High-throughput genotyping of short tandem repeat DNA markers with time-of-flight mass spectrometry

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Abstract

Time-of-flight mass spectrometry (TOF-MS) combined with matrix-assisted laser desorption/ionization (MALDI) can be used to rapidly genotype short tandem repeat (STR) DNA markers. This article reviews various applications and conventional approaches for analysis of STRs and contrasts electrophoretic DNA separation methods with mass spectrometric ones. There is a focus on the advantages and challenges of using MALDI-TOF-MS for reliable genotyping of STRs. Different approaches for reducing DNA size and sample salt content prior to mass spectrometric analysis are discussed as are issues surrounding high volume sample processing such as factors impacting the speed of rapid MS data collection.

Keywords: high-throughput genotyping, short tandem repeats, large DNA molecules, MALDI-TOF-MS, sample salt reduction, PCR product analysis

Short tandem repeat DNA markers and their applications

The measurement of genetic variation is important to a number of different fields in biotechnology. Genetic mapping (*1-3*), disease detection via DNA diagnostics (*4*), agricultural biotechnology (*5*), and human identity testing (*6-7*) use DNA markers that can be highly variable from one individual to another in order to understand similarities and differences between those individuals. This genetic variation occurs as sequence or length differences (polymorphisms) in the actual DNA material of the tested individuals. A widely used category of DNA markers for characterizing genetic variation includes the short tandem repeats, or STRs.

Short tandem repeat DNA markers, also referred to as microsatellites or simple sequence repeats (SSRs), consist of tandemly repeated DNA sequences with a core repeat of (2 to 6) base pairs (bp) (*7*). Dinucleotide repeats have two nucleotides repeated next to each other over and over again. Trinucleotides have three nucleotides in the repeat unit, tetranucleotides have four, pentanucleotides have five, and hexanucleotides have six repeat units in the core repeat. The number of repeats can vary from 3 or 4 repeats to more than 50 repeats. STR repeat sequences are typically named by the length of the repeat unit. Thus, a sample with 6 AATG repeats on one chromosome and 8 AATG repeats on the other for a particular STR marker would be designated by the genotype "6,8".

Measurement of STRs is performed using the polymerase chain reaction (PCR) to copy the DNA region containing the repeat sequence to a detectable level followed by a

length-based separation (*7*). The overall length of the PCR product is then related back to the number of repeat units present in the sample based on where the primers bind in conserved regions of the genomic DNA flanking the repeat region. The number of repeats, and hence the size of the PCR product, may vary among samples in a population making STR markers useful in identity testing or genetic mapping studies. It should be noted that different PCR primers may be used to target the same STR repeat region and thus PCR product sizes may vary for the identical allele at a particular marker with different primer sets (**Figure 1**). The information content of a STR marker is in the number of repeats not the overall size of the PCR product.

Thousands of polymorphic microsatellite markers have been identified in human DNA. STR markers are scattered throughout the genome and occur on average every 10,000 nucleotides (*8*). Academic and commercial laboratories have characterized a large number of STR markers for use in disease gene location studies. For example, the Marshfield Medical Research Foundation in Marshfield, Wisconsin has gathered genotype data on over 8,000 STRs that are scattered across the 23 pairs of human chromosomes for the purpose of developing human genetic maps (*2,3*). Such large-scale studies encourage the use of automation and high-throughput genotyping methods.

Conventional Approaches to STR Genotype Analysis

Technologies for measuring DNA variation, both length and sequence polymorphisms, have advanced rapidly in the last decade. Numerous methods for genotyping STRs have been described using techniques that include gel electrophoresis (*9*), capillary

electrophoresis (*10,11*), microchip capillary electrophoresis (*12*), capillary array electrophoresis (*13,14*), electronic stringency hybridization microarrays (*15*), and mass spectrometry (*16-24*).

Some genotyping applications require large numbers of individual DNA samples run at a relatively few number of STR markers (e.g., forensic human identity testing) while other applications benefit from a large number of STRs on a relatively small number of different DNA samples (e.g., gene mapping studies). Regardless of the application, the total number of genotypes that must be determined for a particular study is equal to the number of DNA samples times the number of DNA markers tested within each sample.

Genotyping throughput can be increased by either reducing the amount of time to analyze a single sample, increasing the number of genotypes obtained with a single analysis, or a combination of these two approaches. To reduce analysis cost and sample consumption and to meet the demands of higher sample throughputs, PCR amplification and detection of multiple markers in the sample analysis, an approached termed multiplex STR analysis, has become a standard technique in many DNA typing laboratories.

The most commonly used methodologies involve gel or capillary electrophoresis with laser-induced fluorescence detection (*7*). STR multiplexing is performed using spectrally distinguishable fluorescent tags and/or non-overlapping PCR product sizes. An example of a STR multiplex produced from a commercially available kit is shown in **Figure 2**. Fluorescently labeled primers are incorporated into amplified PCR products whereupon

the different STR alleles can be characterized by size and color. The STR alleles from these multiplexed PCR products typically range in size from 100 bp to 450 bp. An internal standard, shown in the bottom panel of Figure 2, is used to size the PCR products from the various STR markers.

Conversion of PCR product size back to an actual number of repeats for a particular STR marker is often performed with an allelic ladder (*25*). This ladder is produced by mixing common alleles observed in a population genotypic survey and is used as a sizing/genotyping standard to relate the size of an unknown allele back to its number of repeats. An advantage of storing the information from a STR marker in its genotype form is that results from different primer sets or even different technologies can be readily compared between different laboratories. Thus, genotype information is independent of the platform used for analysis while PCR product size is not.

Advantages of Mass Spectrometry Approaches

The primary reason that mass spectrometry is explored as an alternative approach to conventional STR genotyping is the speed at which data may be collected from individual DNA samples. Ion flight times on the order of microseconds with total data collection times of only a few seconds in duration make mass spectrometry extremely attractive to those who have need for high-throughput genotyping. However, it is important to keep in mind that mass spectrometry involves sequential sample processing whereas other approaches, such as gel electrophoresis or capillary array electrophoresis,

can process DNA samples in parallel leading to high sample throughputs relative to the separation time involved.

Another important advantage of mass spectrometry is that the DNA is directly measured without the need to attach detection labels such as the fluorescent dyes used in conventional analysis of DNA. In addition, the actual mass of the DNA molecule is being measured, making it inherently a more accurate technique than a relative size measurement as in electrophoresis. Electrophoretic separations must always use a size standard to calibrate the relative mobilities of unknown DNA fragments to molecules of known size (*7*).

The high degree of accuracy in sizing STR alleles using time-of-flight mass spectrometry is such that this approach permits reliable genotyping without the use of an allelic ladder (*17*). Allelic ladders as well as internal sizing standards are necessary in electrophoretic separation systems to adjust for minor variations in peak migration times due to fluctuations in temperature and voltage (*10,11,25*).

In order to get the DNA molecules into the gas phase for analysis in the mass spectrometer, either matrix-assisted laser desorption-ionization (MALDI) or electrospray ionization (ESI) may be used. Single charged species are the primary ions produced in MALDI, which is beneficial to STR typing since samples are often heterozygous and contain more than one peak. ESI generates multiply charged species making deconvolution of DNA STR samples containing more than one allele more complex.

While some approaches using ESI are not as fast as MALDI-TOF, ESI is inherently more accurate in sizing the STR alleles (*18,19,21,22*). Electrospray ionization is typically combined with ion trap (*19*) or Fourier-transform ion-cyclotron resonance (FTICR) (*18,21,22*) mass analyzers for this application. **Table 1** includes a brief review of the mass spectrometric methods used for analyzing STR markers. This article will focus on MALDI-TOF approaches, as they have been most widely used for high-throughput genotyping applications.

Steps Involved in MALDI-TOF-MS of DNA

The analysis of DNA using MALDI-TOF-MS proceeds as follows. A liquid DNA sample is combined with an excess of a matrix compound, such as 3-hydroxypicolinic acid (*26,27*). These samples are spotted onto a metal or silicon plate. As the sample air dries, the DNA and matrix co-crystallize. The sample plate is then introduced into the vacuum environment of the mass spectrometer for analysis. A rapid laser pulse initiates the ionization process. The matrix molecules that surround the DNA protect it from fragmentation during the ionization process.

Each pulse of the laser initiates ionization of the sample and the subsequent separation of ions in the flight tube. The DNA ions travel to the detector in a matter of several hundred microseconds as they separate based on their velocity, which is directly related to their mass. However, it takes several seconds to analyze each sample because multiple laser pulses are taken and averaged or summed to form the final mass spectrum. Samples are analyzed sequentially by moving the sample plate underneath a fixed laser beam. Sample

plates are now commercially available that can hold 384 (or more) samples at a time. Each sample plate can be analyzed in less than one hour depending on the number of laser shots collected for each sample and the pulse rate of the laser.

Challenges for Successful Mass Spectral Analysis of STRs

Detection of Large DNA Molecules

Several major challenges impact analysis of PCR products including short tandem repeat DNA markers using MALDI-TOF-MS. Both mass resolution and sensitivity are diminished when DNA size increases. Results are typically best if DNA molecules can be kept smaller than approximately 30,000 Da[§], or about 100 nucleotides. Reduction of STR allele sizes is possible by redesigning PCR primers to bind close to the repeat region (*16,17*). In this manner, the resolution and detectability of PCR products is enhanced in the mass spectrometer. Therefore, projects involving the use of mass spectrometry to genotype STRs involve significant effort in designing and testing new PCR primers to produce smaller PCR product sizes for the DNA markers of interest than would be commonly used in conventional electrophoretic separation systems.

Detection of fairly large DNA molecules has been reported (*28,29*) but resolution is not sufficient to resolve STR alleles differing by 2 to 6 base pairs. For example, a single-stranded DNA molecule 468 nucleotides long with a mass of 146,000 has been measured with a mass resolution of only 30 (*29*). This mass resolution corresponds to a peak width of approximately 5000 Da or about 16 nucleotides. Thus, even if routine ionization of

[§] One Dalton (Da) is equivalent to 1 g/mol.

400 bp PCR products was possible, fragmentation, adduct formation, and isotopic distributions broaden peaks to the point that alleles differing by 4 bp would not be distinguished from one another. While the initial results with infrared MALDI appear promising for ionizing DNA molecules of several hundred base pairs in length (*28*), it remains to be demonstrated whether or not sufficient resolution can be obtained to resolve closely spaced STR alleles with PCR product sizes of up to 300 bp or 400 bp.

Sample Salt Content Reduction

The salt content of a DNA sample impacts its ionization efficiency with MALDI (*30*). Since salts are important to the molecular biology of preparing the DNA sample, solid-phase cleanup approaches have been developed to remove or drastically reduce the amount of salt present in a DNA sample prior to it being introduced to the mass spectrometer (*16,31,32*).

MALDI Artifacts

The MALDI ionization process introduces several artifacts to mass spectra of DNA molecules that are important to be aware of and factored into the analysis or sample preparation steps. First, under typical UV MALDI conditions double-stranded DNA denatures and if the mass resolution is insufficient to resolve the two complementary strands, they are detected as a single broad peak. Best results are obtained when examining STR alleles if one of the two complementary strands is removed prior to mass spectral analysis so that only one single strand of DNA is measured for each allele.

Adducts contribute to peak broadness and reduce mass resolution. The common adducts seen with DNA molecules, which possess a negatively charged phosphate backbone, include the alkali salts sodium (+22 Da) and potassium (+39 Da). These two salts are prevalent in PCR and other molecular biology buffers used to manipulate DNA samples. Additionally, with some DNA sequences matrix adducts (+139 Da for 3-HPA) appear. The addition of these adducts can impact the mass accuracy because as large DNA molecules are analyzed, adducts become more prevalent and instrument resolving power and mass resolution are diminished causing adducts to not be fully resolved from the primary molecular ion (*30*). Thus, the centroid of these peaks is shifted to a higher mass than what actually exists for the molecular ion.

Fragmentation is also an issue with DNA. Depurination is the most common form of fragmentation and involves the loss of a single nucleotide base, an adenine (-117 Da) or guanine (-130 Da), from the molecular ion of interest. Depurination results most likely because of the acidic matrix environment as well as the relatively high laser power used to ionize the large DNA molecules present in STR alleles.

Another important issue is the fact that ionization efficiency differences exist with DNA molecules that vary by more than a few thousand Daltons. Thus, if a heterozygous sample contains two alleles that are wide spread in size, the larger allele is less efficiently ionized and may not be detected at all. Software solutions, such as mass dependent intensity scaling, can reduce the impact of sensitivity loss with DNA size.

While MALDI primarily produces singly charged ions, multiple-charged ions can be created depending on a number of factors including the laser power and matrix used. In addition, gas-phase dimers of smaller DNA molecules, such as remaining PCR primers, can fall within the expected mass range for the STR alleles under consideration. In either case, doubly-charged ions and gas-phase dimers typically exhibit a weaker signal than the singly-charged molecular ion for a STR allele.

Calibration Issues

The conversion of data points in mass spectrometry spectral channels from a time value to a mass value is typically performed with linear interpolation between two standards of known mass or mass-to-charge ratio. This mass calibration is most accurate for peaks that fall within the range defined by the two calibrants. With DNA molecules larger than approximately 20 kDa, peak broadness and mass shifting due to adduct formation impacts the ability to accurately define the standard peak centroid leading to poorer precision and accuracy as DNA size increases (*33*). Likewise, creating a pure synthetic oligonucleotide that is 100 or more nucleotides in length that can serve as a calibrant becomes a challenge. Failure sequences become significant as the number of nucleotides in a synthetic oligonucleotide increases. Thus, both synthetic and measurement issues present obstacles in producing a well-defined DNA standard that will work in a DNA ionizing matrix. For this reason, some STR measurement work has used protein standards to calibrate the time-to-mass within the mass range of interest (*16,34*). Fortunately, most alleles in STR systems are several hundred to more than a thousand Daltons apart (e.g., a

four base repeat is equivalent to ≈1200 Da) and thus even a moderate level of mass accuracy can be sufficient to distinguish neighboring alleles from one another.

Impact of Non-template Addition

An artifact of the polymerase chain reaction amplification reaction also introduces some ambiguity to STR measurements. DNA polymerases, particularly the *Taq* polymerase used in PCR, often add an extra nucleotide to the 3'-end of a PCR product as the DNA template strand is being copied. This non-template addition, which is most often an adenine hence the term "adenylation", can be favored by adding a final incubation step at 60 ^{o}C or 72 ^{o}C after the temperature cycling steps in PCR (*9,35*). However, the degree of adenylation is dependent on the sequence of the template strand, which in the case of PCR results from the 5'-end of the reverse primer. Thus, every locus will have different adenylation properties because the primer sequences are different.

From a measurement standpoint, it is best to have all of the molecules as similar as possible for any given STR allele. Partial adenylation, where some of the PCR products do not have the extra adenine (i.e., -A peaks) and some do (i.e., +A peaks), can contribute to peak broadness if the separation system's resolution is poor (**see Figure 4 top panel**). Sharper peaks improve the likelihood that a system's genotyping software can make accurate calls. Variation in the adenylation status of an allele across multiple samples can have an impact on accurate sizing and therefore genotyping of STR alleles. Therefore, it is beneficial if all PCR products for a particular amplification are either +A or –A rather than a mixture (e.g., ±A).

A number of methods may be used to control the adenylation status of a PCR-amplified STR allele (*7*). For example, some DNA polymerases produce PCR products with only the –A peaks. **Figure 5** compares the MS results obtained at a tetranucleotide STR locus using two different polymerases (*36*). The *Tsp* polymerase (previously sold by Life Technologies; now Invitrogen, Carlsbad, CA) produced PCR products with only the –A peaks while *AmpliTaq* Gold DNA polymerase (Applied Biosystems, Foster City, CA) showed partial adenylation with these TH01 primers. Thus, the *Tsp* polymerase has the potential to produce sharper peaks (i.e., no partial adenylation) and allele masses that can be more easily predicted (i.e., all PCR products would be non-adenylated).

Solutions for Size and Salt Reductions Prior to Mass Spectrometry

DNA Size Reduction Approaches

Several size reduction techniques involving or following PCR amplification that have been described for use prior to mass spectral analysis are illustrated in **Figure 3**. The most successful techniques involve both the design and use of PCR primers that anneal in close proximity to the STR repeat region and the use of post-amplification size reduction methods. For example, the cleavable primer approach reduces the measured DNA size further than just using PCR amplification alone (Figure 3C versus 3A).

The primer oligo base extension (PROBE) reaction is illustrated in Figure 3B (*31,37*). A primer that anneals adjacent to the repeat region is extended using a mixture of three

deoxynucleotide triphosphates (dNTP) and one dideoxynucleotide triphosphate (ddNTP). The three dNTPs match the bases present in the repeat region. For example, if a GATA repeat is being examined then dGTP, dATP, and dTTP would be used along with ddCTP. The extension would then be terminated at the first C nucleotide that occurs on the opposite side of the repeat from the primer. While this method is quite effective in most cases, it requires that only three nucleotides are present in the repeat region. If a compound repeat structure exists for the STR marker (e.g., both TCTA and TCTG repeat units are present), then the PROBE reaction would be unable to extend through the entire repeat region and some of the polymorphic genotypic information would be lost.

For the cleavable primer approach (Figure 3C), the cleavable base is typically placed in the second or third position from the 3'-end of the primer in order to remove as much of the modified primer as possible (*36,38*). Thus, the cleavage step reduces the overall PCR product size by the length of the cleavable primer minus two or three nucleotides. Typically this size reduction is approximately 20 bases. The portion of the DNA product on the other side of the repeat region from the cleavable primer can be reduced in one of three possible ways: (a) moving the opposite primer as much as two full repeats onto the repeat region (*36*), (b) using a restriction enzyme (*39*) or (c) performing a nested linear amplification with a terminating nucleotide (*36,37*). These methods only work for particular situations, but these size reduction methods can play a role in multiplex STR work (*36,40*).

The combination of a cleavable primer approach (Figure 3C) with the PROBE approach (Figure 3B) can result in further size reduction and eliminate the problems of partial non-templated addition. **Figure 4** illustrates the advantage of a ddG termination on a D8S1179 heterozygous sample containing 11 and 13 TATC repeats (*36*). In the bottom panel, 23 nucleotides have been removed as compared to the top panel, which corresponds to a mass reduction of almost 7500 Da. The peaks are sharper in the lower panel as the products are blunt ended and are in a lower mass range. Identical genotypes were obtained with both approaches illustrating that the ddG termination is occurring at the same point on the two different sized alleles.

To summarize, STR sample sizes can be reduced using primers that have been designed to bind close to the repeat region or even partially on the repeat itself. One approach uses a cleavable primer that is incorporated into the PCR product to allow post-PCR chemical cleavage and subsequent mass reduction. Two additional post-PCR methods may also be used to further reduce the measured DNA size. These methods included restriction enzyme digestion in the flanking region on the other side of the repeat region from the cleavable primer (*39*) and a primer extension through the repeat region with a single dideoxynucleotide terminator (*37*).

The advantages of these size reduction approaches to shorten the overall DNA product mass can be illustrated with the tetranucleotide STR locus TPOX (*36*). Using a conventional electrophoresis primer set, a sample containing 11 repeats would measure 232 bp or ≈66,000 Da. If redesigned primers are used to anneal close to the repeat region

(*17*), a PCR product size of 89 bp is obtained. With the cleavable primer, the size is reduced to 69 nucleotides (nt) or ≈21,500 Da. By incorporating a ddC termination reaction to extend through the AATG repeat region, another 20 nt are removed leaving only 49 nt or ≈12,000 Da (primarily only the repeat region). The repeat region alone is 44 nt (4 nt x 11 repeats) or ≈10,500 Da.

By designing the PCR primers to bind close to the repeat region, the STR allele sizes are reduced so that resolution and sensitivity of the PCR products are benefited. Where possible, primers are designed to produce PCR products that are less than 100 bp in size. Unfortunately, due to long and complex repeat structures of some STR markers, it is not always possible to design primers that produce small enough PCR products. In addition, the flanking sequences immediately adjacent to the STR repeat region may not always be conducive to PCR primer annealing. It still remains difficult to resolve neighboring tetranucleotide STR alleles that are larger than about 140 bp in size (*36,41-43*).

Salt Reduction Approaches

Conventional molecular biology approaches of reducing the salt content of DNA samples include ethanol precipitation, size-exclusion microfiltration, and membrane dialysis (*16,43*). However, many procedures for salt reduction used in conjunction with mass spectrometry involve solid-phase purification using streptavidin-coated magnetic beads to capture biotinylated PCR products primarily because this technique is more amenable to automation (*16,17,32,37*).

Biotin is synthetically placed on the 5'-end of one of the PCR primers so that it is incorporated into the PCR product as the amplification reaction occurs. The biotinylated PCR product can then be captured at the surface of a streptavidin-coated magnetic bead because of the strong interaction between biotin and streptavidin. Next, the beads are pulled to the bottom or side of the sample tube with a strong magnet allowing the supernatant to be easily removed. The captured PCR product can be denatured and the non-biotinylated strand removed for analysis. Either before or after the non-biotinylated strand is removed, additional solutions, such as ammonium acetate, can be added to the remaining captured strand anchored to the magnetic beads and a buffer exchange can be performed. For example, the DNA counter-ions of sodium, potassium, and magnesium ions that interfere with the MALDI ionization process (*30*) can be replaced with ammonium ions that improve the DNA signals obtained with MALDI. Appropriate care must be taken to prevent samples from being contaminated with salts both during and after any sample purification procedure.

STR Typing Using Cleavable Primer Chemistry

Several years ago a cleavable primer chemistry was developed by GeneTrace Systems (formerly of Alameda, CA) that is amenable to high-throughput sample processing (*32*). A biotinylated, cleavable oligonucleotide is used as a primer in each assay and is incorporated through standard DNA amplification (e.g., PCR) methodologies into the final product, which is measured in the mass spectrometer. The use of a cleavable primer technology permits both a post-amplification DNA size reduction and sample purification through salt removal using a solid-phase magnetic bead approach (*32,38*).

The process of STR detection and genotype analysis with a cleavable primer can best be illustrated with an example. The STR locus TH01 is a tetranucleotide repeat commonly used for human identity testing purposes (*7,44,45*). While the PCR conditions listed below are specific to the primers designed for the TH01 STR marker, the sample cleanup and mass spectrometry details are fairly universal.

PCR Amplification

Several different primer sets were used for amplification of the TH01 STR marker. Primers were designed to anneal as close as possible to the repeat region and to exhibit a predicted annealing temperature of (57 to 63) ^{o}C. The positions of two different primer pairs for the TH01 locus are illustrated on the complement of the GenBank reference sequence (accession number D00269), which contains 9 AATG repeat units (Figures 6 and 7). The PCR product size for allele 9 was 79 bp with the following primers:

5’-biotin-GGGAACACAGACTCCATGG(T)G-3’

5’-CCTGTTCCTCCCTTATTTCCC-3’

and 88 bp with a second set of primers:

5’-biotin-AGGTCACAGGGAACACAGAC(T)CC-3’

5’-GCCTGTTCCTCCCTTATTTCCC-3’

The chemically cleavable nucleotide is shown here in parentheses and is a 3'-(S)-phosphorothioate that is incorporated into the oligonucleotide primer via standard phosphoramidite chemistry (*38*). Typically the PCR reactions were performed in 20 μL volumes with 20 pmol (1 μM)[§] both forward and reverse primers using 1 unit of AmpliTaq Gold™ DNA polymerase (Applied Biosystems), 200 μM dNTPs, 50 mM KCl, 10 mM Tris-HCl, 5% (v/v) glycerol, and 2 mM $MgCl_2$. With the exception of substituting one of the primers for a cleavable primer, these PCR reaction materials are commonly used in molecular biology protocols.

The thermal cycling was performed in a GeneAmp 9700 (Applied Biosystems) with the following cycling parameters: 95 ^{o}C for 11 min (to activate the TaqGold DNA polymerase), 40 cycles of 94 ^{o}C for 30 s, 55 ^{o}C for 30 s, and 72 ^{o}C for 30 s, followed by 60 ^{o}C for 15 min (to promote non-templated addition). Higher PCR product yields improve MS detection of STR alleles. Thus, many protocols for STR typing with mass spectrometry require more cycles (e.g., 40 instead of 25 or 30 with fluorescent dye labeling methods) or a two-stage amplification (*36*).

Sample Purification and Size Reduction

The cleavable primer sample cleanup method involves washing the PCR-amplified DNA with a series of chemical solutions intended to remove or reduce the high levels of sodium, potassium, and magnesium present from the PCR reaction (*38*). First, the complementary (non-biotinylated) DNA strand is removed with sodium hydroxide and

[§] The accepted SI unit of concentration, mol/L, has been represented by the symbol M in order to conform to the conventions of this publication.

then the beads are washed with ammonium acetate followed by deionized water to reduce the levels of sodium in the sample. The PCR products were then released from the bead with a chemical cleavage step that breaks the covalent bond between the 5'-biotinylated portion of the DNA product and the remainder of the extension product, which contains the STR repeat region. This chemical cleavage is performed with a 0.1 mM silver nitrate solution at 48 ^{o}C for 15 minutes to break the thiophosphate bond in the PCR product backbone.

The cleavage point in each assay is illustrated with the vertical arrow in Figures 6 and 7. The overall size of the DNA molecule that is measured is reduced by 20 nucleotides with the cleavable primer shown in Figure 6 (from 79 to 59 nucleotides) and 21 nucleotides with the cleavable primer shown in Figure 7 (from 88 to 67 nucleotides). With chemical cleavage, the supernatant contains the DNA molecules of interest and therefore can separated from the magnetic beads via a liquid transfer.

In the final step prior to mass spec analysis, samples are combined with dithiothreitol (DTT), evaporated to dryness, and then reconstituted with 0.5 μL of matrix (manual protocol) or 2 μL of matrix (robotic protocol) and spotted on the sample plate. A mixed matrix of 3-hydroxypicolinic acid (3-HPA) and picolinic acid at a molar ratio of 5:1 also benefited detection of DNA molecules greater than ~20 kDa as compared to 3-HPA alone (*33,36*).

TOF-MS Analysis

Linear mode is typically used with STR alleles as masses are too large for reflectron mode to be of much value. Delayed extraction (*46*) and mass gating ("blanking") are used to improve peak resolution and sensitivity, respectively. A delay time of 500 to1000 or more nanoseconds is important for successful STR ionization as is eliminating ions below ≈8,000 Da. An off-axis flight tube was also used to benefit sensitivity (*47*).

DNA samples were processed on a custom built mass spectrometer designed for high-throughput sample processing (*47*). The instrument settings for the mass spectrum shown in Figure 8B were as follows: extraction voltage: +20.5 kV; delayed extraction at 1.12 μs (microseconds) with a +3.6 kV pulse; signal "blanking" below 5.84 μs; and 400 laser pulses summed from 355 nm of Nd:YAG laser.

Data Processing and Genotyping

Raw data files were converted to "smoothed" data files using custom software developed at GeneTrace Systems that involved several multipoint Savitzky-Golay averages along with a baseline subtraction algorithm (*48*) to improve data quality and enhance resolution of closely spaced peaks. In addition, mass dependent intensity scaling was often used to artificially balance allele peak signals between heterozygous individuals.

Genotyping of STR samples involved comparing observed peak masses with expected masses calculated for each STR allele. The expected STR allele masses are determined using the mass of a reference sequence, the mass of the repeat unit, and a preset spread of

alleles. STR reference sequences may be obtained from GenBank (http://www.ncbi.nlm.nih.gov/). For example, the STR locus TH01 is found through GenBank accession number D00269, which contains 2838 bp of the *Homo sapiens* gene for tyrosine hydroxylase. The GenBank sequence for TH01 (located in intron 1 of the tyrosine hydroxylase gene) contains 9 AATG repeat units on the complementary strand, which are shown in Figures 6 and 7 with some of the flanking sequence information. The repeat unit of AATG has a mass of 1260 Da. Thus, once the reference allele mass is calculated based on the PCR primer positions (and post-PCR manipulations) the other common allele masses can be calculated by adding or subtracting the repeat mass of 1260 Da (see Figure 6). Some STR markers contain microvariant alleles that differ from neighboring alleles by less than a full repeat unit. The TH01 9.3 allele is a good example and differs from allele 10 by the deletion of a single adenine in the seventh repeat unit (*44*).

Results

A comparison of TH01 allele peak masses to the calculated masses is shown in Table 2 for 7 different alleles. All allele measurements were less than 30 Da, or ~0.1 nucleotides, from the expected peak masses (see Figure 6). Ion flight times for alleles containing 5 to 10 repeat units ranged from (170 to 205) μs with alleles 9.3 and 10 that differ by only a single base separated by 1.5 μsec. The measurement of allelic ladder, which in this case contains the alleles 5, 6, 7, 8, 9, 9.3, and 10, effectively demonstrates that all alleles can be detected and resolved from one another (**Figure 8**). In the case of the TH01 allelic ladder results shown in Table 2, mass differences between each peak demonstrates that

each allele differs from its neighboring allele by one AATG repeat unit with the exception of alleles 9.3 and 10 that differ by a single adenine (*17*). In addition, mass difference measurements can be helpful in verifying the repeat content in stutter products, identifying the extra base added by the DNA polymerase, and confirming the repeat spread in heterozygous samples (*49,50*).

In MALDI-TOF-MS it is often difficult to obtain mass accuracies of much better than 0.1% for STR alleles because of matrix crystal height variation, which also impacts peak broadening. The matrix crystal height may only vary by a fraction of a millimeter, but this does impact the spatial distribution of ions that are accelerated off the surface of the MALDI target. However, what may appear to be significant relative mass errors in conventional mass spectrometry (e.g., 30 Da or 0.1%) do not impact accurate and reliable genotyping because each allele differs from its adjacent allele by several hundred to over a thousand Daltons (e.g., 1260 Da for an AATG repeat). In fact, a comparison study of MALDI-TOF-MS results with over 1,000 STR alleles measured by conventional fluorescent methods with capillary electrophoresis separation demonstrated an excellent correlation between the two methods (*36*).

Multiplex STR Analysis

Due to the DNA size constraints of mass spectrometry, a different approach must be taken to analyze multiple STR loci simultaneously (*36,51*). Primers are designed such that the PCR product size ranges overlap between multiple loci but have alleles that interleave and are resolvable in the mass spectrometer (**Figure 9**).

Some limited STR multiplexes can be designed by construction of virtual allelic ladders or "mass simuplexes" that involve the predicted mass of all known alleles for a particular locus. STR markers are interleaved based on mass with all alleles between loci being distinguishable (**Figure 9**). STR multiplexes work best if alleles are below (20,000 to 25,000) Da in mass due to the improved sensitivity and resolution that is obtainable in the mass spectrometer. As described above in the section on size reduction, a restriction enzyme or a dideoxynucleotide terminator may be used to shorten the STR allele sizes. For multiplex design, locating a restriction enzyme with cut sites common to all STR loci involved in the multiplex complicates the design process and makes the choice of possible marker combinations more limited. The use of a common dideoxynucleotide terminator is much easier. For example, with the STR loci CSF1PO, TPOX, and TH01 a multiplex was developed using a ddC terminator and primer extension along the AATG strand (*36*).

Issues Involved in High-throughput STR Genotyping

One approach that has been taken for high-throughput STR genotyping is the combination of robotic sample preparation and mass spectrometry (*17,36,41,42,52*) with STR samples processed as singleplex reactions (although some limited multiplex reactions have been performed). The general steps in this approach are illustrated in **Figure 10**. DNA samples are processed in parallel on 96 or 384 well plates through the PCR amplification and PCR product purification steps. The samples are then robotically spotted onto a 384-position MALDI plate and introduced manually into the mass

spectrometer. Rapid data collection is conducted on each spot in a sequential manner followed by conversion of peak masses to STR repeat numbers, i.e., genotypes, with off-line computer programs. **Figure 11** compares the various steps involved in genotyping STR markers using conventional electrophoretic techniques versus the MALDI-TOF-MS approach described here.

Sample Flow

High-throughput operations will be most successful if there is a constant flow of samples moving through the various steps. Thus, it is important to balance the time that the DNA samples spend at each step along the way. Some steps, such as PCR amplification, involve processing samples in parallel while other steps in the operation, such as data collection on the mass spectrometer, are done in a sequential fashion. Significant deviations from this balance will not permit taking full advantage of the speed of one of the steps. For example, if data can be collected from the mass spectrometer at a rate of one sample every 5 seconds, then genotyping software must be able to process each sample at a speed of less than 5 seconds in order to prevent a backlog from developing at the genotyping step (when running at full capacity).

In calculations of theoretical throughput and sample flow, instrument maintenance and downtime are important issues to consider. To optimize the flow of samples through a high-throughput operation, multiple instruments can be inputted into a particular step. For example, if the PCR reaction takes two hours but a plate of 384 samples can be analyzed in the mass spectrometer in one hour, then two 384 well thermal cyclers could be used to

make the process more efficient. Thermal cyclers capable of handling either (96 or 384) well trays are commonly used in molecular biology laboratories and are available from a number of manufacturers. Likewise, 96-tip robots, such as the Beckman Coulter Multimek™, are also used in high-throughput laboratories.

The sample preparation chemistry is important to adapting manually prepared STR genotyping assays to routine robotic processing 96 or 384 samples in parallel without adding significant time to the overall process. While a number of approaches to STR analysis using mass spectrometry have been published, these approaches are not equally amenable to automation and thus may not be used effectively in high-throughput genotyping operations. Cleavable primer technology (*32,38*) has been used to demonstrated the capability of typing 3000-4000 DNA samples per day per high-throughput mass spectrometer when operating at full capacity (*36*).

Rapid MS Data Collection

Data collection on the mass spectrometer must be totally automated in order to operate in a high-throughput environment. Effective rapid and automated data collection is dependent on computer software solutions, instrument hardware, and sample preparation. High quality mass spectra can be collected in an automated fashion using “peak picking” software, such as the “fuzzy logic” algorithm (*53*).

However, even with automated peak picking software capable of distinguishing a useful signal from little-to-no signal, collecting mass spectra in an automated fashion is

challenging with MALDI. Ionization efficiencies can vary widely across a sample spot. This variation is due to non-uniform crystallization with commonly used DNA matrices, such as 3-hydroxypicolinic acid (*26*). Depending on where the laser strikes on the matrix-DNA sample crystal as well as what laser power is used, the signal observed can vary from none at all to excellent within the same sample spot. So called "sweet spots" exist in a typical matrix-DNA sample crystal where the best results are obtained. Manually locating the sweet spots is not an option if high volume sample processing is desired. Thus, the automated data collection software should be able to select "good" over "bad" mass spectra and not save spectra that contains no signal in the expected mass range. If poor signal is saved into the final summed spectrum, data quality suffers.

Equally important is properly locating the sample on the MALDI target plate so that its position is correlated to where the laser strikes the crystal. The MALDI plate must move automatically from one sample to the next with each sample crystal being irradiated by the laser pulses. If the laser light strikes next to a matrix-DNA crystal, no sample ionization will occur regardless of the quality of the DNA sample. Thus, samples need to be uniformly prepared and in exact, specific locations on the MALDI target in order for the peak picking software to quickly locate and acquire the best signal.

Prestructured hydrophilic sample supports on MALDI targets make defining the exact locations of the matrix crystals possible (*54*). With this approach, photolithographic masks are used to generate small, circular gold spots, each measuring 200 μm in diameter that are surrounded by Teflon coating. The water-repellant Teflon helps define the exact

position of drying matrix spots as they shrink during the evaporation/crystallization process. In such a manner, 384 or even 1536 spots may be defined on a MALDI target at specific locations. The x-y sample stage in the mass spectrometer can then be correlated to move from one position to the next as the entire batch of samples on the sample plate is processed.

Factors Impacting Speed

The speed at which a mass spectrum is collected is dependent on a number of factors including mechanical speed of the MS system and sample quality. The total time for collecting data from each sample is made up of three segments: data collection, data storage, and mechanical x-y stage movement to the next sample. Fast computers and fairly sophisticated electronics for signal processing have reduced the time necessary to store each final (summed) mass spectrum to a second or less. Likewise, mechanical stage movements from sample to sample can be a fraction of a second to a few seconds depending on the distance between samples. Thus, the majority of the time involved examining a sample lies in the data collection portion.

Each sample mass spectrum in MALDI-TOF-MS is created by averaging or summing multiple individual spectra. Most time-of-flight mass spectrometers are set up to collect data from the sample ionization that occurs with each laser shot. Thus, data collection time is a factor of the total number of laser shots averaged (summed) to form the final mass spectrum multiplied by the amount of time between each laser pulse.

Commercial MALDI-TOF-MS instruments commonly come with a 337 nm nitrogen laser capable of up to 10 shots per second. However, higher duty cycle lasers capable of 100 or more shots per second are available with solid-state lasers such as the Nd:YAG (typically used at 355 nm for UV MALDI). The advantage of using a faster laser is that more shots can be collected in a certain period of time, a fact that can be used to increase the data quality and signal-to-noise ratio in a given period of time or reduce the amount of time needed to collect a fixed number of laser shots. Therefore, sample throughput increases if a 100 Hz laser can collect 200 shots in two seconds compared with a 10 Hz laser that takes 20 seconds to collect the same number of shots. However, sample quality and sample spot positioning also play an important role in the speed of data collection.

A comparison of data from four different sets of 384 samples collected on a high-throughput mass spectrometer is found in **Table 3**. The laser on this particular system was operated at a speed of 100 shots per second. Peak picking software was programmed to collect 200 good quality mass spectra ("good shots") per sample and to allow up to 1000 laser shots producing spectra that did not meet the selection criteria of a peak occurring within a specified mass range with a specified signal-to-noise ("bad shots"). The total number of laser pulses ("total shots") shown below is the combination of all good shots and all bad shots for all 384 samples on a plate.

Under ideal conditions with all laser pulses collecting useful signal, the minimum time for collecting data on a plate of 384 samples would be 16.4 minutes, which corresponds to an average of 2.6 seconds per sample. In reality though, the time required to begin

acquiring good shots varies from plate to plate. For example with plate A, the sample quality is pretty good as the number of good shots is close to the ideal situation. Sample spot positioning relative to the x-y table movements and laser pulses is pretty good. All 384 samples were analyzed in 30 minutes for an overall speed of 4.7 seconds per sample (*36*). However, some of the other plates were not analyzed quite as fast. Sample quality was not as good in Plate D as Plate A (or the spots were not positioned as well) leading to a higher number of bad shots, a higher number of total shots, and therefore a slower sample throughput (Table 3). An increase of only 3 seconds per sample increases the total time for analysis of a plate from (30 to 51) minutes (see Table 3 plate A vs. D). Thus, in summary data collection in a rapid and automated fashion requires small and uniform matrix crystals, well-defined spot positions on the sample plate, and a good balance between laser rate and desired spectral quality.

Comparison to Throughput Using Fluorescent Detection Methods

While tremendous sample analysis speeds are possible with mass spectrometry, it is important to keep in mind that MS is a serial technique. Technologies using parallel detection formats, while possessing much slower run times, can compete quite well with mass spectrometry in terms of sample genotyping throughput. The ability to multi-task detection through differentially labeling DNA molecules with spectrally distinguishable fluorescent dyes has been used for a number of years to perform reliable STR typing (*1,7,9*). Gel electrophoresis systems can run up to 96 lanes in parallel and capillary array electrophoresis instruments are now commercially available with 96 capillaries. These parallel separation systems in combination with multi-color fluorescent detection and

multiplex PCR can yield genotyping throughputs on the same time scale as mass spectrometry (**Table 4**). Note for example that a 96-capillary instrument separating multi-colored STR alleles from a 16-locus amplification can measure these alleles at an overall rate of approximately 6.5 seconds per genotype even with a run time of almost 3 hours.

In forensic DNA laboratories today, as many as 16 different DNA markers can been simultaneously PCR-amplified (*55,56*) and measured in a single lane of a gel or a single capillary injection using three colors to detect the PCR products and a fourth color for an internal sizing standard (see Figure 2 for example). If STR markers are run one at a time via mass spectrometry, then it takes 16 sample preparations and the collection and processing of 16 mass spectra to duplicate the results from a single capillary injection or a single lane on a multi-color fluorescence detection platform. It is important to keep these calculations in mind when comparing the rapid serial separation and detection methods of mass spectrometry to slower parallel separation techniques. However, these comparisons do not mean that mass spectrometry does not have a role to play in high-throughput genotyping only that the rapid speeds of mass spectrometry should not be exaggerated.

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Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology (NIST) nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose. The opinions stated in this article are those of the author alone.

Table 1. Review of efforts with STR genotyping and mass spectrometry.

Method	STR Markers	Mass Range	Size-reduction/ Purification Method	Reference
MALDI-TOF	VWF (Siberian huskey)	45-55 kDa	Qiagen PCR purification kit and nitrocellulose film	(*33*)
MALDI-TOF	TH01, FES/FPS, CSF1PO, F13A1	15-30 kDa	Cleavable primer	(*57*)
MALDI-TOF	D2S1384, D2S428, D1S255, GATA145f08	15-30 kDa	Cleavable primer	(*52*)
MALDI-TOF	TH01, TPOX, COL1A	20-30 kDa	Magnetic beads, ethanol precipitation, membrane dialysis	(*16*)
MALDI-TOF	AluVpA microsatellite	11-26 kDa	ddNTP termination; magnetic beads	(*31*)
MALDI-TOF	D21S1260, D21S1893	11-17 kDa	ddNTP termination; magnetic beads	(*58*)
MALDI-TOF	VWFII, VWA, F13B, LPL	35-60 kDa	Sephadex spin columns, ethanol precipitation	(*43*)
MALDI-TOF	TH01, TPOX, COL1A	20-30 kDa	Magnetic beads	(*59*)
MALDI-TOF	TH01, TPOX, CSF1PO, AMEL	15-30 kDa	Cleavable primer	(*17*)
ESI-FTICR	HOXD13 trinucleotide	30-37 kDa	*Not described*	(*60*)
ESI-FTICR	TH01, VWA	50 kDa	Ethanol precipitation and microdialysis	(*18,21,22*)
MALDI-TOF	CAG repeat in androgen receptor gene	56-67 kDa	Ethanol precipitation	(*34*)
MALDI-TOF	TH01, TPOX, FGA, CSF1PO, vWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, DYS19, DYS391, F13A1, FES/FPS, HPRTB, CTT multiplex, D16S2622	10-50 kDa	Cleavable primer cleavable primer with restriction enzyme or ddNTP termination	(*38,40-42,49-51*)
ESI-ion trap	HMS3, HTG6, VHL20 (horse dinucleotides)	20-30 kDa	Reversible binding to magnetic beads	(*19*)
MALDI-TOF	GART, AC1, TGFBR2, MSH3, MSH6	4-9 kDa	Reversible binding to magnetic beads	(*20*)
MALDI-TOF	CA repeats	19-34 kDa	Reversible binding to magnetic beads	(*23*)
MALDI-TOF	Bovine di-, tri-, and tetra-nucleotide repeats	7-20 kDa	RNase cleavage/ membrane filtration	(*24*)

Table 2. Measurement of TH01 alleles in allelic ladder (see Figure 8B for spectrum)

TH01 Allele	PCR Size	Post-Cleavage	Ion Flight Time	Measured Mass	Expected Mass	Mass Offset	Relative Error*
5	63 bp	43 nt	170.1 μsec	13975 Da	13958 Da	17 Da	0.12%
6	67 bp	47 nt	177.6 μsec	15230 Da	15218 Da	12 Da	0.08%
7	71 bp	51 nt	184.8 μsec	16492 Da	16478 Da	14 Da	0.08%
8	75 bp	55 nt	191.7 μsec	17766 Da	17738 Da	28 Da	0.16%
9	79 bp	59 nt	198.4 μsec	19019 Da	18998 Da	21 Da	0.11%
9.3	82 bp	62 nt	203.3 μsec	19973 Da	19945 Da	28 Da	0.14%
10	83 bp	63 nt	204.8 μsec	20280 Da	20258 Da	22 Da	0.11%

*Relative error = (measured-expected)/expected

Table 3. Review of multiple high-throughput runs collected on a GeneTrace Systems custom-built time-of-flight mass spectrometer (*36*). The laser on this particular system was operated at a speed of 100 shots per second. Peak picking software was programmed to collect 200 good quality mass spectra ("good shots") per sample and to allow up to 1000 laser shots producing spectra that did not meet the selection criteria of a peak occurring within a specified mass range with a specified signal-to-noise ("bad shots"). The total number of laser pulses ("total shots") shown below is the combination of all good shots and all bad shots for all 384 samples on a plate. Under ideal conditions with all laser pulses collecting useful signal, the minimum time for collecting data on a plate of 384 samples would be 16.4 min, which corresponds to an average of 2.6 s per sample.

	Good shots (goal: 200/sample)	Bad shots (up to 1000/sample)	Total number of laser shots	Total Time for 384 samples	Average Time per Sample
Ideal result	*76800*	*0*	*76800*	*16.4 min*	*2.6 s*
Plate A	74277	66063	140340	30 min	**4.7 s**
Plate B	68283	108968	177251	37 min	5.8 s
Plate C	68749	133360	202109	41 min	6.4 s
Plate D	53839	192144	245983	51 min	8.0 s

Table 4. Estimated time per genotype for collecting STR data using multi-color fluorescence detection with STR samples generated by multiplex PCR amplification.

# STR Markers Multiplexed	Single capillary (ABI 310) **Each run: 30 min**	96 capillary array (ABI 3700) **Each run: 2 h 46 min**
1	1800 s (30 min)	104 s (1.7 min)
8	225 s (3.8 min)	13 s (0.2 min)
16	113 s (1.9 min)	**6.5 s** (0.1 min)

Figure 1. Schematic of PCR primer positions for amplification of a short tandem repeat DNA marker (*7*). The single-headed arrows represent the primer positions. The double-headed arrows illustrate the overall PCR product size using a particular set of primers. The PCR product size is measured and converted back to the number of repeat units present in the sample for genotyping purposes.

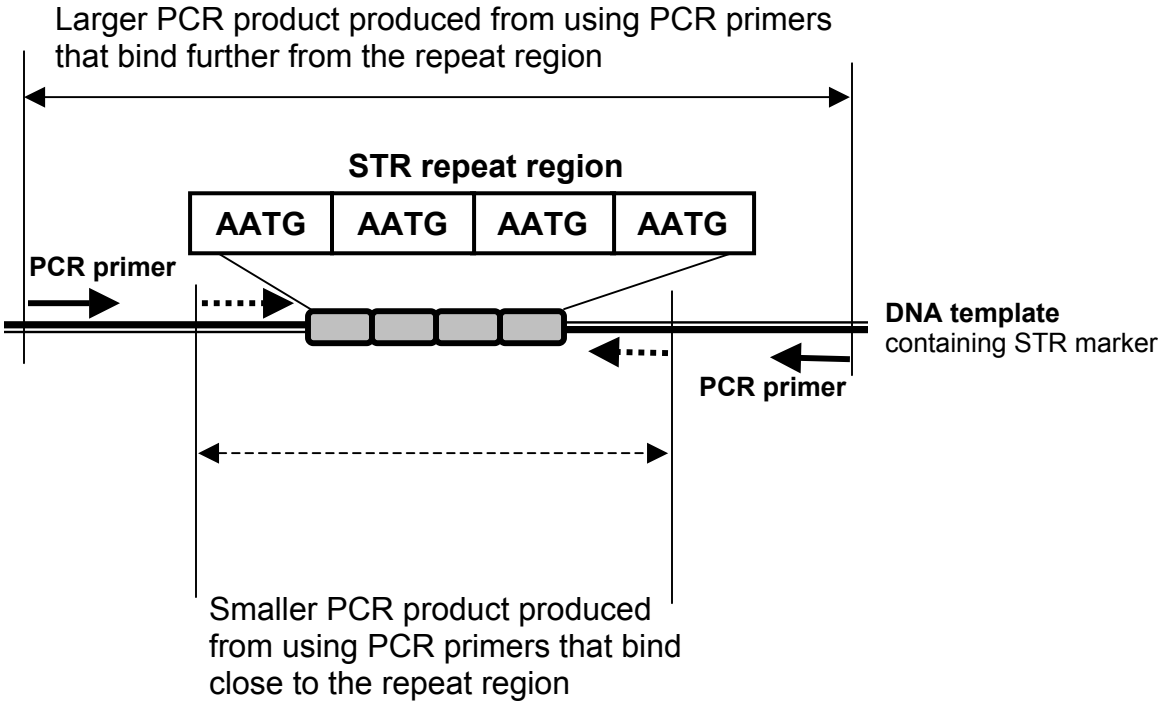


Figure 2. STR analysis at 9 different loci and the sex-typing marker amelogenin (shown in the boxed area) using capillary electrophoretic separation of multiple dye labeled PCR products. These PCR products were generated from a single multiplex PCR reaction using the AmpFlSTR® Profiler Plus™ kit (Applied Biosystems) with separation and detection on the ABI Prism 310 Genetic Analyzer. Each panel illustrates the PCR products labeled with the indicated fluorescent dye. PCR product sizes are generated by comparison of peak migration times to the GS500-ROX internal sizing standard (bottom panel). The genotypes (number of repeats) for each locus are determined by comparison of allele sizes to allelic ladders using Genotyper® 2.5 software.

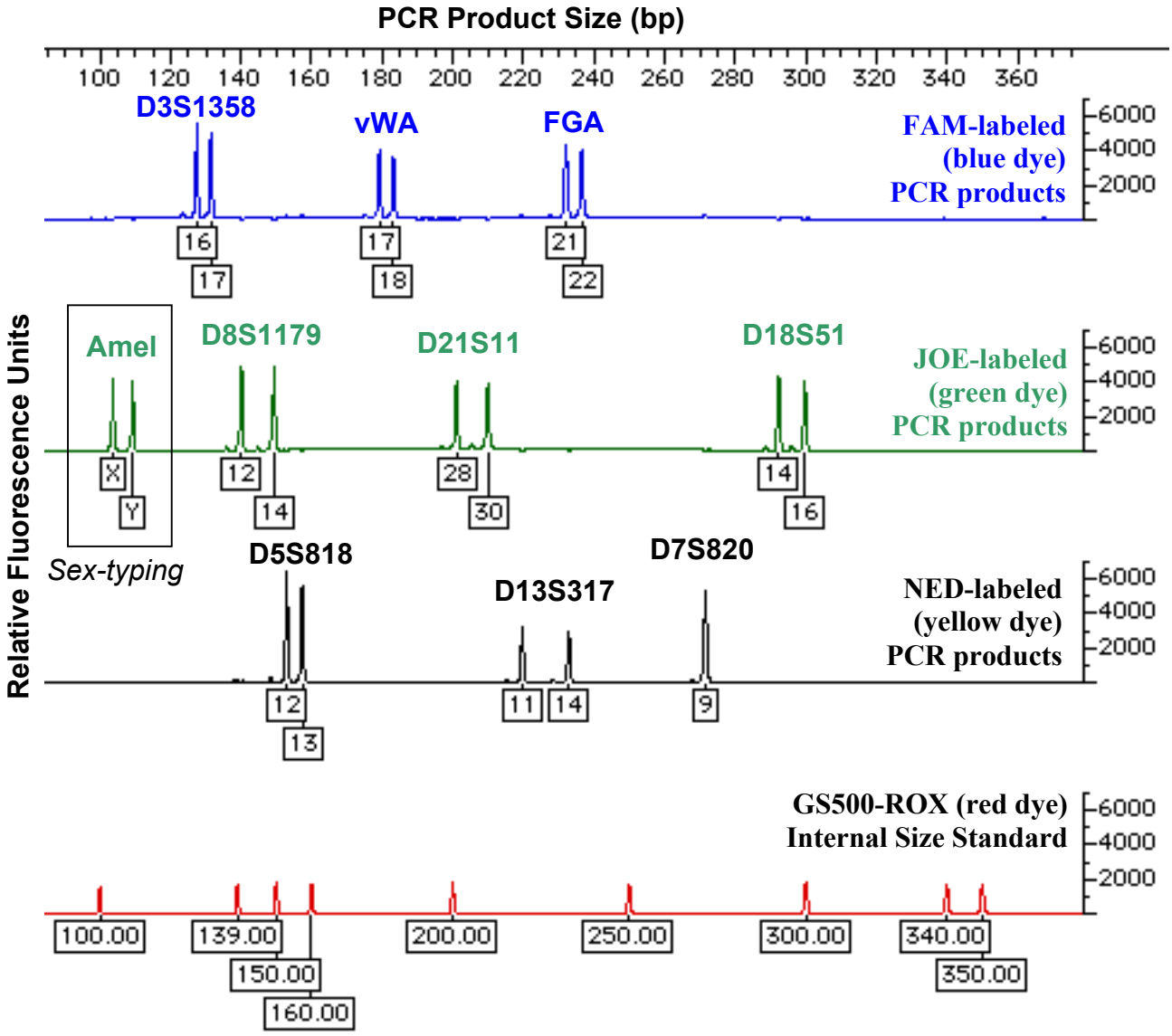
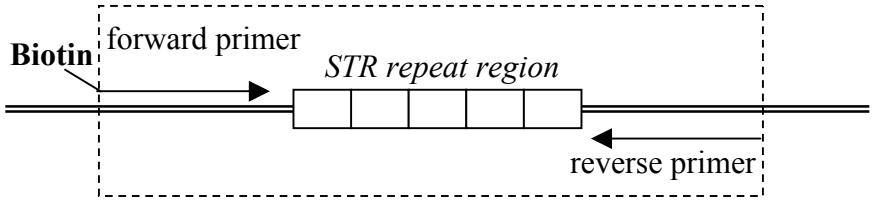
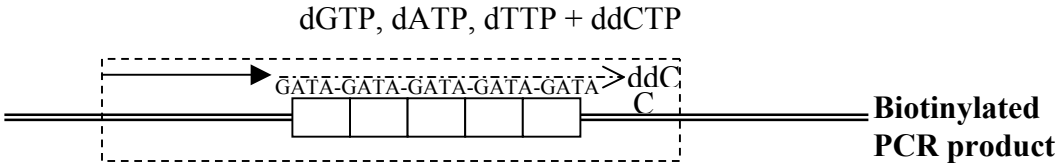


Figure 3. Methods for size reduction of STR alleles prior to mass spectral analysis. The boxed areas show the region that is actually measured in the mass spectrometer.

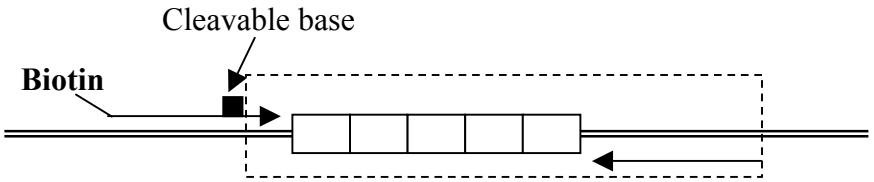
(A) Redesign of PCR primers to make smaller amplicons (*16*)



(B) Primer Oligo Base Extension (PROBE) assay (*31*)



(C) Cleavable primer assay (*32,38*)



(D) Restriction digestion beyond the repeat region (*39*)

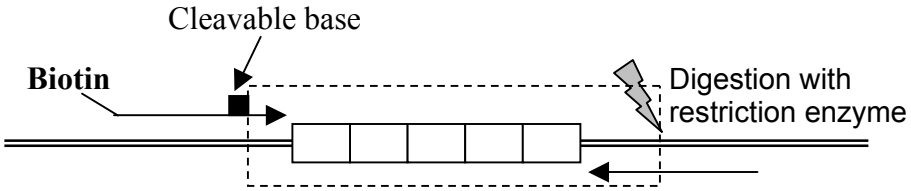


Figure 4. Mass spectra of a D8S1179 sample illustrating the benefit of a dideoxynucleotide termination approach (*36*). The top panel displays a result from a regular PCR product while the bottom panel contains the same sample treated with a linear amplification mix containing a ddG terminator with dA, dT, and dC deoxynucleotides. With the ddG approach, the problem of incomplete adenylation (both –A and +A forms of a PCR product) is eliminated and the amplicons are smaller which improves their sensitivity and resolution in the mass spectrometer. Note that the genotype (i.e., 11 and 13 repeats) is identical between the two approaches, even though almost 7,500 Da are removed with the ddG termination.

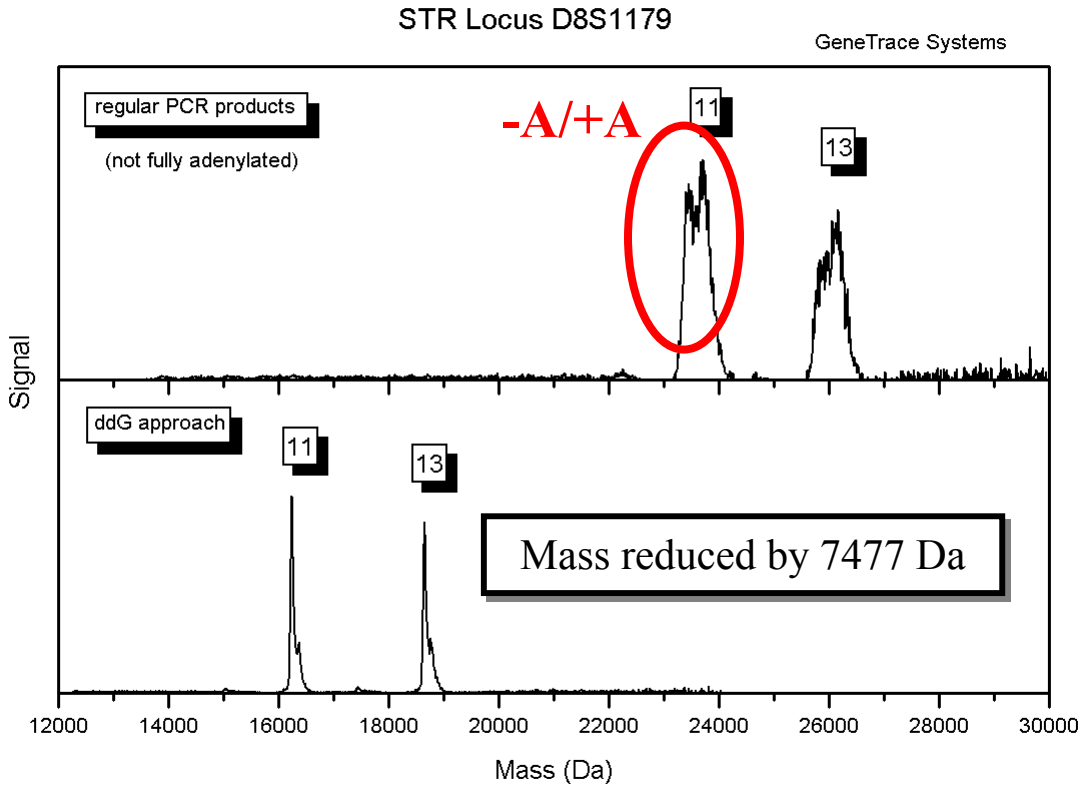


Figure 5. Mass spectra of the same sample with TH01 genotype 6,8 amplified with the TH01 PCR primer set and two different DNA polymerases (*36*). In the top panel, the TaqGold DNA polymerase (Applied Biosystems) produces split peaks for each allele due to partial adenylation of the STR alleles. In the bottom panel, the *Tsp* polymerase (Life Technologies) does not exhibit non-templated nucleotide addition and thus yields only a single peak for each allele. The peak masses in Daltons are shown with each peak.

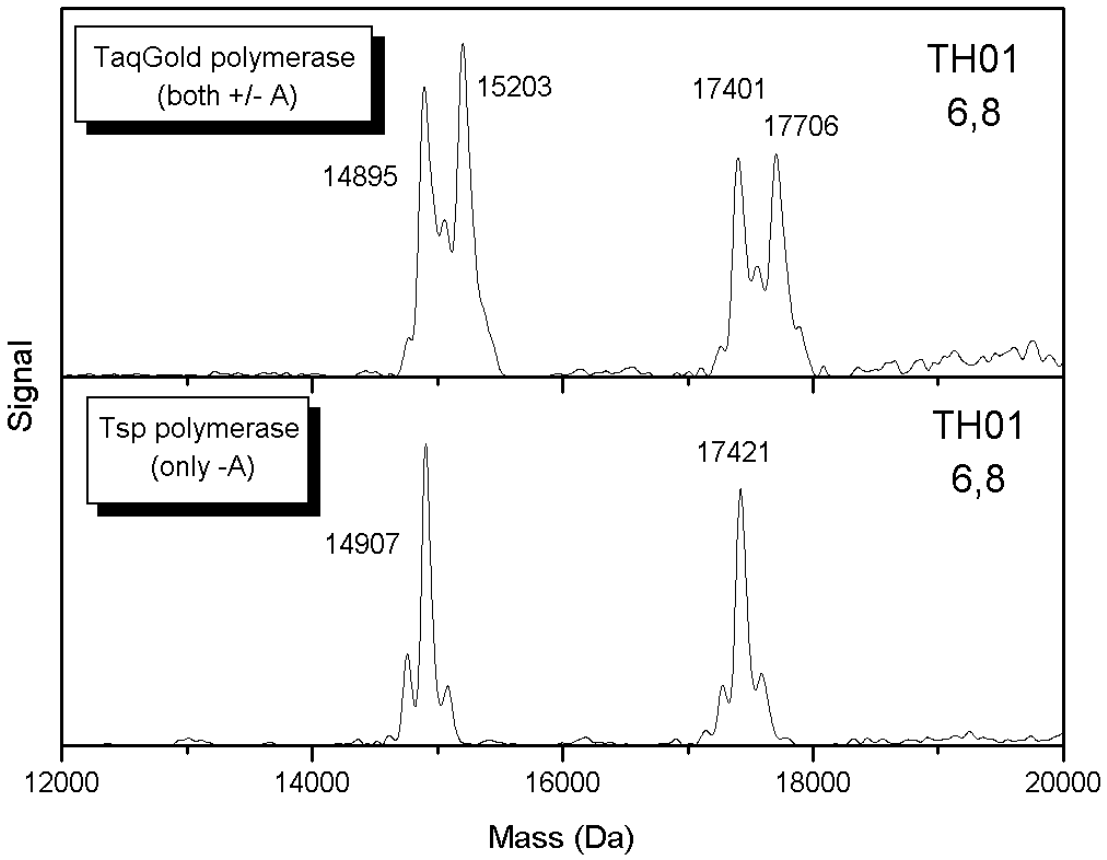
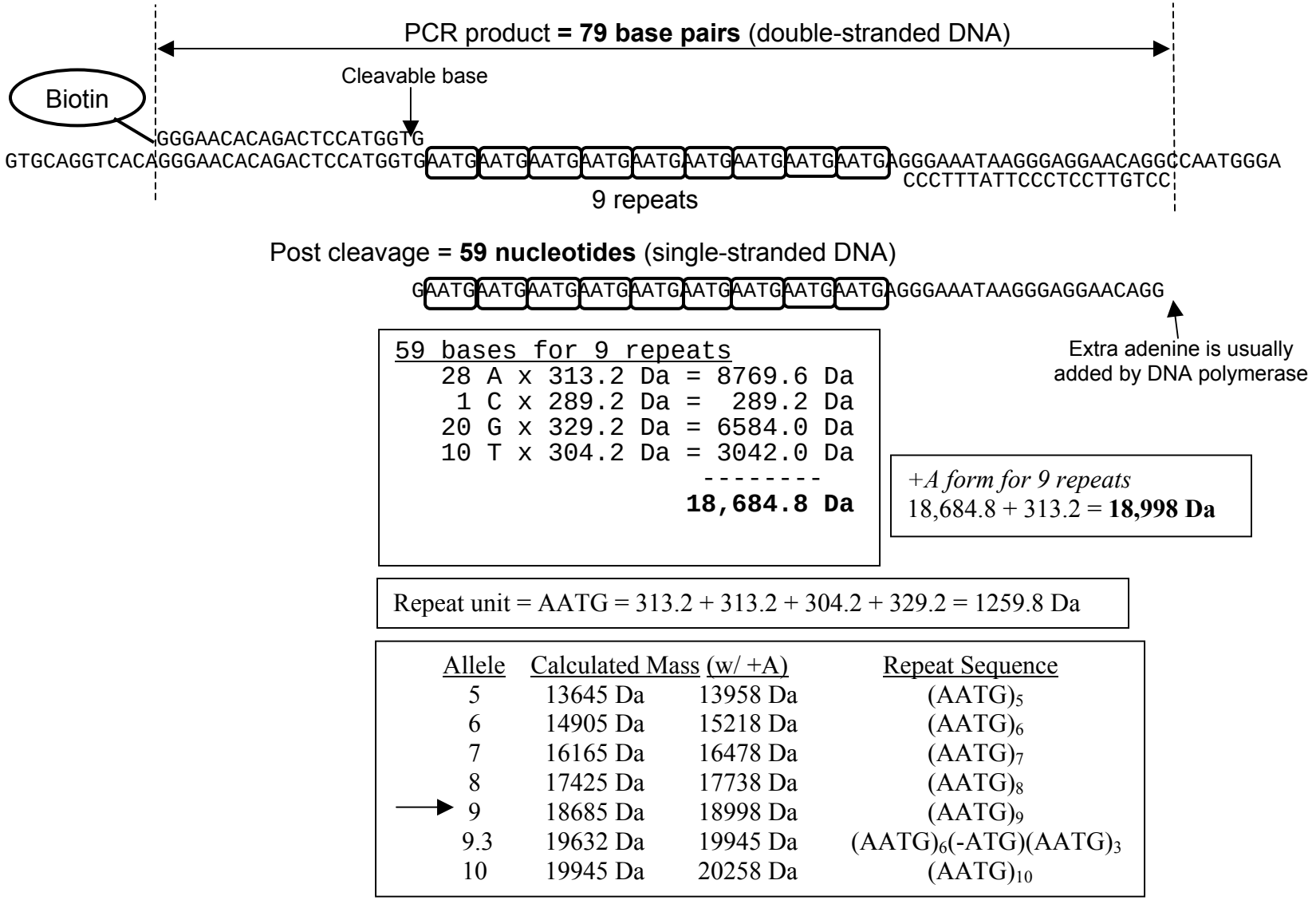


Figure 6. Illustration of steps involved in calculating allele masses from a reference sequence for TH01 (GenBank #D00269).



Allele	Calculated Mass	(w/ +A)	Repeat Sequence
5	13645 Da	13958 Da	$(AATG)_5$
6	14905 Da	15218 Da	$(AATG)_6$
7	16165 Da	16478 Da	$(AATG)_7$
8	17425 Da	17738 Da	$(AATG)_8$
→ 9	18685 Da	18998 Da	$(AATG)_9$
9.3	19632 Da	19945 Da	$(AATG)_6(-ATG)(AATG)_3$
10	19945 Da	20258 Da	$(AATG)_{10}$

Figure 7. TH01 sequence with 9 repeats and different primers to illustrate calculation of allele masses with ddC termination approach.

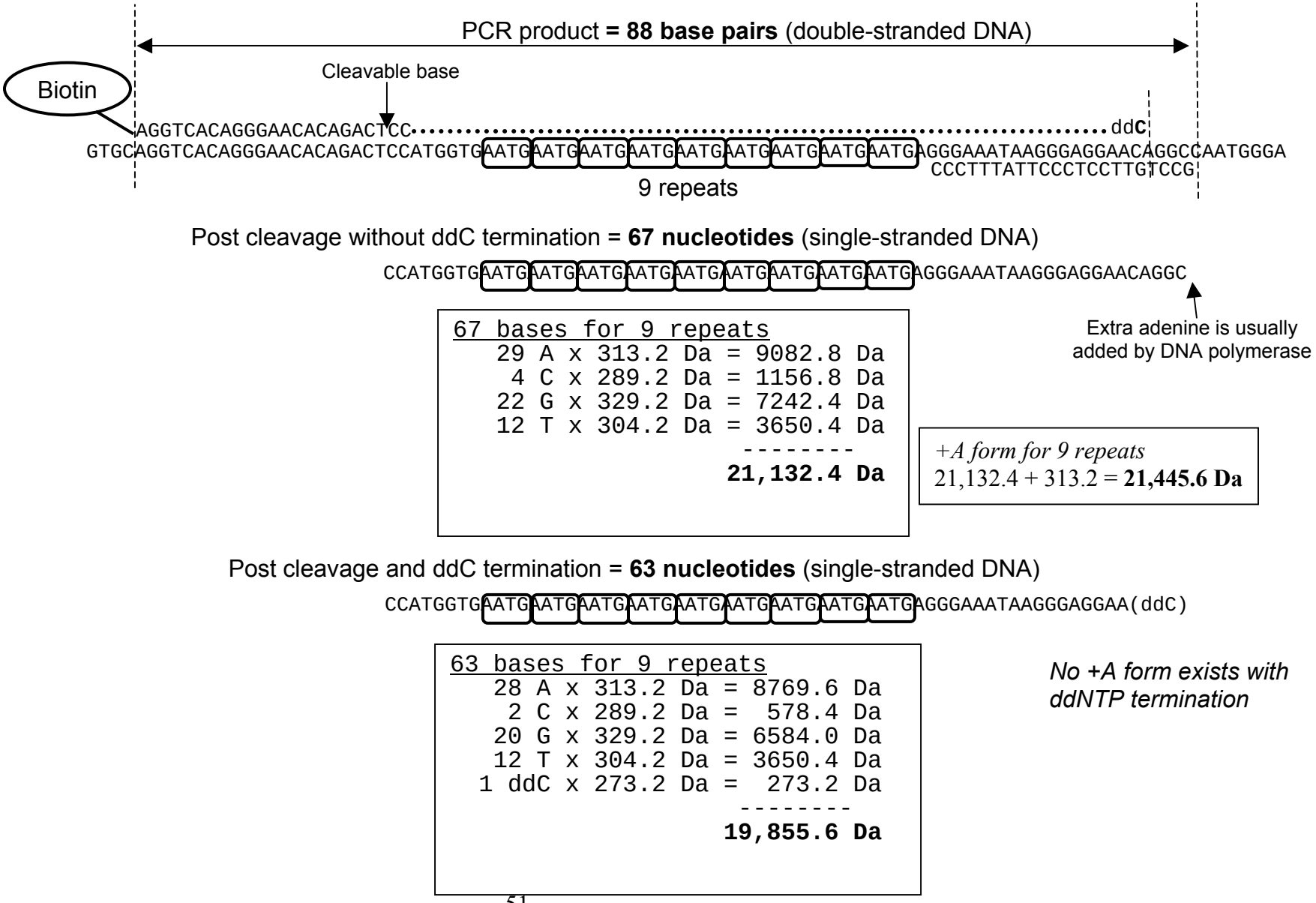


Figure 8. Separation and detection of STR alleles in the TH01 allelic ladder (AmpFlSTR® COfiler™ STR kit) using (A) capillary electrophoresis via the ABI Prism 310 Genetic Analyzer and (B) time-of-flight mass spectrometry. The PCR product sizes for each of the TH01 alleles are more than 100 bp smaller with the mass spectrometry approach and are further reduced using the cleavable primer approach (*17*). The TH01 STR locus contains the 4 bp repeat sequence AATG with common alleles ranging in size from 5 to 10 repeats. A microvariant allele known as 9.3 is also common and included in the allelic ladder shown here. Allele 9.3 differs from allele 10 by a single base deletion of adenine (*44*).

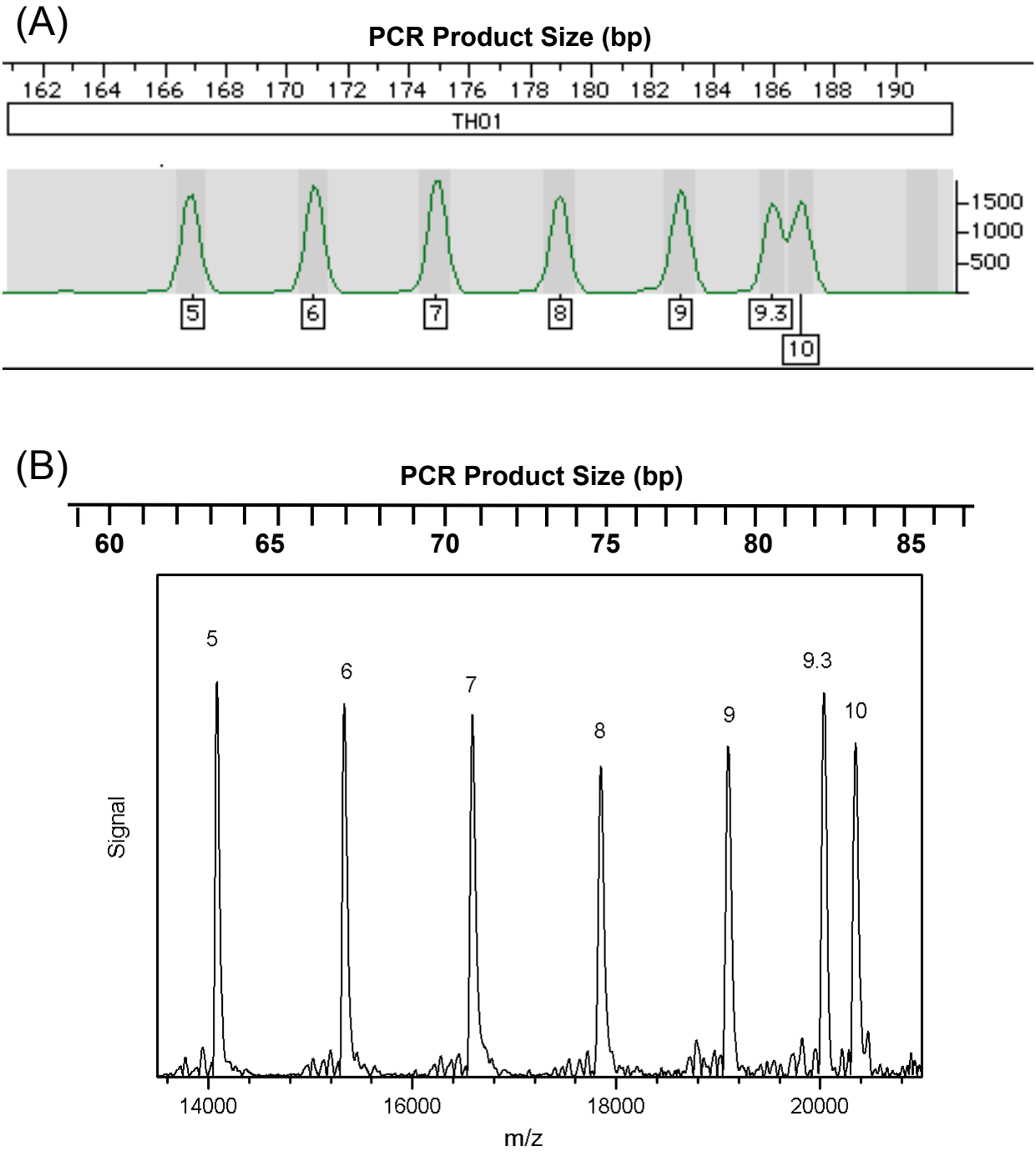


Figure 9. Expected allele masses for 3 STR markers with overlapping size ranges but distinguishable alleles that are interleaved in order to keep a compressed mass range (*38*).

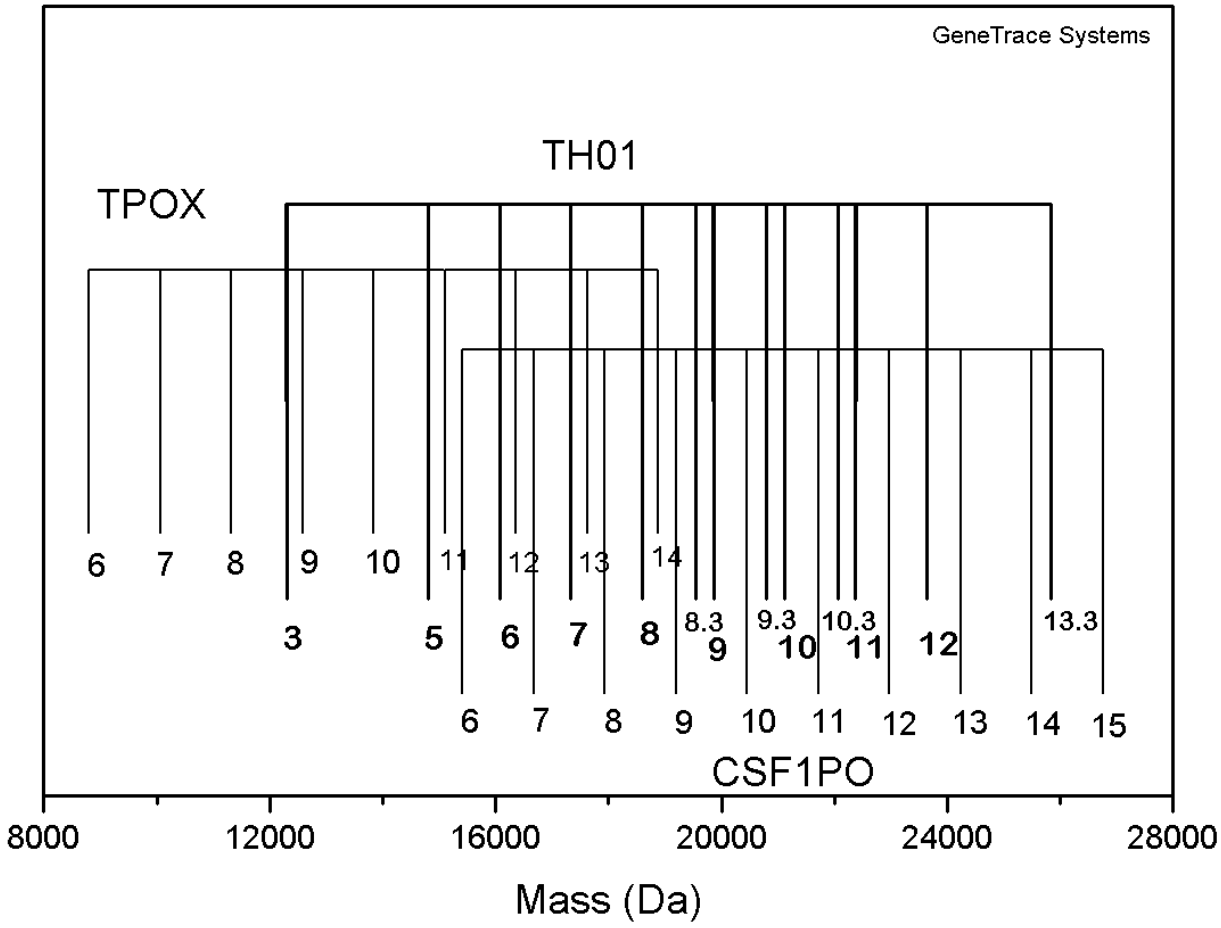


Figure 10. Schematic of high-throughput DNA sample processing using robotic sample preparation, automated mass spectrometry, and computer-assisted genotyping.

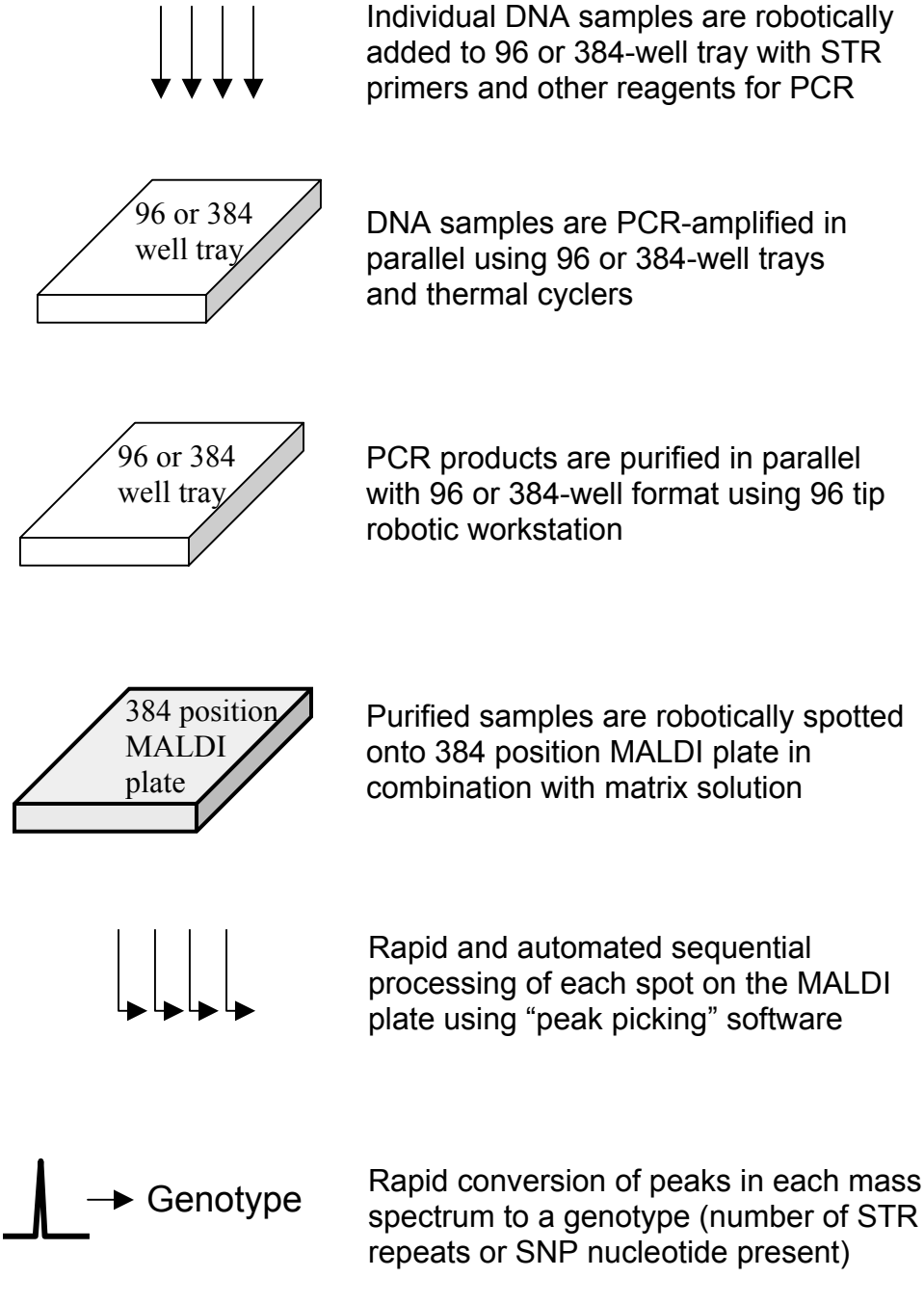


Figure 11. Comparison of steps involved in genotyping STR markers using conventional electrophoretic techniques versus a time-of-flight mass spectrometry approach.