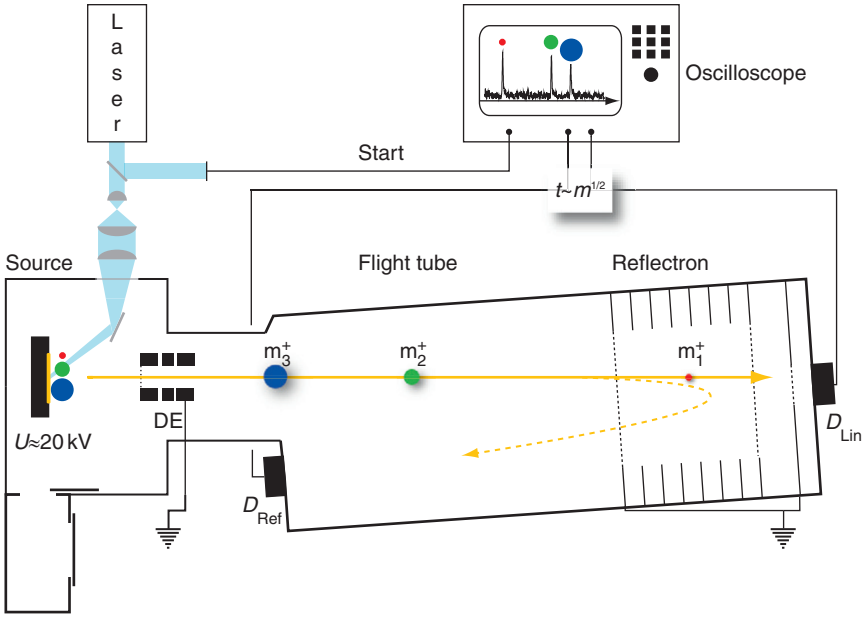

FIG. 1. MALDI-TOF-MS. MALDI-TOF instruments basically consist of three major components: a vacuum chamber housing the source and the flight tube, a pulsed UV laser, and a fast oscilloscope. The sample placed on a stage inside the source is irradiated by a short UV-laser pulse of a few nanoseconds. The matrix/analyte molecules are desorbed/ionized, and accelerated by an electric field of about $U = 20$ kV into a field-free drift path inside the flight tube. The oscilloscope is started by the laser pulse for measurement of the flight time. As all molecules are accelerated to the same kinetic energy $E = zeU = 1/2mv^2$, ions of different masses ($m_1 < m_2 < m_3$) are separated from each other during the flight and hit the detector D_{Lin} after $t = L(m/2E)^{1/2}$ (L = length of the flight path). Typically 20–100 laser shots are accumulated for a MALDI spectrum, and the detector signals are registered by the oscilloscope. Linear time-of-flight MS (registration at D_{Lin}) has become the standard mode for most genotyping applications by MALDI-TOF-MS. Mass resolution can be optimized by application of delayed ion extraction (DE). Reflectron TOF MS instruments can further increase mass resolution (at the expense of signal intensity) by reflecting the ions at the end of the flight tube back on a second detector D_{Ref}.

development requires accurate evaluation of the instrumental aspects, matrix, purification, sample preparation and analysis, and primer design in order to achieve highest degree of multiplexing at sufficient signal quality.

4.2.1. *Instrumental Aspects*

The mass range, the mass resolution, and the mass accuracy are central parameters of a mass analyzer that define the limits of an MS-based assay. Common MALDI-TOF-MS instruments use UV lasers, which allow the identification of oligonucleotides, for example, sequencing ladder products, in a mass range of up to 30,000 Da (ca. 100 nucleotides) [44]. This upper mass

limit is caused by size-dependent fragmentation of DNA during desorption, which results in loss of signal intensity toward higher masses. RNAs are more stable than DNA in MALDI because the additional 2′-hydroxyl group stabilizes the glycosidic bond and reduces depurination and fragmentation of the entire oligomer [45]. Large DNA fragments such as PCR products of up to 1400 bp can be detected using infrared MALDI [46,47], which is known to be considerably softer than UV-MALDI. Further increase of the mass range requires new types of detectors, as the sensitivity of microchannel plate detectors, commonly used in TOF instruments, decreases with molecule velocity. Cryogenic detectors have shown to decrease the detection limit by several orders of magnitudes [48].

Although MALDI-TOF-MS allows the principal detection of small PCR products <100 bp, mass resolving power of TOF analyzers is insufficient to identify point mutations and SNPs by direct comparison of the PCR products. Hence, MALDI-based genotyping has been optimized with respect to a window from 1000 to 9000 Da [15]. Reflectron TOF-MS instruments offer higher mass resolution and mass accuracy than linear types in this mass range. But the majority of instruments used for multiplex genotyping are of linear type, because these instruments have generally higher transmission, are cheaper than reflectron analyzers and enable very compact bench top designs.

4.2.2. *Matrices*

About one dozen of different matrices have been tested for the optimal analysis of oligonucleotides by MALDI [34]. Generally, a MALDI matrix must be able to embed the analyte molecules, must be stable in the vacuum and must promote desorption/ionization of the analyte. Matrices are classified by the terms "hot" and "cool." "Hot" matrices results in excessive fragmentation and thus are suitable for the detection of small nucleic acids or RNA. The latter type is suitable for the analysis of larger oligonucleotides and produces little fragmentation during MALDI. The use of matrix additives as sugar has been shown to decrease fragmentation and increases signal resolution by an additional "cooling" effect.

Two matrices have been proven to be optimal for the analysis of nucleic acids. A mixture of 2,3,4- and 2,4,6-trihydroxyacethophenone (THAP) is well suited for RNA analysis [49], while 3-hydroxypicolinic acid (3-HPA) is the preferred matrix for the detection of DNA [50] and has become the "golden standard" for oligonucleotide analysis so far.

4.2.3. *Sample Purification*

A challenging problem in the detection of oligonucleotides by MALDI is the negative charge of the sugar-phosphate backbone in solution. The high affinity to sodium and potassium from different buffers used results in

adducts formation during the ionization process and lowers the quality and intensity of the signals. Several homogenous and heterogeneous purification protocols have been applied to remove the salt from reaction products and matrices. These include dialysis [51], ethanol precipitation [52], and reversed-phase [53] or size-exclusion chromatography [54]. The use of biotin-linked terminators in combination with streptavidin-coated magnetic particles is an elegant and effective solid phase purification, as this method results in highly cleaned reaction products by removing salts, PCR as well as unextended primer products [55]. However, for high-throughput applications with a capacity of many thousands of samples per day multiple washing steps by liquid handlers represent a bottleneck, and consumption of coated magnetic particles substantially increases assay costs. Alternatively, samples can be incubated with NH_4^+-conditioned ion-exchange resins for 1 h, which replace alkali-metal ions by ammonium ions [56,57]. Optimization of resin types and dilution of the sample can decrease incubation time to 10 min [58]. Although the method is not as efficient as the usage of magnetic particles, this homogenous purification scheme enables easy, inexpensive, and rapid removal of salt adducts for various high-throughput genotyping assays. Other approaches involve on-target purification using commercial polymers, self-assembled monolayers, ultrathin polymer films, and surface coatings with nucleic acid-binding properties [59–62]. These techniques may introduce several problems as spreading of sample droplets and low homogeneity, but are promising procedures for highly purified samples at medium throughput.

4.2.4. *Sample Preparation and Analysis*

The universal preparation method for MALDI matrices is the droplet procedure, where matrix and sample are mixed together and spotted onto a MALDI target plate. Alternatively, the matrix can be dispensed first and the sample is spotted onto the dried matrix crystals. This method, which is the standard preparation for 3-HPA, results in matrix/sample spots of ca. 1–2 mm diameter and requires searching for a "sweet spot," as crystal morphology and variation in the local matrix-to-sample ratio dramatically influences signal intensity and quality. Several preparation techniques have been tested to enable homogenous crystallization for fast and automated MALDI analyses. The so-called thin-layer method uses other matrices than 3-HPA, which are soluble in organic solvents [63,64]. After the matrix is spotted or sprayed onto the plate, the sample is dispensed onto the matrix. As the sample is diluted in a water-based solvent, the matrix is not completely dissolved and the analyte is homogenously embedded into the crystal surface. However, the majority of techniques have focused on optimized dried droplet preparation for 3-HPA. These methods have the ability to produce a single,

homogenous matrix crystal by the application of different miniaturization techniques. Two commercial methods are widely used, available as part of different assay formats developed by Bruker Daltonics [65] and Sequenom [66]. Bruker uses Teflon coated sample plate with arrays up to 1536 hydrophilic spots. The spot size varies from 200 to 800 μm and arrays can be spotted by matrix of choice using classical automated liquid handlers. Samples are upconcentrated on the anchor spots during solvent evaporation. The plate can be washed and reused many times before the coating is degraded. Sequenom's SpectroCHIPs are based on silicon dioxide and are prestructured with 384 spots with 4 nl 3-HPA. The chips can be used once only and require a special nanoliter dispensing device, which dispenses 384 samples in less than 15 min. On-target plate can hold up to 10 SpectroCHIPs and represents the actually highest number of sample loading for MALDI-TOF-MS.

MALDI analyses are routinely controlled by fuzzy controlled data acquisition software, allowing fast sample analysis under optimal conditions. The laser beam is directed over the matrix/sample spot on a defined track in a spiral or S-curve while laser intensity is held on an adequate level to obtain high-quality spectra. Acquisition time is 3–5 s per sample and 384 samples are analyzed within 30 min. As only a small amount of the matrix spots is consumed after irradiation by typically 20–50 laser shots, samples can be genotyped twice if higher assay accuracy is required. Commercial software environments as Bruker's Compass/GenoTools or Sequenom's MassARRAY Typer packages enable highly automated sample tracking, data acquisition and analysis, and quality control and facilitate rapid genotyping of several hundreds to 100,000 genotypes per day.

5. Multiplex Genotyping by MALDI-TOF-MS

5.1. SNP-Genotyping Using Primer Extension Assays

Due to its robustness, flexibility, and easy design, primer extension has become the most widely used format for SNP-genotyping—not only in mass spectrometry [67,68]. Several MALDI-based primer extension formats have been published using single- or multibase extension; they differ by speed, cost, complexity of sample preparation, quality of allele separation, and level of multiplexing. These methods usually begin with the amplification of a target region by PCR. A primer for detection of the SNP is then annealed next to the polymorphic side and extended by a polymerase. The extension is terminated either on the polymorphic side or a few bases behind. PCR reagents and products, which can disturb the extension reaction in

heterogeneous and homogenous assays, are often removed by either washing or enzymatic cleavage, respectively.

Although poorly discussed in the literature, optimization of multiplex genotyping assays based on primer extension is always a crucial step and strongly determines the efficiency, robustness, and accuracy of the method. Even, if primer characteristics can be roughly predicted by various primer calculation and simulation programs, assay development remains an empirical process and efforts are generally proportional to the number of SNPs investigated per reaction [69]. Common problems include unbalanced template PCR amplification, self-priming, or annealing to an incorrect location, that is, on a pseudogene. In the case of incompatible PCR primers, reactions can be run in parallels for different sets of PCR-templates and the products are mixed together before the allele-specific reaction is performed. The design of extension primers can become even more difficult, as the lengths of the extension primers influence the primer annealing temperatures and have to be different and evenly distributed in a mass window of ca. 5000 Da. This step of optimization can be done either manually or by sophisticated software solutions as Sequenom's MassARRAY designer, which support quick assay development with success rates of $>95\%$ (15-plex) [70].

The first and most simple approach was a single-base extension assay called PinPoint developed by Haff and Smirnov in 1997 [71], which terminates the extension by conventional ddNTPs on the polymorphic site (Fig. 2). Allele discrimination is based on the mass difference between the four terminators (9 Da for ddA/ddT; 15 Da for ddT/ddC; 16 Da for ddA/ddG; 24 Da for ddC/ddA; 25 Da for ddT/ddG; and 40 Da for ddC/ddG). The level of multiplexing could be increased to 20 by careful assay design with "mass-tuning" of the extension primers [72]. Typical mass spectra of a 20-plex PinPoint assay are illustrated in Fig. 3. Sample purification is based either on reversed-phase columns or on ion-exchange resins. However, the PinPoint suffers from ambiguous identification of A/T heterozygotes in routine analyses due to the low mass difference of 9 Da between the extension products for masses >5000 Da. Thus, this approach has mainly been used in assays, which did not analyze A/T transversions (lowest frequency of ca. 7% of all single nucleotide changes [73]), or if a short primer length below 5000 Da was compatible with assay design. A simple way to increase the mass difference for A/T, but also other heterozygotes, is the usage of ddNTPs/dNTPs mixtures or mass-modified terminators.

The PROBE assay was introduced by Little and coworkers in 1997 [74,75] and uses one ddNTP and three dNTPs for extension reaction (Fig. 2). This multibase extension approach enables clear distinction between all reaction products, in the way that mixtures are optimized to always result in at least one nucleotide (ca. 300 Da) mass difference between two allele-specific

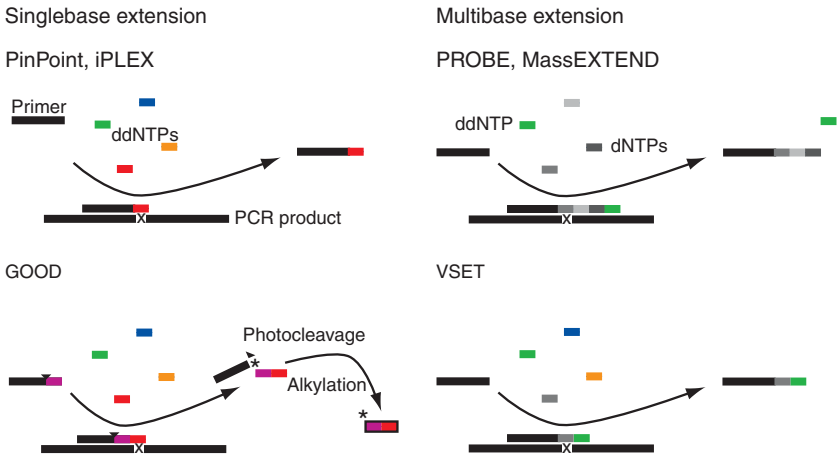

FIG. 2. SNP-genotyping by primer extension assays. Primer extension can be performed as single- or multibase extension reactions. All types of reactions require purified PCR products. Single-base extension by the PinPoint and iPLEX assays represent the simplest approaches. Here, a primer is annealed next to the mutation site (X) and extended by a ddNTP. While the PinPoint uses natural ddNTPs and suffers from insufficient allele separation of A/T heterozygotes and mass interferences by salt adducts, the iPLEX reaction is performed using mass-modified terminators, which enable unambiguous allele identification. The GOOD assay represents the most advanced single-base extension assay and includes charge-tagging and backbone neutralization. Primers are modified by photocleavable *o*-nitrobenzyl moieties for primer shortening and charge-tagging. Extension reaction uses synthetic alpha-thio ddNTPs. After extension primers are cleaved by UV-light and are alkylated in the last step to avoid cationization by salt adducts. Multibase extension assays use mixtures of ddNTPs and dNTPs (gray tones) and have the advantage of larger mass differences between signals of different alleles or primers and salt adducts. The PROBE and the MassEXTEND use three dNTPs and one ddNTP for allele determination. Dependent on the nucleotide sequence, primers can be extended by several bases before reactions are terminated by a ddNTP. The length of extension products is reduced in average by usage of three ddNTPs and one dNTP as demonstrated by the VSET assay.

extension products. However, this method can also lead to misinterpretation of genotyping results due to polymerase-pausing artifacts, which can have the same mass as "real" termination products. This weakness can be minimized by the use of three ddNTPs and one dNTP, which results in mainly short extensions as demonstrated by the VSET assay [52]. Another drawback of the PROBE assay is the use of magnetic beads for primer immobilization, which restricts throughput and increases cost and was the reason that the PROBE, but also other heterogeneous assays as the Solid Phase Capture Single-Base Extension [55] and the GenoSNIP [76], were replaced by homogenous techniques. However, heterogeneous assays produce highly purified

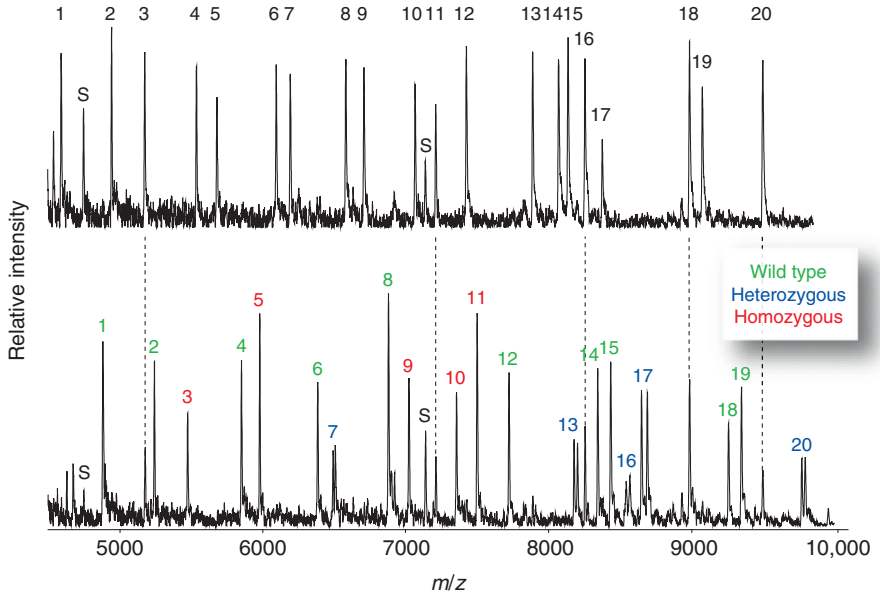

FIG. 3. MALDI-TOF spectra of a primer extension assay. Typical MALDI-TOF-MS spectra of a primer extension reaction are illustrated. The upper panel shows the spectrum of a 20-plex primer mixture before extension reaction, which spans a mass range from 4500 to nearly 10,000 Da. Primers are numbered from 1 to 20 and standards for mass calibration are labeled (S). The lower panel shows the spectrum after reaction (PinPoint), and wild type, heterozygous, and homozygous mutant genotypes are assigned. Peaks of residual reaction primers are connected to the upper spectrum.

extension products and, thus, have demonstrated the highest level of multiplexing so far with 50 SNPs per reaction [77].

A very popular homogenous multiplexing assay for SNP-genotyping by MALDI is the MassEXTEND assay by Sequenom [58,78]. It is an improvement of the PROBE and uses ion-exchange resins for desalting. In addition, the assay uses sample dilution with deionized water for optimization of the crystallization and desorption/ionization process, as the homogenous assay design cannot completely remove contaminations from buffers and reaction components. The MassEXTEND is routinely performed with up to 12 simultaneous SNPs, but higher multiplexing levels can be obtained by careful design of the extension primers. The assay is performed with microarrayed MALDI targets and nanoliter dispensing devices. The reduction of the processed sample volume lowers assay costs and makes the MassEXTEND a very cost-effective choice for 2–10 plexes when processed in 384 plates.

For higher multiplexing of SNPs, Sequenom's iPLEX represents a more cost-effective approach and routinely genotypes between 25 and 29 loci per reaction [70,79]. In contrast to the MassEXTEND, this assay is a single-base extension format and uses exclusively mass-modified ddNTPs. The assay creates mass differences large enough to differentiate between all bases compared to natural ddNTPs (ddA/ddT: 56 vs. 9 Da; ddC/ddT: 80 vs. 16 Da; and ddG/ddT: 40 vs. 25 Da). In contrast to the MassEXTEND, the iPLEX produces smaller mass gaps between primers and extension products and, therefore, enables a higher multiplexing level of up to 40 SNPs per reaction.

While the iPLEX assay is the result of advanced desalting techniques and new types of terminators, other approaches aim to handle the difficulties of oligonucleotides detection at the level of primer synthesis. These approaches use neutralization of the sugar-phosphate backbone to avoid purification from salts [80], charge-tagging to increase signal–noise ratio [81], and primer cleavage to utilize the lower mass range [76]. These concepts have been merged and realized in different versions of a homogenous approach called "GOOD assay" by Sauer *et al.* [82,83]. Common to other assay formats, the target region is amplified by PCR and the mutation site identified by primer extension. The extension primer carries a charge-tag close to the 3′ end and a phosphorthioate bridge on the 5′ side of the charge tag. Primer extension is performed using alpha-thio ddNTPs. Digestion by 5′-specific phosphodiesterase cleaves the primer down to the first phosphorthiodate, which results in a short oligonucleotide including a charge tag and the SNP-specific nucleotide. Finally, alkylation by methyl iodide of the phosphorthiodate bridge neutralizes the backbone and prevents cationization by sodium or potassium. Recently, the assay protocol has been improved by less-toxic reagents for alkylation and photocleavable *o*-nitrobenzyl moieties for primer shortening and charge-tagging [84]. So far, the GOOD assay has not become as popular as the MassEXTEND or iPLEX due to relatively low multiplexing level of <10 SNPs per reaction.

5.2. SNP-Genotyping Using Nonprimer Extension Assays

Several MALDI-based methods for genotyping were developed already in the 1990s, using other techniques than primer extension such as hybridization, ligation, or cleavage reaction (Fig. 4). Although many of these methods include novel approaches to overcome various limitations related to amplification by PCR or nucleic acid analysis by MALDI, none of these methods have so far been widely used for high-throughput genotyping. This might be related to either heterogeneous assay design or usage of expensive or toxic reagents.

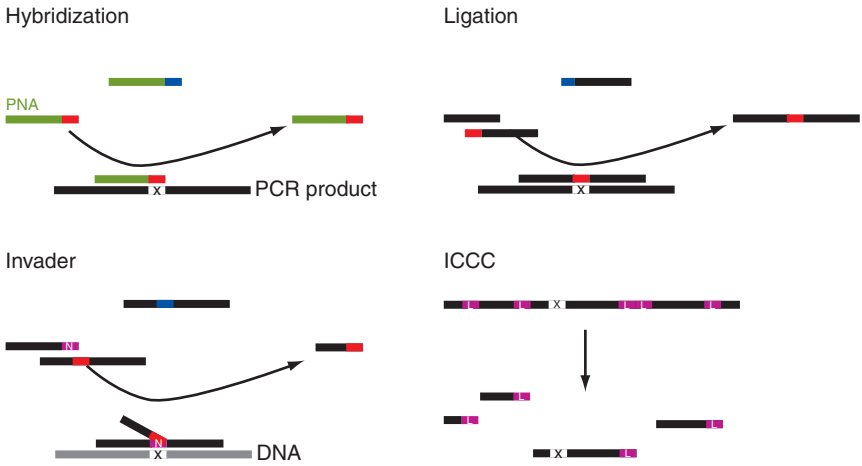

FIG. 4. SNP-genotyping by nonprimer extension assays. Common nonprimer extension assays are based on hybridization, ligation, or cleavage reactions. For the hybridization assay, two different peptide nucleic acid (PNA) probes are designed for the template sequence and the matching probe is hybridized to the PCR products. The DNA–PNA hybrid is immobilized on a solid support and the unbound probe removed by washing. Ligation assays use two different allele-specific oligonucleotides, which are enzymatically ligated with a second primer when fully complementary to the target sequence. The invader assay uses a "probe" and an "invader" oligonucleotide, which both hybridize to the target DNA. The probe is the downstream oligonucleotide, the invader the upstream oligonucleotide, which includes a noncomplementary nucleotide at the 3′ end (N). Both oligonucleotides form a duplex, which includes the polymorphic site and a noncomplementary 5′ overhang called "flap." The invader nucleotide creates a sequence overlap that is recognized by a DNA repair enzyme. The flap is released by the cleavage reaction and detected. For each SNP, genotyping is performed using two probes with different sequences in the flap. The Incorporation and Complete Chemical Cleavage (ICCC) assay uses a chemically labile nucleotide (L), which replaces one of the four dNTPs during PCR. The generated amplicon is cleaved after PCR at the incorporation sites and the genotype can be determined from the specific cleavage products in the mass spectrum.

Peptide nucleic acid (PNA) hybridization probes have several advantages in MALDI-based genotyping [85,86]. PNAs are more resistant to fragmentation and, due to the uncharged backbone, less prone to alkali-metal adduct formation than DNA. They have been successfully applied in DNA sequence determination, including SNP-genotyping. However, complicated reaction conditions, expensive PNA probes, and the need for streptavidin-coated magnetic beads have so far hindered the use of PNAs in high-throughput approaches. In a recent work, Boontha *et al*. have described an improved and simplified procedure using pyrrolidinyl-PNA probes [87]. In combination with ion-exchange capture, this PNA-based method has demonstrated potential of rapid and inexpensive high-throughput multiplex genotyping.

Oligonucleotide ligation was also tested in the early reports on the use of MALDI-MS for SNP-genotyping. Here, two oligonucleotides are ligated when fully complementary to the target sequence and the genotype is identified by mass determination of the ligation product [88]. To date, this technique has not been developed beyond this prove-of-principle study.

The invader assay uses two sequence-specific oligonucleotides, a "probe" and an "invader" oligonucleotide, which hybridize to the target DNA [89]. The probe is the downstream oligonucleotide, the invader the upstream oligonucleotide. Both oligonucleotides form a duplex, which includes the polymorphic site and a noncomplementary 5′ overhang called "flap." The invader nucleotide creates a sequence overlap that is recognized by a DNA repair enzyme. The flap is released by the cleavage reaction and detected. For each SNP, genotyping is performed using two probes with different sequences in the flap. The process is isothermally run near melting temperature using thermostable enzymes and has the main advantage that it does not require PCR thermocycling for amplification. Griffin *et al.* combined the invader assay for genotyping with MALDI with the so-called "Invader Squared assay" [90]. Here, the cleaved flap serves as an invader oligonucleotide for a second invader reaction, which is performed with a biotin-labeled flap for sample desalting using streptavidin-coated magnetic beads. So far, the heterogeneous assay design and the relatively large amount of required genomic DNA have hindered the MALDI-based Invader assay to become as popular as the commercial Invader assays using fluorescent probe detection [91].

The Incorporation and Complete Chemical Cleavage (ICCC) assay introduced by Wolfe *et al.* uses a chemically labile nucleotide, which replaces one of the four dNTPs during PCR [92]. The generated amplicon is cleaved after PCR at the incorporation sites and the genotype can be determined from the specific cleavage products in the mass spectrum. As PCR and genotyping is combined in one reaction, the assay does not require a cleanup step and is well suited for automation. In addition, double-strand amplicons are used for genotyping and cleavage products are generated from both directions. This process delivers redundant information for genotype determination and provides highly accurate genotyping. A disadvantage of the assay is low amplification yields for larger templates due to the use of synthetic nucleotides and it is not well suited for high-throughput applications due to the use of toxic agents for cleavage reaction.

5.3. Genotyping of Other Polymorphic Variants

In addition to SNP-genotyping, various MALDI-based assay formats have been developed for the determination of other common genetic variants, which include RFLPs [93], sequence variation as insertions or deletions (indels) [94], STRs [95], and alternative splicing variants (ASVs) [96], as well as CNVs [97].

RFLPs are DNA-based variations at restriction sites, which alter the fragment length pattern after cleavage by a restriction endonuclease enzyme. RFLPs are of biallelic nature and can be defined as a subset of SNPs in the recognition sequence for a restriction site. Primer extension based assays are difficult to develop for RFLP genotyping, as the polymorphic region is characterized by multiple closely located polymorphisms. The restriction fragment mass polymorphism (RFMP) assay developed by Kim *et al.* [17] enables genotyping of polymorphic regions by incorporation of IIS restriction endonuclease recognition sites during PCR amplification and cleavage of the amplicons using IIS restriction endonuclease. This homogenous assay allows rapid genotyping also for SNPs and offers high accuracy by simultaneous genotyping of both DNA strands. The RFMP has been mostly applied in screening studies for genotyping of hepatitis B, C, and papilloma viruses, but also for SNP identification from pooled human DNA [98–101].

Sequence variations by insertion or deletion of one or more nucleotides represent another important class of polymorphisms. Similar to SNPs, insertions and deletions can alter the structure or the expression of a protein. Single-base indels are easy to genotype using a simple primer extension approach. Longer sequences can be determined by semiquantitative probing of the junction or a specific SNP inside the insertion [102]. More importantly, genotyping of indels is a powerful application for determination of STRs and ASVs. STRs or microsatellite are stretches of DNA, which consist of repeating units of two, three, or four nucleotides, and the different repeat length represents the alleles of the polymorphism. STRs are valuable markers for genetic linkage studies, but also in cancer diagnostics, as microsatellite instability is related to deficiency of the mismatch repair system. Determination of indels/STRs in repeating sequences is difficult, as partly hybridization often occurs in the repetitive sequence. Two different approaches have been developed using primer extension as well as cleavage reaction for allele identification. The primer extension method by Bonk *et al.* [18] uses primers that directly anneal at the 5′ end of short mononucleotide repeats, and extension results in specific peak patterns. For cleavage reaction, two different formats have been published. In the method by Krebs *et al.*, RNA fragments were produced by cutting transcripts using ribozyme [19] or G-specific ribonuclease [103]. The second approach by Sasayama *et al.* uses *in vitro* transcription of PCR products and cleavage of the 5′ and 3′ ends of the indel site by artificial RNA cutters [20].

Alternative splicing is a significant contributor to transcriptome diversity, as ca. 74% of the human-multiexon genes are alternatively spliced. McCullough *et al.* [104] presented a quantitative primer extension approach, which allows

the identification of different splicing isoforms as exon insertion/deletion, splicing acceptor and donor, and a mutually exclusive exon. Here, reverse transcribed cDNA was PCR amplified using primers that flank the site of alternative splicing. Extension primers were designed to extend from one to a few bases into the sequence differences and ASVs could be determined by the weight of extension product using MALDI.

CNVs are segments of DNA ranging in size from thousands to millions of DNA bases, which differ among individuals by the number of copies. The most well known CNV is the Trisomy 21, a disorder caused by gene duplication in chromosome 21. CNVs play an important role in disease susceptibility and have been related to risk modification in, for example, HIV infection and lung cancer. Different primer extension approaches have been established for relative, absolute, and allele-specific determination of CNVs. For relative quantification, a previously identified SNP is genotyped in a given population by a single-base extension assay as PinPoint [72] or iPLEX [105] and the relative allele signals are investigated by a scatter plot. As the allele ratio for a heterozygous individual is 1:1, heterozygotes with allele ratios above or below 1:1 indicate a potential CNV. Different statistical tools have been developed for identification of CNV from SNP data [106,107]. Absolute CNV quantification has been established on Sequenom's MassARRAY platform in combination with real-competitive PCR [21]. Here, a competitor DNA is spiked in at known concentration and is coamplified during PCR. As the competitor includes an artificial marker, no SNP site is required in this assay. This approach enables multiplexing of up to 20 CNVs and can also be combined to allele-specific PCR.

5.4. Molecular SNP-Haplotyping

In diploid organisms, a collection of SNPs found on a single chromosome can be more informative than individual SNPs for the determination of a phenotypic outcome. These haplotype structures represent powerful markers and can provide additional statistical power in mapping disease genes, as they reflect the sequence of a transcribed protein. The knowledge of haplotypes helps to decrease the number of SNPs that is needed to be genotyped in association studies. Haplotypes can be determined using SNP genotype data and computational algorithms as the EM [108] and PHASE [109]. However, these programs can fail and have a limited degree of accuracy, especially when many heterozygous SNPs occur in close proximity. An alternative to these methods is the direct molecular haplotyping. Several techniques have been developed for the direct determination of haplotypes including mass spectrometry, which has the advantage of multiplexing several SNPs. Three MALDI-TOF based approaches have been recently published using

Sequenom's Mass EXTEND or the GOOD assay. In the first approach, two homologous DNAs are separated by single-molecule dilution to about one genome copy per PCR aliquot, which is genotyped by the Mass EXTEND assay [110]. Amplicons are about 100 bp long and amplified with 90–95% efficiency. The haplotyping efficiency per reaction is 40–45%, and thus four replicates should be sufficient to achieve 90% efficiency. This method is well suited to multiplex SNPs that are far apart from each other. A major drawback of the approach is the sensitivity to contaminations and dependence on template integrity, as genomic DNA fragments of about 25 kb are commonly seen. The two other techniques include the GOOD assay for SNP identification in combination with either allele-specific PCR or clone-based systematic haplotyping. Allele-specific PCR is performed using primers that hybridize with the 3′ end on the polymorphic site and enable the amplification of the desired allele [111]. The multiple SNPs used as PCR anchors have first to be typed or are already available from a database. Using a brute-force approach allele-specific PCR enables simple and effective molecular haplotyping. However, this method can suffer from coamplification of the undesired allele due to primer mismatch. In addition, size of amplicons is limited to about 1000 bp and long-range haplotyping requires unfavorable walking from fragment to fragment. In contrast to allele-specific amplification, clone-based systematic haplotyping enables the study of large genomic regions of $>$30 kb [112]. Cosmid/fosmid libraries were constructed from fragmented isolated DNA covering 10% of the genome and individual clones were genotyped if tested positive in a pool of 96 clones.

5.5. Quantification of Mixed DNA

MALDI-MS has been shown to enable quantification of proteins and peptides [26]. For oligonucleotides, the strong heterogeneity of the 3-HPA crystal morphology makes quantification a demanding challenge, although the more homogenous structure of nucleic acids, consisting of four similarly building units, provides some compensation. The key to achieve high reproducibility and, thus accurate quantification of nucleic acids, is to analyze a large fraction of the sample/matrix by rastering the laser over many sample spots and/or shrinking the preparation to a "single" crystal using miniaturization. Especially, Sequenom's silicon chip based MassARRAY technique has been proven to minimize sample heterozygosity so that the highest impact on accuracy and reproducibility is caused by DNA preparation.

Quantitative MALDI analyses in genomics are based on the calculation of the ratio of allele-specific primer extension products, which enables the relative and absolute quantification of allele frequencies. A popular genotyping application using relative allele frequencies is screening of pooled DNA to validate

SNPs in association studies and to characterize their allele frequency in various ethnic groups [113–115]. Pooling decreases the number of necessary genotypes in large-scale studies and represents a shortcut to identify associations between genetic loci and phenotypes. Only SNPs that show significant difference between two pools of, that is, case and controls are selected for individual genotyping. As a drawback in pooling, rare alleles may be missed and individual information as genotypes and haplotypes is lost. Ross *et al.* were the first who described the relative quantification of allele-specific extension products in mixed DNA by MALDI and found that frequencies down to 5% could be accessed routinely [22]. Further studies demonstrated a limit of detection of about 2% and a standard deviation of 2–3% for allele frequencies between 10% and 90% [23,116,117]. Detailed analysis has shown that this deviation is mainly caused by unbalanced amplification during PCR. When allele frequencies determined from the pooled samples were compared to the real frequencies calculated, a deviation of $<4\%$ was measured. This effect is caused by numerous factors like pool preparation or preferential DNA amplification. Skewed distributions of two alleles are another frequent phenomena in individual DNA samples and is caused by unbalanced amplification during PCR and different incorporation rates of ddNTPs during primer extension. This deviation from the expected 1:1 ratio has been reported for natural and mass-modified terminators [72] and can be calculated into a correction factor which improves allele frequency estimation from DNA pools [118]. Several comparative pooling studies have shown that multiplex primer extension by MALDI-TOF-MS represents an accurate and cost-effective technique for allele frequency estimation in DNA pools. Results by MALDI performed as well as or better than RFLP, Pyrosequencing, Taqman, SNuPe, or SNaPshot, and all methods were suited for determination of SNP allele frequencies in DNA pools [119–121]. As first genome-wide study, Buetow *et al.* applied allele-frequency estimations by MALDI-TOF-MS in DNA pools and genotyped 9000 SNPs of 95 individuals [116]. Other groups have confirmed that this approach allows for identification of genetic contributors to complex diseases [122–125].

Recently, the quantitative feature of MALDI-TOF-MS and its high sensitivity and specificity when combined with PCR allow the detection of genetic variants in fetal DNA from maternal plasma and make MALDI a valuable tool for noninvasive prenatal diagnosis [126,127]. During pregnancy, fetal DNA amounts to 3–6% of the total DNA in maternal plasma [128,129]. Several studies have described that detection of fetal point mutations by conventional PCR-based assays is difficult due to the high amount of maternal DNA sequences [127,130]. Ding and coworkers were the first showing that fetal single gene differences could be correctly discriminated in maternal plasma using MALDI-TOF-MS for typing of β-thalassemia mutations [131]. In addition to the MassEXTEND assay, they combined MALDI with the

so-called single allele base extension reaction (SABER) protocol. The advantage of the SABER assay over the MassEXTEND is higher sensitivity, as only the fetal-specific allele is extended in the primer extension reaction. The SABER assay has also been successfully proven for noninvasive fetal blood group genotyping to determine fetal RhD status with respect to improved management of RhD-negative pregnant women [132]. In addition to single point mutations, the MassEXTEND assay can also be used in noninvasive detection of CNVs in fetal DNA for the identification of abnormalities in chromosome numbers as Trisomy 21 [133,134]. Enrichment of cell-free fetal DNA by size fractionation can be applied prior to MALDI analysis in order to increase assay sensitivity [135]. On the basis of these assays, Sequenom has recently launched a commercial prenatal screening technology called SEQureDX [136], which will facilitate diagnosis of high-risk familial disorders by typing both fetal DNA as well as fetal RNA in maternal blood. Besides prenatal diagnosis, detection of free circulating DNA may also enable new methods for monitoring of cancer, diabetes, trauma, and stroke [137].

6. Conclusions

During the past years, MALDI-TOF-MS has been proven to be a versatile tool for rapid multiplex genotyping in different field of applications from pharmacogenetic and genomics [138–141], disease association studies [125, 142–147], clinical diagnostic testing [14,15,140,148], controlling in agriculture [149,150] and breeding [151–154], and bacterial and viral typing [155,156]. Determination of the molecular mass offers high flexibility in assay design for investigation of SNPs and other polymorphic sequence variants by analysis of DNA and RNA fragments, using commercial systems from Bruker or Sequenom, or custom-built platforms. Especially, when combined to primer extension assays, MALDI has been demonstrated to provide highly accurate and robust genotyping.

Although MALDI-based assay have been successfully applied in whole-genome scans for discovery of susceptibility genes, the method has lost its position in the past years. Multiplexing of about 40 SNPs per reaction enables throughput levels of 150,000 genotypes per day, which have difficulties to compete with levels of up to one million genotypes per sample generated by commercially available microarray genotyping technologies from Affymetrix or Illumina, respectively [7]. Even, if the level of multiplexing could be doubled by optimal utilization of the mass range accessible by nowadays instruments, assay throughput by MALDI-MS remains on a low level. As a complementary method to array and bead-based technologies, the advantage of MALDI-TOF-MS is the high throughput of up to

3840 individual samples per day. This makes MALDI-TOF-MS highly suited for fine-mapping studies and replication of findings from genome-wide scans and an interesting alternative to other medium multiplexing platforms as SNPlex from Applied Biosystems.

Due to the decrease in price for genotyping of down to actually 1–10 cents per SNP [6], platforms can only compete by implementation of new applications, improvement of assay efficiency to decrease genotyping costs, and development of simple-to-use/low-maintenance equipment. Several more demanding MALDI-based applications have been developed in the recent years for genomic research using its high sensitivity and ability to quantify. Besides the simple genotyping of SNPs, gene expression analysis [157], DNA methylation analysis [158,159], and resequencing [160] demonstrate the potential of MALDI-TOF-MS as flexible technique for DNA/RNA analysis. Assay costs could be further reduced by development of new tools for design of more efficient high-level multiplex PCRs as well as by reduction of sample volumes as already demonstrated by Sequenom's iPLEX and SpectroCHIP techniques. Today PCR and primer extension reaction take place in 5 μl volumes while only 10 nl are require for MALDI analysis. Integration of the PCR and primer extension processes into microfluid devices [161] or directly onto the MALDI-chip [162] could further lower sample volumes and would increase the level of automation. Ideally, primer design, sample processing, and analysis will merge together in one instrument to facilitate fully automated genotyping. Development of one-push-button systems on the basis of compact platforms as Bruker's Microflex, Sequenom's MassCOMPACT, or Waters's microMX will strengthen the position of MALDI-TOF-MS as high-throughput genotyping technique for basic applications as well as clinical diagnostics.

References

[1] B.S. Shastry, SNPs: impact on gene function and phenotype, Methods Mol. Biol. 578 (2009) 3–22.
[2] I.N. Day, dbSNP in the detail and copy number complexities, Hum. Mutat. 31 (1) (2010) 2–4.
[3] S.T. Sherry, M.H. Ward, M. Kholodov, J. Baker, L. Phan, E.M. Smigielski, et al., dbSNP: the NCBI database of genetic variation, Nucleic Acids Res. 29 (1) (2001) 308–311.
[4] K.A. Frazer, D.G. Ballinger, D.R. Cox, D.A. Hinds, L.L. Stuve, R.A. Gibbs, et al., A second generation human haplotype map of over 3.1 million SNPs, Nature 449 (7164) (2007) 851–861.
[5] A.C. Syvanen, Toward genome-wide SNP genotyping, Nat. Genet. 37 (Suppl.) (2005) S5–S10.
[6] S. Kim, A. Misra, SNP genotyping: technologies and biomedical applications, Annu. Rev. Biomed. Eng. 9 (2007) 289–320.

[7] J. Ragoussis, Genotyping technologies for genetic research, Annu. Rev. Genomics Hum. Genet. 10 (2009) 117–133.
[8] C. Ding, S. Jin, High-throughput methods for SNP genotyping, Methods Mol. Biol. 578 (2009) 245–254.
[9] H. Oberacher, On the use of different mass spectrometric techniques for characterization of sequence variability in genomic DNA, Anal. Bioanal. Chem. 391 (1) (2008) 135–149.
[10] J. Tost, I.G. Gut, Genotyping single nucleotide polymorphisms by mass spectrometry, Mass Spectrom. Rev. 21 (6) (2002) 388–418.
[11] W. Pusch, J.H. Wurmbach, H. Thiele, M. Kostrzewa, MALDI-TOF mass spectrometry-based SNP genotyping, Pharmacogenomics 3 (4) (2002) 537–548.
[12] S. Sauer, I.G. Gut, Genotyping single-nucleotide polymorphisms by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 782 (1–2) (2002) 73–87.
[13] I.G. Gut, DNA analysis by MALDI-TOF mass spectrometry, Hum. Mutat. 23 (5) (2004) 437–441.
[14] W. Pusch, M. Kostrzewa, Application of MALDI-TOF mass spectrometry in screening and diagnostic research, Curr. Pharm. Des. 11 (20) (2005) 2577–2591.
[15] J. Tost, I.G. Gut, Genotyping single nucleotide polymorphisms by MALDI mass spectrometry in clinical applications, Clin. Biochem. 38 (4) (2005) 335–350.
[16] S. Sauer, R. Reinhardt, H. Lehrach, I.G. Gut, Single-nucleotide polymorphisms: analysis by mass spectrometry, Nat. Protoc. 1 (4) (2006) 1761–1771.
[17] Y.J. Kim, S.O. Kim, H.J. Chung, M.S. Jee, B.G. Kim, K.M. Kim, et al., Population genotyping of hepatitis C virus by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of short DNA fragments, Clin. Chem. 51 (7) (2005) 1123–1131.
[18] T. Bonk, A. Humeny, J. Gebert, C. Sutter, M. von Knebel Doeberitz, C.M. Becker, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry-based detection of microsatellite instabilities in coding DNA sequences: a novel approach to identify DNA-mismatch repair-deficient cancer cells, Clin. Chem. 49 (4) (2003) 552–561.
[19] S. Krebs, D. Seichter, M. Forster, Genotyping of dinucleotide tandem repeats by MALDI mass spectrometry of ribozyme-cleaved RNA transcripts, Nat. Biotechnol. 19 (9) (2001) 877–880.
[20] T. Sasayama, M. Kato, H. Aburatani, A. Kuzuya, M. Komiyama, Simultaneous genotyping of indels and SNPs by mass spectroscopy, J. Am. Soc. Mass Spectrom. 17 (1) (2006) 3–8.
[21] Sequenom, CNV Analysis Using MassARRAY. Sequenom QuickGuide, (2008).
[22] P. Ross, L. Hall, L.A. Haff, Quantitative approach to single-nucleotide polymorphism analysis using MALDI-TOF mass spectrometry, Biotechniques 29 (3) (2000) 620–626, 8–9.
[23] M. Werner, M. Sych, N. Herbon, T. Illig, I.R. Konig, M. Wjst, Large-scale determination of SNP allele frequencies in DNA pools using MALDI-TOF mass spectrometry, Hum. Mutat. 20 (1) (2002) 57–64.
[24] Y.M. Lo, Recent developments in fetal nucleic acids in maternal plasma: implications to noninvasive prenatal fetal blood group genotyping, Transfus. Clin. Biol. 13 (1–2) (2006) 50–52.
[25] D.I. Papac, Z. Shahrokh, Mass spectrometry innovations in drug discovery and development, Pharm. Res. 18 (2) (2001) 131–145.
[26] M.W. Duncan, H. Roder, S.W. Hunsucker, Quantitative matrix-assisted laser desorption/ionization mass spectrometry, Brief Funct. Genomic Proteomic 7 (5) (2008) 355–370.
[27] C.M. Whitehouse, R.N. Dreyer, M. Yamashita, J.B. Fenn, Electrospray interface for liquid chromatographs and mass spectrometers, Anal. Chem. 57 (3) (1985) 675–679.

[28] M. Karas, D. Bachmann, U. Bahr, F. Hillenkamp, Matrix-assisted ultraviolet laser desorption of non-volatile compounds, Int. J. Mass Spectrom. Ion Process. 78 (1987) 53–68.
[29] G.L. Glish, R.W. Vachet, The basics of mass spectrometry in the twenty-first century, Nat. Rev. Drug Discov. 2 (2) (2003) 140–150.
[30] S. Sauer, Typing of single nucleotide polymorphisms by MALDI mass spectrometry: principles and diagnostic applications, Clin. Chim. Acta 363 (1–2) (2006) 95–105.
[31] I.G. Gut, Automation in genotyping of single nucleotide polymorphisms, Hum. Mutat. 17 (6) (2001) 475–492.
[32] M. Kussmann, P. Roepstorff, Sample preparation techniques for peptides and proteins analyzed by MALDI-MS, Methods Mol. Biol. 146 (2000) 405–424.
[33] G. Bolbach, Matrix-assisted laser desorption/ionization analysis of non-covalent complexes: fundamentals and applications, Curr. Pharm. Des. 11 (20) (2005) 2535–2557.
[34] S. Sauer, The essence of DNA sample preparation for MALDI mass spectrometry, J. Biochem. Biophys. Methods 70 (2) (2007) 311–318.
[35] R. Knochenmuss, Photoionization pathways and free electrons in UV-MALDI, Anal. Chem. 76 (11) (2004) 3179–3184.
[36] L.V. Zhigilei, E. Leveugle, B.J. Garrison, Y.G. Yingling, M.I. Zeifman, Computer simulations of laser ablation of molecular substrates, Chem. Rev. 103 (2) (2003) 321–348.
[37] H. Ehring, M. Karas, F. Hillenkamp, Matrix-assisted ultraviolet laser desorption of non-volatile compounds, Int. J. Mass Spectrom. Ion Process. 78 (1987) 53–68.
[38] M. Karas, M. Gluckmann, J. Schafer, Ionization in matrix-assisted laser desorption/ionization: singly charged molecular ions are the lucky survivors, J. Mass Spectrom. 35 (1) (2000) 1–12.
[39] R. Knochenmuss, Ion formation mechanisms in UV-MALDI, Analyst 131 (9) (2006) 966–986.
[40] R.H. Perry, R. Graham Cooks, R.J. Noll, Orbitrap mass spectrometry: instrumentation, ion motion and applications, Mass Spectrom. Rev. 27 (2008) 661–699.
[41] C. Weickhardt, F. Moritz, J. Grotemeyer, Time-of-flight mass spectrometry: state-of-the-art in chemical analysis and molecular science, Mass Spectrom. Rev. 15 (1996) 139–162.
[42] P. Juhasz, M.T. Roskey, I.P. Smirnov, L.A. Haff, M.L. Vestal, S.A. Martin, Applications of delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry to oligonucleotide analysis, Anal. Chem. 68 (6) (1996) 941–946.
[43] B.A. Mamyrin, Laser assisted reflectron time.of.flight mass spectrometry, Int. J. Mass Spectrom. Ion Processes 131 (1994) 1–19.
[44] N.I. Taranenko, S.L. Allman, V.V. Golovlev, N.V. Taranenko, N.R. Isola, C.H. Chen, Sequencing DNA using mass spectrometry for ladder detection, Nucleic Acids Res. 26 (10) (1998) 2488–2490.
[45] F. Kirpekar, E. Nordhoff, K. Kristiansen, P. Roepstorff, A. Lezius, S. Hahner, et al., Matrix assisted laser desorption/ionization mass spectrometry of enzymatically synthesized RNA up to 150 kDa, Nucleic Acids Res. 22 (19) (1994) 3866–3870.
[46] S. Berkenkamp, F. Kirpekar, F. Hillenkamp, Infrared MALDI mass spectrometry of large nucleic acids, Science 281 (5374) (1998) 260–262.
[47] F. Kirpekar, S. Berkenkamp, F. Hillenkamp, Detection of double-stranded DNA by IR- and UV-MALDI mass spectrometry, Anal. Chem. 71 (13) (1999) 2334–2339.
[48] P. Christ, S. Rutzinger, W. Seidel, S. Uchaikin, F. Pro, C. Koy, et al., High detection sensitivity achieved with cryogenic detectors in combination with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry, Eur. J. Mass Spectrom. Chichester Eng. 10 (4) (2004) 469–476.

[49] Y.F. Zhu, C.N. Chung, N.I. Taranenko, S.L. Allman, S.A. Martin, L. Haff, et al., The study of 2,3,4-trihydroxyacetophenone and 2,4,6-trihydroxyacetophenone as matrices for DNA detection in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 10 (3) (1996) 383–388.
[50] K.J. Wu, A. Steding, C.H. Becker, Matrix-assisted laser desorption time-of-flight mass spectrometry of oligonucleotides using 3-hydroxypicoline acid as an ultraviolet-sensitive matrix, Rapid Commun. Mass Spectrom. 7 (1993) 142–146.
[51] Z. Fei, L.M. Smith, Analysis of single nucleotide polymorphisms by primer extension and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 14 (11) (2000) 950–959.
[52] X. Sun, H. Ding, K. Hung, B. Guo, A new MALDI-TOF based mini-sequencing assay for genotyping of SNPS, Nucleic Acids Res. 28 (12) (2000) E68.
[53] M. Gilar, A. Blenky, B.H. Wang, High-throughput biopolymer desalting by solid-phase extraction prior to mass spectrometric analysis, J. Chromatogr. A 921 (2001) 3–13.
[54] I.P. Smirnov, L.R. Hall, P.L. Ross, L.A. Haff, Application of DNA-binding polymers for preparation of DNA for analysis by matrix-assisted laser desorption/ionization mass spectrometry, Rapid Commun. Mass Spectrom. 15 (16) (2001) 1427–1432.
[55] S. Kim, J.R. Edwards, L. Deng, W. Chung, J. Ju, Solid phase capturable dideoxynucleotides for multiplex genotyping using mass spectrometry, Nucleic Acids Res. 30 (16) (2002) e85.
[56] E. Nordhoff, A. Ingendoh, R. Cramer, A. Overberg, B. Stahl, M. Karas, et al., Matrix-assisted laser desorption/ionization mass spectrometry of nucleic acids with wavelengths in the ultraviolet and infrared, Rapid Commun. Mass Spectrom. 6 (12) (1992) 771–776.
[57] A. Harksen, P.M. Ueland, H. Refsum, K. Meyer, Four common mutations of the cystathionine beta-synthase gene detected by multiplex PCR and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Clin. Chem. 45 (8 Pt 1) (1999) 1157–1161.
[58] N. Storm, B. Darnhofer-Patel, D. van den Boom, C.P. Rodi, MALDI-TOF mass spectrometry-based SNP genotyping, Methods Mol. Biol. 212 (2003) 241–262.
[59] K.C. Hung, H. Rashidzadeh, Y. Wang, B. Guo, Use of paraffin wax film in MALDI-TOF analysis of DNA, Anal. Chem. 70 (14) (1998) 3088–3093.
[60] K.C. Hung, H. Ding, B. Guo, Use of poly(tetrafluoroethylene)s as a sample support for the MALDI-TOF analysis of DNA and proteins, Anal. Chem. 71 (2) (1999) 518–521.
[61] Y. Xu, M.L. Bruening, J.T. Watson, Non-specific, on-probe cleanup methods for MALDI-MS samples, Mass Spectrom. Rev. 22 (6) (2003) 429–440.
[62] P. Kepper, R. Reinhardt, A. Dahl, H. Lehrach, S. Sauer, Matrix-assisted laser desorption/ionization mass spectrometric analysis of DNA on microarrays, Clin. Chem. 52 (7) (2006) 1303–1310.
[63] B.A. Garcia, P.J. Heaney, K. Tang, Improvement of the MALDI-TOF analysis of DNA with thin-layer matrix preparation, Anal. Chem. 74 (9) (2002) 2083–2091.
[64] O. Vorm, P. Roepstorff, M. Mann, Improved resolution and very high sensitivity in MALDI-TOF of matrix surfaces made by fast evaporation, Anal. Chem. 66 (1994) 3281–3287.
[65] M. Schuerenberg, C. Luebbert, H. Eickhoff, M. Kalkum, H. Lehrach, E. Nordhoff, Prestructured MALDI-MS sample supports, Anal. Chem. 72 (15) (2000) 3436–3442.
[66] C. Jurinke, D. van den Boom, C.R. Cantor, H. Koster, The use of MassARRAY technology for high throughput genotyping, Adv. Biochem. Eng. Biotechnol. 77 (2002) 57–74.
[67] A.C. Syvanen, From gels to chips: ''minisequencing'' primer extension for analysis of point mutations and single nucleotide polymorphisms, Hum. Mutat. 13 (1) (1999) 1–10.

[68] M. Nikolausz, A. Chatzinotas, A. Tancsics, G. Imfeld, M. Kastner, The single-nucleotide primer extension (SNuPE) method for the multiplex detection of various DNA sequences: from detection of point mutations to microbial ecology, Biochem. Soc. Trans. 37 (Pt 2) (2009) 454–459.
[69] D. Podini, P.M. Vallone, SNP genotyping using multiplex single base primer extension assays, Methods Mol. Biol. 578 (2009) 379–391.
[70] P. Oeth, M. Beaulieu, C. Park, D. Kosman, G. Del Mistro, D. Van den Boom, et al., iPLEX^{TM} Assay: Increased Plexing Efficiency and Flexibility for MassARRAY System Through Single Base Primer Extension with Mass-Modified Terminators. Sequenom Application Note, (2005).
[71] L.A. Haff, I.P. Smirnov, Single-nucleotide polymorphism identification assays using a thermostable DNA polymerase and delayed extraction MALDI-TOF mass spectrometry, Genome Res. 7 (4) (1997) 378–388.
[72] K. Meyer, A. Fredriksen, P.M. Ueland, MALDI-TOF MS genotyping of polymorphisms related to 1-carbon metabolism using common and mass-modified terminators, Clin. Chem. 55 (1) (2009) 139–149.
[73] C. Jiang, Z. Zhao, Mutational spectrum in the recent human genome inferred by single nucleotide polymorphisms, Genomics 88 (5) (2006) 527–534.
[74] A. Braun, D.P. Little, H. Köster, Detecting CFTR gene mutations by using primer oligo base extension and mass spectrometry, Clin. Chem. 43 (1997) 1151–1158.
[75] D.P. Little, A. Braun, B. Darnhofer-Demar, H. Koster, Identification of apolipoprotein E polymorphisms using temperature cycled primer oligo base extension and mass spectrometry, Eur. J. Clin. Chem. Clin. Biochem. 35 (1997) 545–548.
[76] T. Wenzel, T. Elssner, K. Fahr, J. Bimmler, S. Richter, I. Thomas, et al., Genosnip: SNP genotyping by MALDI-TOF MS using photocleavable oligonucleotides, Nucleosides Nucleotides Nucleic Acids 22 (5–8) (2003) 1579–1581.
[77] A. Misra, J.Y. Hong, S. Kim, Multiplex genotyping of cytochrome p450 single-nucleotide polymorphisms by use of MALDI-TOF mass spectrometry, Clin. Chem. 53 (5) (2007) 933–939.
[78] M. Beaulieu, Multiplexing the Homogeneous MassEXTEND Assay. Sequenom Application Note, (2003).
[79] S. Gabriel, L. Ziaugra, D. Tabbaa, SNP genotyping using the Sequenom MassARRAY iPLEX platform, Curr. Protoc. Hum. Genet, Chapter 2:Unit 2 (2009) 12.
[80] I.G. Gut, S. Beck, A procedure for selective DNA alkylation and detection by mass spectrometry, Nucleic Acids Res. 23 (8) (1995) 1367–1373.
[81] K. Berlin, I.G. Gut, Analysis of negatively 'charge tagged' DNA by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 13 (17) (1999) 1739–1743.
[82] S. Sauer, D. Lechner, K. Berlin, H. Lehrach, J.L. Escary, N. Fox, et al., A novel procedure for efficient genotyping of single nucleotide polymorphisms, Nucleic Acids Res. 28 (5) (2000) E13.
[83] S. Sauer, D. Lechner, K. Berlin, C. Plancon, A. Heuermann, H. Lehrach, et al., Full flexibility genotyping of single nucleotide polymorphisms by the GOOD assay, Nucleic Acids Res. 28 (23) (2000) E100.
[84] S. Sauer, I.G. Gut, Extension of the GOOD assay for genotyping single nucleotide polymorphisms by matrix-assisted laser desorption/ionization mass spectrometry, Rapid Commun. Mass Spectrom. 17 (12) (2003) 1265–1272.
[85] T.J. Griffin, W. Tang, L.M. Smith, Genetic analysis by peptide nucleic acid affinity MALDI-TOF mass spectrometry, Nat. Biotechnol. 15 (13) (1997) 1368–1372.

[86] P.L. Ross, K. Lee, P. Belgrader, Discrimination of single-nucleotide polymorphisms in human DNA using peptide nucleic acid probes detected by MALDI-TOF mass spectrometry, Anal. Chem. 69 (20) (1997) 4197–4202.
[87] B. Boontha, J. Nakkuntod, N. Hirankarn, P. Chaumpluk, T. Vilaivan, Multiplex mass spectrometric genotyping of single nucleotide polymorphisms employing pyrrolidinyl peptide nucleic acid in combination with ion-exchange capture, Anal. Chem. 80 (21) (2008) 8178–8186.
[88] C. Jurinke, D. van den Boom, A. Jacob, K. Tang, R. Worl, H. Koster, Analysis of ligase chain reaction products via matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry, Anal. Biochem. 237 (2) (1996) 174–181.
[89] R.W. Kwiatkowski, V. Lyamichev, M. de Arruda, B. Neri, Clinical, genetic, and pharmacogenetic applications of the Invader assay, Mol. Diagn. 4 (4) (1999) 353–364.
[90] T.J. Griffin, J.G. Hall, J.R. Prudent, L.M. Smith, Direct genetic analysis by matrix-assisted laser desorption/ionization mass spectrometry, Proc. Natl. Acad. Sci. USA 96 (11) (1999) 6301–6306.
[91] A. Mast, M. de Arruda, Invader assay for single-nucleotide polymorphism genotyping and gene copy number evaluation, Methods Mol. Biol. 335 (2006) 173–186.
[92] J.L. Wolfe, T. Kawate, D.A. Sarracino, M. Zillmann, J. Olson, V.P. Stanton Jr., et al., A genotyping strategy based on incorporation and cleavage of chemically modified nucleotides, Proc. Natl. Acad. Sci. USA 99 (17) (2002) 11073–11078.
[93] B. de Martinville, A.R. Wyman, R. White, U. Francke, Assignment of first random restriction fragment length polymorphism (RFLP) locus (D14S1) to a region of human chromosome 14, Am. J. Hum. Genet. 34 (2) (1982) 216–226.
[94] N.A. Chuzhanova, E.J. Anassis, E.V. Ball, M. Krawczak, D.N. Cooper, Meta-analysis of indels causing human genetic disease: mechanisms of mutagenesis and the role of local DNA sequence complexity, Hum. Mutat. 21 (1) (2003) 28–44.
[95] G.R. Taylor, J.S. Noble, J.L. Hall, A.D. Stewart, R.F. Mueller, Hypervariable microsatellite for genetic diagnosis, Lancet 2 (8660) (1989) 454.
[96] P.A. Sharp, Split genes and RNA splicing, Cell 77 (6) (1994) 805–815.
[97] R. Redon, S. Ishikawa, K.R. Fitch, L. Feuk, G.H. Perry, T.D. Andrews, et al., Global variation in copy number in the human genome, Nature 444 (7118) (2006) 444–454.
[98] H.B. Oh, S.O. Kim, C.H. Cha, S.P. Hong, W.R. Folk, K.M. Kim, et al., Identification of hepatitis C virus genotype 6 in Korean patients by analysis of 5' untranslated region using a matrix assisted laser desorption/ionization time of flight-based assay, restriction fragment mass polymorphism, J. Med. Virol. 80 (10) (2008) 1712–1719.
[99] S.Y. Kwon, W.H. Choe, C.H. Lee, J.E. Yeon, K.S. Byun, Rapid re-emergence of YMDD mutation of hepatitis B virus with hepatic decompensation after lamivudine retreatment, World J. Gastroenterol. 14 (27) (2008) 4416–4419.
[100] S.P. Hong, S.I. Ji, H. Rhee, S.K. Shin, S.Y. Hwang, S.H. Lee, et al., A simple and accurate SNP scoring strategy based on typeIIS restriction endonuclease cleavage and matrix-assisted laser desorption/ionization mass spectrometry, BMC Genomics 9 (2008) 276.
[101] C.H. Lee, S.O. Kim, K.S. Byun, M.S. Moon, E.O. Kim, J.E. Yeon, et al., Predominance of hepatitis B virus YMDD mutants is prognostic of viral DNA breakthrough, Gastroenterology 130 (4) (2006) 1144–1152.
[102] K. Meyer, A. Fredriksen, P.M. Ueland, High-level multiplex genotyping of polymorphisms involved in folate or homocysteine metabolism by matrix-assisted laser desorption/ionization mass spectrometry, Clin. Chem. 50 (2) (2004) 391–402.
[103] S. Krebs, I. Medugorac, D. Seichter, M. Forster, RNaseCut: a MALDI mass spectrometry-based method for SNP discovery, Nucleic Acids Res. 31 (7) (2003) e37.

[104] R.M. McCullough, C.R. Cantor, C. Ding, High-throughput alternative splicing quantification by primer extension and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Nucleic Acids Res. 33 (11) (2005) e99.
[105] C. Jurinke, Copy Number Variance using MassARRAY, in: SEQUENOM's 4th European User Group Meeting, October 8–9, 2007, Hamburg (2007).
[106] L. Winchester, C. Yau, J. Ragoussis, Comparing CNV detection methods for SNP arrays, Brief Funct. Genomic Proteomic 8 (5) (2009) 353–366.
[107] C. Yau, C.C. Holmes, CNV discovery using SNP genotyping arrays, Cytogenet. Genome Res. 123 (1–4) (2008) 307–312.
[108] J. Ott, Counting methods (EM algorithm) in human pedigree analysis: linkage and segregation analysis, Ann. Hum. Genet. 40 (4) (1977) 443–454.
[109] M. Stephens, N.J. Smith, P. Donnelly, A new statistical method for haplotype reconstruction from population data, Am. J. Hum. Genet. 68 (4) (2001) 978–989.
[110] C. Ding, C.R. Cantor, Direct molecular haplotyping of long-range genomic DNA with M1-PCR, Proc. Natl Acad. Sci. USA 100 (13) (2003) 7449–7453.
[111] J. Tost, O. Brandt, F. Boussicault, D. Derbala, C. Caloustian, D. Lechner, et al., Molecular haplotyping at high throughput, Nucleic Acids Res. 30 (19) (2002) e96.
[112] C. Burgtorf, P. Kepper, M. Hoehe, C. Schmitt, R. Reinhardt, H. Lehrach, et al., Clone-based systematic haplotyping (CSH): a procedure for physical haplotyping of whole genomes, Genome Res. 13 (12) (2003) 2717–2724.
[113] P. Sham, J.S. Bader, I. Craig, M. O'Donovan, M. Owen, DNA Pooling: a tool for large-scale association studies, Nat. Rev. Genet. 3 (11) (2002) 862–871.
[114] N. Norton, N.M. Williams, M.C. O'Donovan, M.J. Owen, DNA pooling as a tool for large-scale association studies in complex traits, Ann. Med. 36 (2) (2004) 146–152.
[115] J. Knight, S.F. Saccone, Z. Zhang, D.G. Ballinger, J.P. Rice, A comparison of association statistics between pooled and individual genotypes, Hum. Hered. 67 (4) (2009) 219–225.
[116] K.H. Buetow, M. Edmonson, R. MacDonald, R. Clifford, P. Yip, J. Kelley, et al., High-throughput development and characterization of a genomewide collection of gene-based single nucleotide polymorphism markers by chip-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Proc. Natl. Acad. Sci. USA 98 (2) (2001) 581–584.
[117] K.L. Mohlke, M.R. Erdos, L.J. Scott, T.E. Fingerlin, A.U. Jackson, K. Silander, et al., High-throughput screening for evidence of association by using mass spectrometry genotyping on DNA pools, Proc. Natl. Acad. Sci. USA 99 (26) (2002) 16928–16933.
[118] K. Tang, P. Oeth, S. Kammerer, M.F. Denissenko, J. Ekblom, C. Jurinke, et al., Mining disease susceptibility genes through SNP analyses and expression profiling using MALDI-TOF mass spectrometry, J. Proteome Res. 3 (2) (2004) 218–227.
[119] S. Le Hellard, S.J. Ballereau, P.M. Visscher, H.S. Torrance, J. Pinson, S.W. Morris, et al., SNP genotyping on pooled DNAs: comparison of genotyping technologies and a semi automated method for data storage and analysis, Nucleic Acids Res. 30 (15) (2002) e74.
[120] A. Rautanen, M. Zucchelli, S. Makela, J. Kere, Gene mapping with pooled samples on three genotyping platforms, Mol. Cell. Probes 19 (6) (2005) 408–416.
[121] S. Shifman, A. Pisante-Shalom, B. Yakir, A. Darvasi, Quantitative technologies for allele frequency estimation of SNPs in DNA pools, Mol. Cell. Probes 16 (6) (2002) 429–434.
[122] A. Bansal, D. van den Boom, S. Kammerer, C. Honisch, G. Adam, C.R. Cantor, et al., Association testing by DNA pooling: an effective initial screen, Proc. Natl. Acad. Sci. USA 99 (26) (2002) 16871–16874.
[123] K.B. Beckman, K.J. Abel, A. Braun, E. Halperin, Using DNA pools for genotyping trios, Nucleic Acids Res. 34 (19) (2006) e129.

[124] K. Abel, R. Reneland, S. Kammerer, S. Mah, C. Hoyal, C.R. Cantor, et al., Genome-wide SNP association: identification of susceptibility alleles for osteoarthritis, Autoimmun. Rev. 5 (4) (2006) 258–263.
[125] Y. Bosse, F. Bacot, A. Montpetit, J. Rung, H.Q. Qu, J.C. Engert, et al., Identification of susceptibility genes for complex diseases using pooling-based genome-wide association scans, Hum. Genet. 125 (3) (2009) 305–318.
[126] X.Y. Zhong, W. Holzgreve, MALDI-TOF MS in Prenatal Genomics, Transfus Med Hemother. 36 (2009) 263–272.
[127] B. Vlkova, T. Szemes, G. Minarik, J. Turna, P. Celec, Advances in the research of fetal DNA in maternal plasma for noninvasive prenatal diagnostics, Med. Sci. Monit. 16 (4) (2010) RA85–RA91.
[128] Y.M. Lo, N. Corbetta, P.F. Chamberlain, V. Rai, I.L. Sargent, C.W. Redman, et al., Presence of fetal DNA in maternal plasma and serum, Lancet 350 (9076) (1997) 485–487.
[129] Y.M. Lo, M.S. Tein, T.K. Lau, C.J. Haines, T.N. Leung, P.M. Poon, et al., Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis, Am. J. Hum. Genet. 62 (4) (1998) 768–775.
[130] R.W. Chiu, Y.M. Lo, Application of fetal DNA in maternal plasma for noninvasive prenatal diagnosis, Expert Rev. Mol. Diagn. 2 (1) (2002) 32–40.
[131] C. Ding, R.W. Chiu, T.K. Lau, T.N. Leung, L.C. Chan, A.Y. Chan, et al., MS analysis of single-nucleotide differences in circulating nucleic acids: application to noninvasive prenatal diagnosis, Proc. Natl. Acad. Sci. USA 101 (29) (2004) 10762–10767.
[132] Y. Li, K. Finning, G. Daniels, S. Hahn, X. Zhong, W. Holzgreve, Noninvasive genotyping fetal Kell blood group (KEL1) using cell-free fetal DNA in maternal plasma by MALDI-TOF mass spectrometry, Prenat. Diagn. 28 (3) (2008) 203–208.
[133] D.J. Huang, M.R. Nelson, B. Zimmermann, L. Dudarewicz, F. Wenzel, R. Spiegel, et al., Reliable detection of trisomy 21 using MALDI-TOF mass spectrometry, Genet. Med. 8 (11) (2006) 728–734.
[134] N.B. Tsui, R.W. Chiu, C. Ding, A. El-Sheikhah, T.N. Leung, T.K. Lau, et al., Detection of trisomy 21 by quantitative mass spectrometric analysis of single-nucleotide polymorphisms, Clin. Chem. 51 (12) (2005) 2358–2362.
[135] Y. Li, F. Wenzel, W. Holzgreve, S. Hahn, Genotyping fetal paternally inherited SNPs by MALDI-TOF MS using cell-free fetal DNA in maternal plasma: influence of size fractionation, Electrophoresis 27 (19) (2006) 3889–3896.
[136] A. Cullinan, C. Cantor, Sequenom, Inc, Pharmacogenomics 9 (9) (2008) 1211–1215.
[137] A.S. Giasuddin, K.A. Jhuma, A.M. Haq, Applications of free circulating nucleic acids in clinical medicine: recent advances, Bangladesh Med. Res. Counc. Bull. 34 (1) (2008) 26–32.
[138] F.B. Kohlrausch, C.S. Gama, M.I. Lobato, P. Belmonte-de-Abreu, A. Gesteira, F. Barros, et al., Molecular diversity at the CYP2D6 locus in healthy and schizophrenic southern Brazilians, Pharmacogenomics 10 (9) (2009) 1457–1466.
[139] R.A. Wilke, R.U. Simpson, B.N. Mukesh, S.V. Bhupathi, R.A. Dart, N.R. Ghebranious, et al., Genetic variation in CYP27B1 is associated with congestive heart failure in patients with hypertension, Pharmacogenomics 10 (11) (2009) 1789–1797.
[140] J.E. Zhang, A.L. Jorgensen, A. Alfirevic, P.R. Williamson, C.H. Toh, B.K. Park, et al., Effects of CYP4F2 genetic polymorphisms and haplotypes on clinical outcomes in patients initiated on warfarin therapy, Pharmacogenet. Genomics 19 (10) (2009) 781–789.
[141] J.R. Weiss, M.R. Baer, C.B. Ambrosone, J.G. Blanco, A. Hutson, L.A. Ford, et al., Concordance of pharmacogenetic polymorphisms in tumor and germ line DNA in adult patients with acute myeloid leukemia, Cancer Epidemiol. Biomark. Prev. 16 (5) (2007) 1038–1041.

[142] O. Bergman, A. Hakansson, L. Westberg, A.C. Belin, O. Sydow, L. Olson, et al., Do polymorphisms in transcription factors LMX1A and LMX1B influence the risk for Parkinson's disease? J. Neural Transm. 116 (3) (2009) 333–338.
[143] J.W. Han, H.F. Zheng, Y. Cui, L.D. Sun, D.Q. Ye, Z. Hu, et al., Genome-wide association study in a Chinese Han population identifies nine new susceptibility loci for systemic lupus erythematosus, Nat. Genet. 41 (11) (2009) 1234–1237.
[144] R.J. Martin, A.J. McKnight, C.C. Patterson, D.M. Sadlier, A.P. Maxwell, A rare haplotype of the vitamin D receptor gene is protective against diabetic nephropathy, Nephrol. Dial. Transplant. 25 (2) (2010) 497–503.
[145] H. Stefansson, R.A. Ophoff, S. Steinberg, O.A. Andreassen, S. Cichon, D. Rujescu, et al., Common variants conferring risk of schizophrenia, Nature 460 (7256) (2009) 744–747.
[146] L.A. Weiss, D.E. Arking, M.J. Daly, A. Chakravarti, A genome-wide linkage and association scan reveals novel loci for autism, Nature 461 (7265) (2009) 802–808.
[147] J. Zerbib, J.M. Seddon, F. Richard, R. Reynolds, N. Leveziel, P. Benlian, et al., rs5888 variant of SCARB1 gene is a possible susceptibility factor for age-related macular degeneration, PLoS ONE 4 (10) (2009) e7341.
[148] Y. Li, G. Altarescu, P. Renbaum, T. Eldar-Geva, E. Levy-Lahad, E.J. Margalioth, et al., Non-invasive prenatal diagnosis using cell-free fetal DNA in maternal plasma from PGD pregnancies, Reprod. Biomed. Online 19 (5) (2009) 714–720.
[149] N. Appleby, D. Edwards, J. Batley, New technologies for ultra-high throughput genotyping in plants, Methods Mol. Biol. 513 (2009) 19–39.
[150] P.C. Bundock, F.G. Eliott, G. Ablett, A.D. Benson, R.E. Casu, K.S. Aitken, et al., Targeted single nucleotide polymorphism (SNP) discovery in a highly polyploid plant species using 454 sequencing, Plant Biotechnol. J. 7 (4) (2009) 347–354.
[151] S.H. Wood, X. Ke, T. Nuttall, N. McEwan, W.E. Ollier, S.D. Carter, Genome-wide association analysis of canine atopic dermatitis and identification of disease related SNPs, Immunogenetics 61 (11–12) (2009) 765–772.
[152] W.W. Laegreid, M.P. Heaton, J.E. Keen, W.M. Grosse, C.G. Chitko-McKown, T.P. Smith, et al., Association of bovine neonatal Fc receptor alpha-chain gene (FCGRT) haplotypes with serum IgG concentration in newborn calves, Mamm. Genome 13 (12) (2002) 704–710.
[153] J.G. Kim, T.P. Smith, W.M. Snelling, J.L. Vallet, R.K. Christenson, Linkage mapping of the bovine bone morphogenetic protein receptor-1B (BMPR1B) to chromosome 6, Anim. Genet. 34 (4) (2003) 311.
[154] E.M. Campbell, D. Nonneman, G.A. Rohrer, Fine mapping a quantitative trait locus affecting ovulation rate in swine on chromosome 8, J. Anim. Sci. 81 (7) (2003) 1706–1714.
[155] G. Amexis, P. Oeth, K. Abel, A. Ivshina, F. Pelloquin, C.R. Cantor, et al., Quantitative mutant analysis of viral quasispecies by chip-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Proc. Natl. Acad. Sci. USA 98 (21) (2001) 12097–12102.
[156] S.S. Jiang, T.C. Chen, J.Y. Yang, C.A. Hsiung, I.J. Su, Y.L. Liu, et al., Sensitive and quantitative detection of severe acute respiratory syndrome coronavirus infection by real-time nested polymerase chain reaction, Clin. Infect. Dis. 38 (2) (2004) 293–296.
[157] C. Ding, C.R. Cantor, A high-throughput gene expression analysis technique using competitive PCR and matrix-assisted laser desorption ionization time-of-flight MS, Proc. Natl. Acad. Sci. USA 100 (6) (2003) 3059–3064.
[158] J. Tost, P. Schatz, M. Schuster, K. Berlin, I.G. Gut, Analysis and accurate quantification of CpG methylation by MALDI mass spectrometry, Nucleic Acids Res. 31 (9) (2003) e50.

[159] M. Ehrich, M.R. Nelson, P. Stanssens, M. Zabeau, T. Liloglou, G. Xinarianos, et al., Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry, Proc. Natl. Acad. Sci. USA 102 (44) (2005) 15785–15790.
[160] M. Ehrich, S. Bocker, D. van den Boom, Multiplexed discovery of sequence polymorphisms using base-specific cleavage and MALDI-TOF MS, Nucleic Acids Res. 33 (4) (2005) e38.
[161] Y. Zhang, P. Ozdemir, Microfluidic DNA amplification–a review, Anal. Chim. Acta 638 (2) (2009) 115–125.
[162] K. Tang, D.J. Fu, D. Julien, A. Braun, C.R. Cantor, H. Koster, Chip-based genotyping by mass spectrometry, Proc. Natl. Acad. Sci. USA 96 (18) (1999) 10016–10020.