Laser mass spectrometry for DNA sequencing, disease diagnosis, and fingerprinting

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ABSTRACT

Since laser mass spectrometry has the potential for achieving very fast DNA analysis, we recently applied it to DNA sequencing, DNA typing for fingerprinting, and DNA screening for disease diagnosis. Two different approaches for sequencing DNA have been successfully demonstrated. One is to sequence DNA with DNA ladders produced from Sanger's enzymatic method. The other is to do direct sequencing without DNA ladders. The need for quick DNA typing for identification purposes is critical for forensic application. Our preliminary results indicate laser mass spectrometry can possibly be used for rapid DNA fingerprinting applications at a much lower cost than gel electrophoresis. Population screening for certain genetic disease can be a very efficient step to reducing medical costs through prevention. Since laser mass spectrometry can provide very fast DNA analysis, we applied laser mass spectrometry to disease diagnosis. Clinical samples with both base deletion and point mutation have been tested with complete success.

1. INTRODUCTION

Since the initiation of the Human Genome Project[1,2] with its goal of achieving the complete mapping and sequencing of the human genome, development of rapid sequencing technology has been considered very critical for the success of this project. Ordinarily, different sizes of DNA ladders, which are produced by either Sanger's enzymatic method[3] or Maxam-Gilbert's chemical cleavage method[4], are separated by gel-electrophoresis to achieve DNA sequencing. These methods involve the use of either radioactive labeling or fluorescent dyes for identification. However, gel electrophoresis is a time-consuming process that normally requires at least a few hours. Recently, capillary gel electrophoresis[5] and ultra-thin gel electrophoresis[6] have made significant progress in increasing sequencing speed. Nevertheless, speed is still limited by gel running time and time required for labeling. In addition to the conventional gel electrophoresis sequencing, several innovative sequencing technologies without the need of gels have been proposed. They include sequencing by hybridization[7], scanning tunneling microscopy[8], single molecule detection using flow cytometry[9], primer walking[10], and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry.[11] Among these new approaches, MALDI is emerging as a promising technology with the potential for very fast sequencing.[12] In addition, MALDI sequencing has no need of labeling for identification, making it inexpensive as compared to other methods.

Since the discovery of MALDI in 1987[13], sequencing of DNA by mass spectrometry has been proposed. However, early detection of DNA by MALDI was limited to very short synthetic oligonucleotides[14] due to the easy fragmentation of this species. A few new matrices suitable for

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DNA have been discovered[15-17], and single-stranded (ss) DNA of 500 nucleotides and double-stranded (ds) DNA longer than 500 base pairs have been detected.[18,19] DNA fragments replicated from polymerase chain reaction (PCR) have also been successfully detected by MALDI.[20] However, the mass resolution of long DNA fragments by MALDI is still not high enough to obtain direct sequencing information. Recent development on delayed-pulsed ion extraction[21-23] significantly improves the resolution of proteins and small DNAs. Sequencing short DNA segments now appears feasible.

There are two different approaches to the use of MALDI for DNA sequencing. One is to achieve sequencing without the need of DNA ladders. The other is to do sequencing with DNA ladders. Pieles et al.[24] and Smirnov et al.[25] used time dependent sequential excision of oligonucleotides by exonuclease and MALDI to obtain sequencing information on short oligonucleotides. McLafferty and his colleagues[26] obtained sequencing information by using collision-induced dissociation and Fourier Transform Mass Spectrometer for short oligonucleotides. Hillenkamp and his co-workers[27] have investigated the DNA fragmentation pattern in MALDI using infrared lasers for desorption. Zhu et al.[28] achieved the sequencing of a 35-mer oligonucleotide by MALDI with a specific matrix for selective fragmentation. However, most direct sequencing approaches without DNA ladders need very high mass resolution to resolve the differences between bases. Due to the natural abundance of ^{13}C isotope, the maximum size of DNA to be sequenced by direct sequencing without DNA ladders will be limited to less than 100 nucleotides. Longer DNAs can possibly be sequenced by MALDI with DNA ladders since the requirement of mass resolution is much less. In this work, we performed sequencing with DNA ladders and without DNA ladders. In 1993, Smith and his colleagues[28] demonstrated a MALDI time-of-flight (TOF) mass spectrometer (MS) analysis of the mock Sanger sequencing reactions containing mixtures of synthetic oligonucleotides. In 1995, Shaler et al.[29] reported the first sequencing of a 45-mer ssDNA with MALDI-TOF with enzymatic preparation of DNA ladders. Recently, Roskey et al.[30] reported the results of sequencing a synthetic ssDNA template of 50 nucleotides. Koster et al.[31] also succeeded in sequencing synthetic ssDNA of 39 nucleotides with magnetic beads coated with streptavidin for purification of DNA ladders. Smith and his coworkers[33] reported MALDI analysis of sequencing reactions from bacteriophage M13. Here we report a complete sequencing work for a ssDNA with 50 bases using both the conventional Sanger method and cycle sequencing processing for DNA ladder preparation. No false stops, no serious fragmentation, and no unidentified peaks that interfere with the determination of sequencing were observed.

In order to speed up sequencing by using mass spectrometry, it is desirable to do the sequencing directly without having to prepare DNA ladders. Recently, we tried to achieve sequencing by MALDI with fragmentation between bases such that sequencing can be achieved without DNA ladders. We called this process matrix-assisted laser-induced desorption/ionization/fragmentation (MALDIF).

There are three preconditions that need to be satisfied in order to obtain full sequence information on DNA by MALDIF. (1) A form of selective bond cleavage should be established to simplify the mass spectrum of fragments. The ideal case of selective bond cleavage for oligonucleotides will be the P-O bond at either 5' or 3' linkage. (2) Secondary fragmentation should be avoided

(i.e., the further fragmentation of the primary fragment). (3) For a selected bond, the bond strength and the probability for collisional dissociation should be nearly identical at all positions in a DNA chain so that the dissociation cross section will be the same at any position of this bond. In this way, the full sequence information by direct fragmentation of an oligonucleotide can be obtained. We found UV-MALDI-TOF mass spectrometry can be suitable for all three conditions. In this approach, the selective bond cleavage can be established through bond weakening by the interactions between DNA and matrix molecules during the sample preparation. The fast desorption of DNA fragments into a collision-free zone in the MALDI process largely eliminates secondary fragmentation. Based on these preconditions, the MALDIF mass spectroscopic method may be the most promising approach for direct DNA sequencing.

Since the bonding energy of P-O is slightly less than that of C-C, C-O and C-N, it is easier to have cleavage of P-O bond during the laser ablation process. However all P-O bonds in DNA have similar dissociation probabilities. This leads to a potential problem of secondary fragmentation. Fortunately, the collision time in MALDI process is so short that primary fragmentation usually dominates. This means that each oligonucleotide can only fragment once. The nearly identical dissociation probability of P-O bonds at all positions result in a fragmentation pattern which carries full sequence information. This can be easily understood by using the illustration in Figs. 1 and 2. Assuming the selective cleavage of P-O bond at 3'-linkage for an oligonucleotide of 5'-CTGTGA-3', the primary fragmentation of such bond breaking will produce two series of fragments. These are labeled as 5'- and 3'-termini. Each series has six members ranging from 1- to 6-mer. The vertical dotted line in Fig. 1 represents the position of cleavage. The fragments in both series can be ionized and resolved in MALDI spectra. Figure 2 gives the simulated mass spectra for these two series. Trace 'a' in Fig. 2 (top trace) is the spectrum of the series with 3'-termini. The mass difference between two adjacent peaks in the series provides the information for each extra base. For example, the first member of this series is A, and the second member of this series is GA. Due to the selective cleavage of 3'-linkage, the mass difference between these two peaks will be exactly 329.2 Daltons, which represents the mass of dGMP. The sequence information can be obtained by analyzing all values of mass difference in this series together with the total mass of 5'-CTGTGA-3'. The same information can also be obtained from the series with 5'-termini (Fig. 2, trace 'b'). If a complete series (either 5'- or 3'- termini) of such fragmentation for a DNA sample can be experimentally resolved, the full sequence information will be obtained. Since the mass information of these two series is identical, the full sequence information still can be obtained when two partially resolved series are clearly observed. Trace 'c' of Fig. 2 shows a mass spectrum of two series from the cleavage of 5'-linkage P-O bond. Generally two or three series can be resolved in the MALDIF experiment. The first digit represents the cleavage position of P-O bond, which can be either 5 or 3 for the 5'- or 3'-linkage. The second digit represents the termini of fragments, which also can be 5 or 3 for 5'- or 3'-termini; e.g., the 53 series represents the fragments of a series produced by the cleavage of 5'-linkage with 3'-termini.

We recently succeed in sequencing several blind, short-synthetic DNA with MALDIF, the results were found to be 100% accurate. We believe MALDIF can be very valuable for sequencing primers.

Although laser desorption mass spectrometry cannot yet be used for routine sequencing of long DNA, it can be applied to fast DNA analysis for disease diagnosis. Recently, we succeeded in detecting both base deletion and point mutation which most commonly occur in genetic diseases.

The application of DNA fingerprinting has become very valuable in forensic analysis since every individual's DNA structure is identical within all tissues of their body. DNA fingerprinting was initiated by the use of restriction fragment length polymorphism (RFLP). In 1987, Nakamura et al[32] pointed out that DNA loci containing variable numbers of tandem repeats (VNTR) can be used as a very reliable tool for the differentiation of individuals. The probability of different persons having the same number of tandem repeats in several different alleles is low. More recently focus has shifted toward the development of small VNTRs known as short tandem repeats (STRs). These polymophic loci contain repeat units of 3 ~ 5 bp in length, and multiple loci may be coamplified via the PCR process. The use of multiplex analysis of STRs makes possible frequency estimates of better than 1 in 10^6 for particular individuals.

There are three major steps in the PCR method for the analysis of DNA samples: (1) specimen processing, which usually involves the chemical extraction of genomic DNA; (2) amplification of selected DNA using polymerase chain reaction (PCR); and (3) gel electrophoresis to identify the size of the DNA fragments. This process may also involve dye tagging for detection. Conventional slab gel electrophoresis can be a very time consuming and labor intensive process. Skillful technicians are always needed to obtain reliable results. Development of new reliable and fast methods for DNA typing is critically needed. With a laser desorption mass spectrometric (LDMS) approach, the analysis time (not including sample preparation) per sample can be less than one second compared to hours for gel electrophoresis. In addition, no dye tagging is required. The LDMS approach is more suitable for automation than the gel method, since there is no need to prepare buffer solution and gel.

We recently developed the LDMS technique which can do DNA analysis in less than one second per sample compared to hours for gel electrophoresis. In addition, no radioactive material labeling or dye tagging is required. Thus, no nuclear or hazardous chemical wastes will be produced. Due to the potential of ultra-fast DNA analysis, screening of DNA fingerprinting can become feasible.

3. RESULTS AND DISCUSSION

Experimental results of cycle sequencing to produce DNA ladders by LDMS is shown in Fig. 3. The composite of all four reactions are shown in Fig. 4, which clearly indicates that LDMS can be used for short DNA sequencing. Similar results were obtained for DNA ladders prepared by Sanger's standard enzymatic method. The primer, template, and all the expected sequence fragments and their assigned molecular masses are represented in Table 1. The spectra of Fig. 3 and Fig. 4 exactly corresponds to the DNA ladders in Table 1.

We also pursued MALDIF for direct DNA sequencing. Figure 5 shows a MALDIF spectrum of a 5'-GTGAGTTGAT-3' oligonucleotide sample. This spectrum serves as a good example for a demonstration of the extraction of sequence information from experimental data. In the MALDI

spectra, the matrix signal is generally much more intense than the fragments signal. The mass range of these matrix signals usually exends to 600 Daltons. Therefore, the fragment signal of 1- and 2-mer will overlap with the matrix signal and cannot be resolved clearly, so sequencing depends on resolving the fragment signals of two uncompleted series. In the spectrum of Fig. 5, these two series have been clearly resolved. The top trace is the experimental MALDI spectrum of a 5'-GTGAGTTGAT-3' oligonucleotide, and the bottom trace is the simulated spectrum of a fragment pattern for the 33- and 35-series of a 5'-GTGAGTTGAT-3' oligonucleotide. The series with high intensity is 33-series, and the series with weak intensity is 35-series. The two series are indicated by a simulated spectrum (bottom trace). The 33-series starts at a lowest discernible member corresponding to the 3-mer fragment peak at the mass position of 945 Daltons (left side of top trace). This is the fragment of GAT-3'. However, we cannot get this sequencing information directly from its mass, so we label it ???-3'. The next member of this series will be the fragment TGAT-3' positioned at mass 1249 Daltons. The mass difference between these two peaks corresponds to the mass of T. Therefore this second member is T???-3'. By extending the analysis of this series to the mass peak of the parent oligonucleotide the sequence information of 5'-GTGAGTT???-3' is obtained, which is indicated by the high intensity series in the simulated spectrum (bottom trace). The identity of ???-3' is the missing information in the 33-series. However, we can obtain this information from the 35-series which represents the fragments due to breaking the 3'-linkage with 5'-termini. The last fragment of the 35-series is 5'-GTGAGTTGA positioned at mass 2856 Daltons. The mass difference between this peak and the parent ion peak shows that the 3'-end terminates with T. Together with the mass information of fragments 5'-GTGAGTTG and 5'-GTGAGTT in 35-series, we immediately know the fragment ???-3' is GAT-3', and its total mass is consistent with the mass of the first member of 33-series (945 Daltons). The 35-series provides a sequence information of 5'-???AGTTGAT-3' which is indicated by the low intensity series in the simulated spectrum. Combining the information from the two series, the sequence of 5'-GTGAGTTGAT-3' oligonucleotide was experimentally obtained from the fragmentation information provided by MALDIF time-of-flight spectroscopy. The experimental spectrum perfectly matches the simulated spectrum. This sequencing method is very reliable since the sequence results from one series can be reconfirmed by the other series. Figure 6 shows the experimental results of sequencing a DNA segment of 35 nucleotides.

From the above results, we believe MALDIF can be used for short DNA sequencing without using DNA ladders. This technique should be very valuable for sequencing short DNA probes. With more improvement on resolution in the near future, MALDI can be used to sequence longer DNAs (> 100 nucleotides) with DNA ladders prepared by Sanger's method.

Since LDMS can do DNA analysis very quickly, we also applied it to disease diagnosis. Figure 7 shows the experimental results on ΔF508 deletion in the cystic fibrosis gene. More recently, we also succeeded in detecting point mutation with G551D mutation in cystic fibrosis. Results are shown in Fig. 8. The primers were designed to overlap at the point mutation site. Primers and templates are shown in Table 2. These results indicate that LDMS is about to rapidly emerge as new technology for disease diagnosis.

Tandem repeats frequently occur in genomic DNA. Since the number of repeats often differ

among different people, it can be used as a forensic application for DNA fingerprinting. Figure 9 shows the results of distinguishing 128 basepairs from 132 basepairs, indicating LDMS should be possible for DNA fingerprinting applications.

4. CONCLUSION

Laser desorption mass spectrometry is expected to be broadly applied to various genome sequencing efforts, disease diagnosis, and DNA fingerprinting in the near future. However, mass resolution, detection sensitivity, and speed of DNA purification still need improvement.

5. ACKNOWLEDGEMENT

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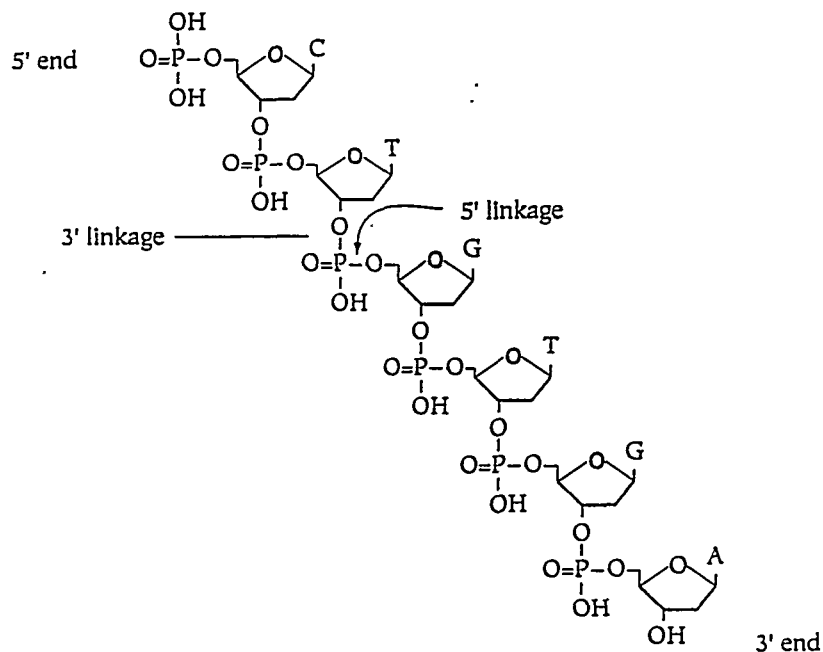17. N. I. Taranenko, K. Tang, S. L. Allman, L. Y. Chang, and C. H. Chen, Rapid Comm. Mass Spectrom. 8, 1001 (1994).

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TABLE 1: Sequence of 50 mer DNA Template and Molecular Weights of Reactions Fragments

	A-reaction	C-reaction	G-reaction	T-reaction
5'-CTAGCGTAATCATAGTCATAGCTGTATCCTGTGTGAAATTGTTATCCGCT-3'				
3'-AGGACACACTTTAACAATAGGCGA-5'				
(25) 3'-TAGGACACACTTTAACAATAGGCGA-5'				7744
(26) 3'-ATAGGACACACTTTAACAATAGGCGA-5'	8057			
(27) 3'-CATAGGACACACTTTAACAATAGGCGA-5'		8346		
(28) 3'-ACATAGGACACACTTTAACAATAGGCGA-5'	8659			
(29) 3'-GACATAGGACACACTTTAACAATAGGCGA-5'			8988	
(30) 3'-CGACATAGGACACACTTTAACAATAGGCGA-5'		9277		
(31) 3'-TCGACATAGGACACACTTTAACAATAGGCGA-5'				9581
(32) 3'-ATCGACATAGGACACACTTTAACAATAGGCGA-5'	9894			
(33) 3'-TATCGACATAGGACACACTTTAACAATAGGCGA-5'				10198
(34) 3'-GTATCGACATAGGACACACTTTAACAATAGGCGA-5'			10527	
(35) 3'-AGTATCGACATAGGACACACTTTAACAATAGGCGA-5'	10840			
(36) 3'-CAGTATCGACATAGGACACACTTTAACAATAGGCGA-5'		11129		
(37) 3'-TCAGTATCGACATAGGACACACTTTAACAATAGGCGA-5'				11433
(38) 3'-ATCAGTATCGACATAGGACACACTTTAACAATAGGCGA-5'	11746			
(39) 3'-TATCAGTATCGACATAGGACACACTTTAACAATAGGCGA-5'				12050
(40) 3'-GTATCAGTATCGACATAGGACACACTTTAACAATAGGCGA-5'			12379	
(41) 3'-AGTATCAGTATCGACATAGGACACACTTTAACAATAGGCGA-5'	12692			
(42) 3'-TAGTATCAGTATCGACATAGGACACACTTTAACAATAGGCGA-5'				12996
(43) 3'-TTAGTATCAGTATCGACATAGGACACACTTTAACAATAGGCGA-5'				13300
(44) 3'-ATTAGTATCAGTATCGACATAGGACACACTTTAACAATAGGCGA-5'	13613			
(45) 3'-CATTAGTATCAGTATCGACATAGGACACACTTTAACAATAGGCGA-5'		13902		
(46) 3'-GCATTAGTATCAGTATCGACATAGGACACACTTTAACAATAGGCGA-5'			14231	
(47) 3'-CGCATTAGTATCAGTATCGACATAGGACACACTTTAACAATAGGCGA-5'		14520		
(48) 3'-TCGCATTAGTATCAGTATCGACATAGGACACACTTTAACAATAGGCGA-5'				14824
(49) 3'-ATCGCATTAGTATCAGTATCGACATAGGACACACTTTAACAATAGGCGA-5'	15137			
(50) 3'-GATCGCATTAGTATCAGTATCGACATAGGACACACTTTAACAATAGGCGA-5'			15466	

TABLE 2. Sequences of two sets of oligonucleotide primers were designed to directly detect the G551D genotype without the use of the restriction enzyme digestion.

NORMAL	MUTANT
Forward N_f (19 bp) 5'-GAATCACACTGAGTGGAGG-3'	Forward M_f (19 bp) 5'-GAATCACACTGAGTGGAGA-3'
Reverse N_R (19 bp) 5'-CAAATTCTTGCTCGTTGAC-3'	Reverse M_R (28 bp) 5'-CTTGGTAAACAAATTCTTG- CTCGTTGAT-3'

5'-GGAGAAGGTGGAATCACACTGAGTGGA<u>GGT</u>CAACGAGCAAGAATTTGTTTAG-
CAAG-3'

Sequence of the CFTR DNA in the area of the 551 mutation. The sequence GGT is mutated to GAT, for G551D mutation.

Fragment pattern of breaking 3 linkage

5' -termini   5' -C⋮T-G-T-G-A-3'
5' -C-T⋮G-T-G-A-3'
5' -C-T-G⋮T-G-A-3'   3' -termini
5' -C-T-G-T⋮G-A-3'
5' -C-T-G-T-G⋮A-3'
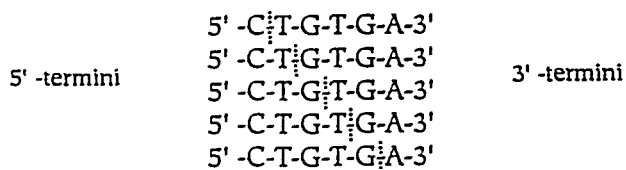
Figure 1. Illustration of the selective cleavage P-O bond and its fragmentation pattern for a 5'-CTGTGA-3' oligonucleotide.
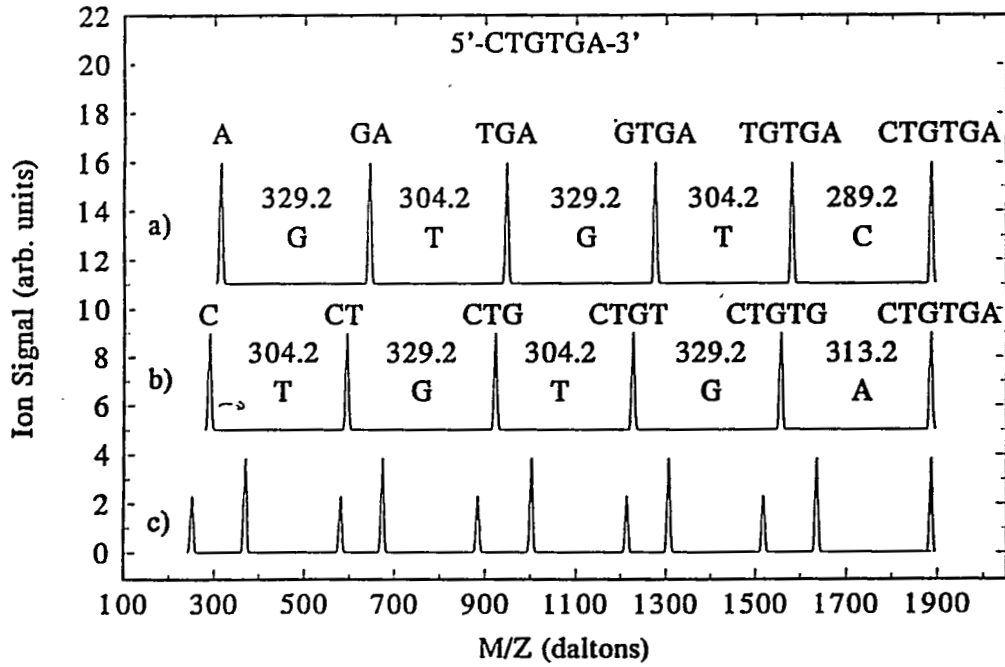
Figure 2. The simulated spectra of the selective cleavage of P-O 3'-linkage and 5'-linkage for 5'-CTGTGA-3' oligonucleotide. Trace a) is the simulated spectrum of fragment series with 3'-termini by the 3'-cleavage. Trace b) is the simulated spectrum of fragment series with 5'-termini by the 3'-cleavage. Trace c) is the simulated spectrum of fragment series with both 5'- and 3'-termini by the 5'-cleavage.
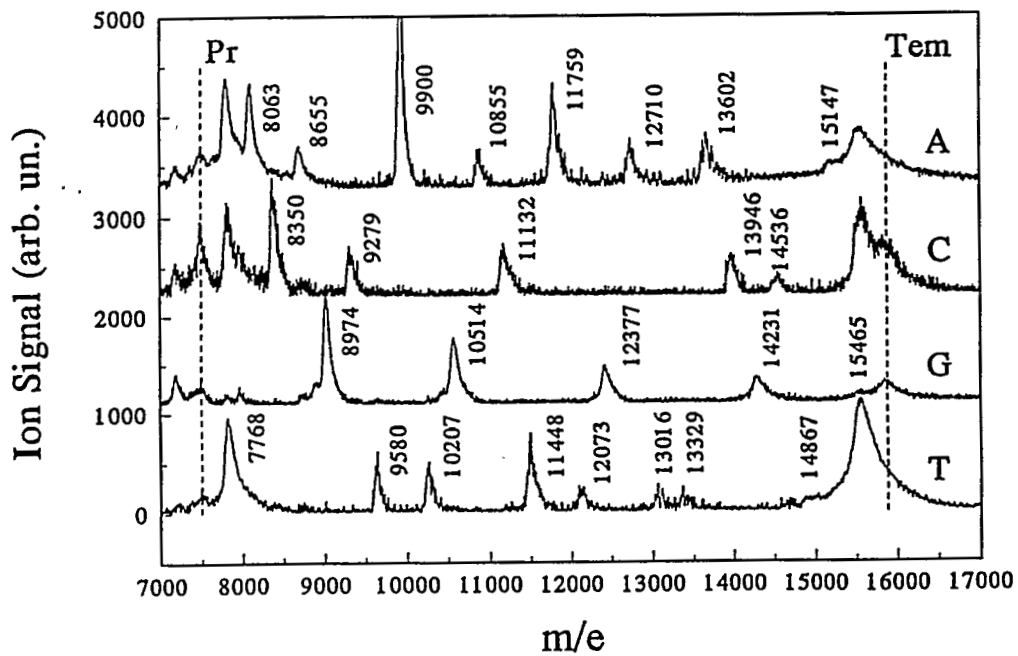

Figure 3. Negative ion mass spectra of the chain termination sequencing ladders by primer extension on the single-stranded, 50 mer template for A-, C-, G-, and T-reactions, respectively.
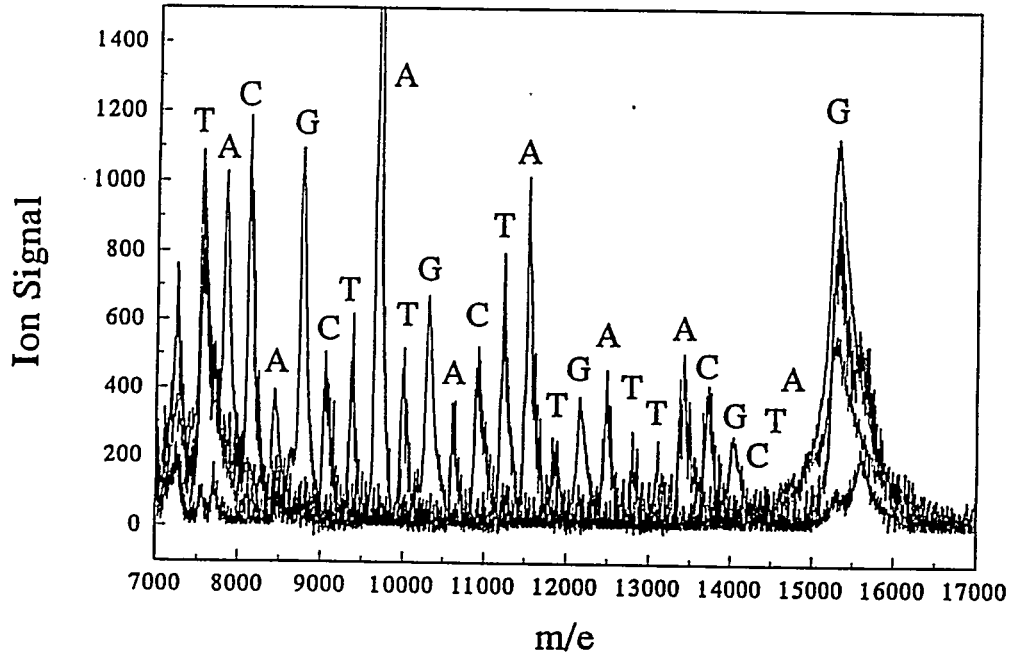
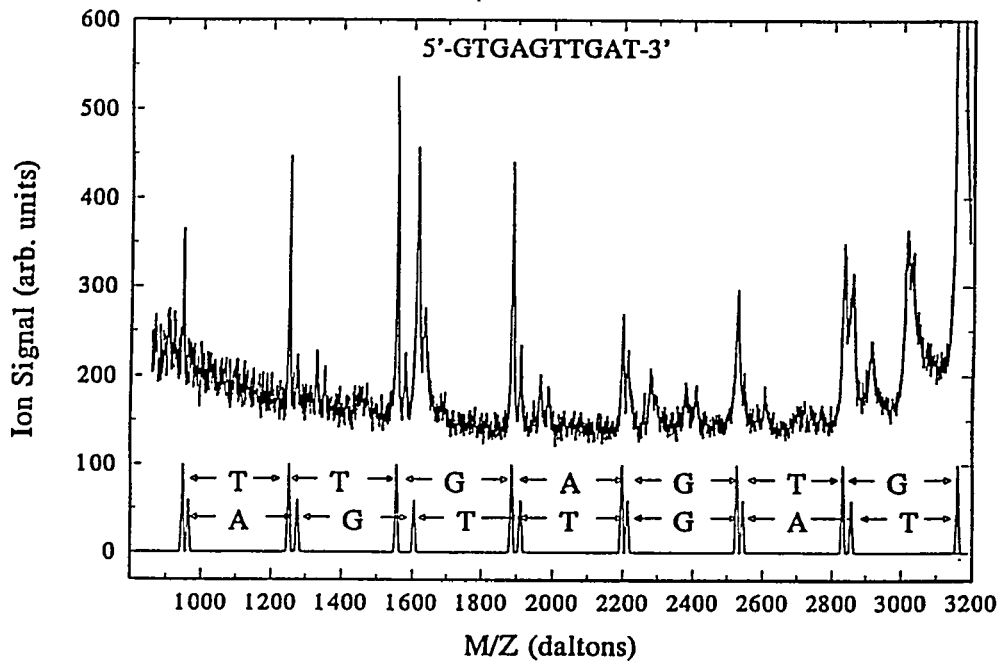**Figure 4.** Composite of four reactions from Fig. 3.

Figure 5. The experimental MALDI spectrum (top trace) of a 10-mer 5'-GTGAGTTGAT-3' oligonucleotide using the cleavage of 3'- and 5'-linkage method, and its simulated spectrum (bottom trace).
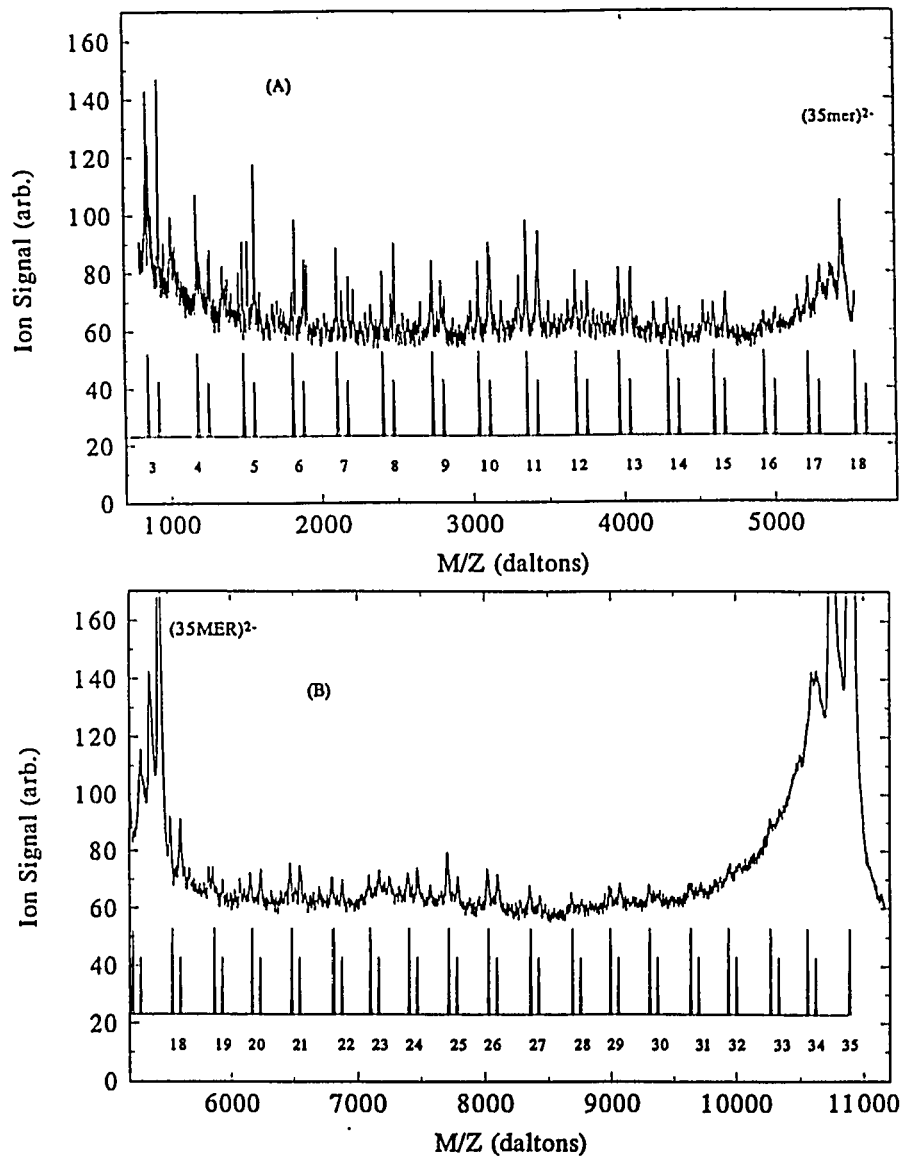
Figure 6. The mass spectrum of experimental MALDI spectrum (top trace) of a 35-mer, 5'-GCGTGATGGAATCGATGACGTGCGATGTCGTGTTT-3', oligonucleotide using the selective cleavage of 3'- and 5'-linkage method, and its simulated spectrum (bottom trace). The high intensity series in the simulated spectra is the 53-series, and the low intensity series is 33*-series. A mixture of 2,4,6-trihydroxyacetophenone (1.5 x 10⁵ moles) was used as the matrix. Quantity of 35-mer DNA loaded was 100 picomoles. (A) and (B) are for low and high mass regions, respectively. The same sample was used for both cases with different laser fluences.
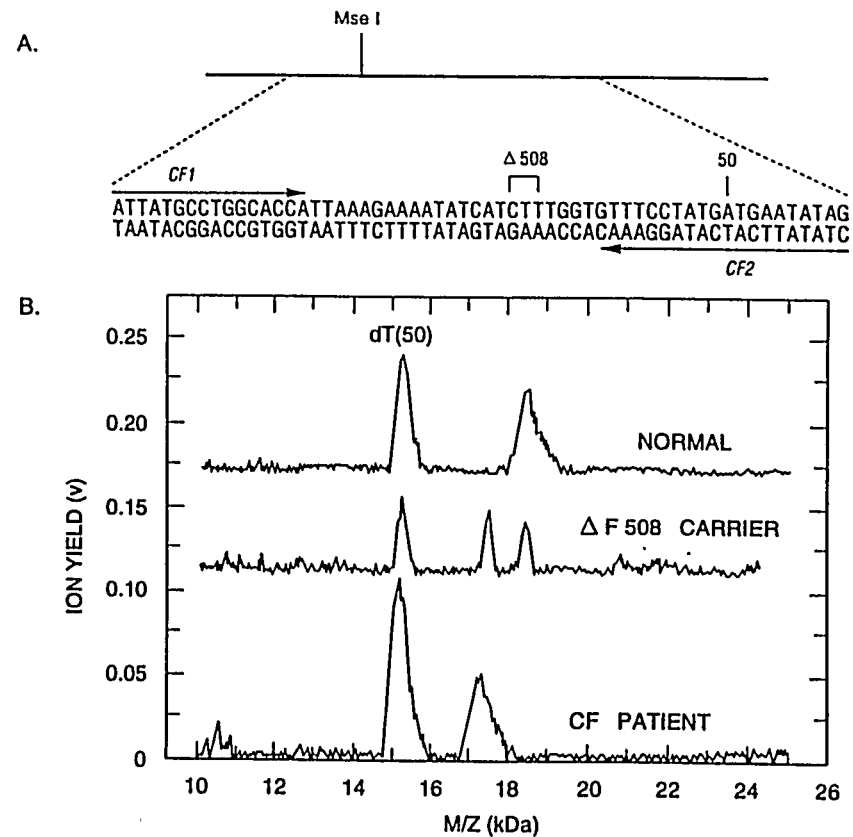

Figure 7. Differential diagnosis of individuals with normal and ΔF508 deletion by laser mass spectrometry. (A) Nucleotide sequence of a part of cystic fibrosis gene with the primers CF and CF₂ for DNA replication. A deletion of 3 base pairs in ΔF508 is marked by the bracket. With this deletion, the patient can have cystic fibrosis disease. (B) Mass spectra from a healthy volunteer, a cystic fibrosis carrier, and a cystic fibrosis patient. This clearly indicates that this new technique can be used for fast diagnosis of cystic fibrosis.
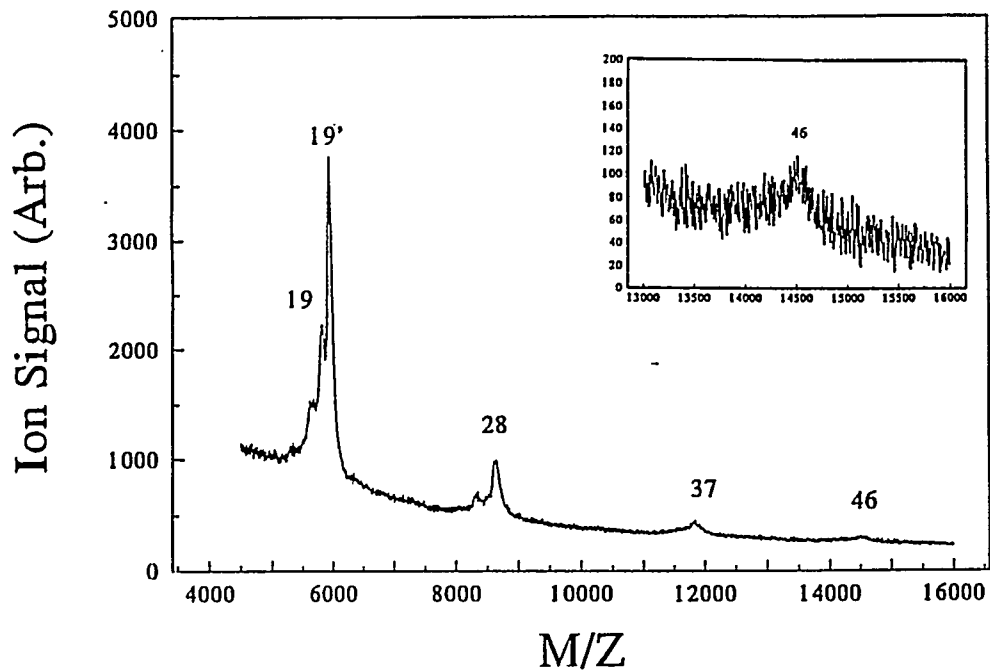
Figure 8. Negative-ion mass spectrum of PCR product in 37 bp and 45 bp (heterozygous template and four primers: two normal primers, 19 mer each, and two mutant primers, 19 and 28 mer). The total amount of DNA was 2 pmol. The matrix was 3-HPA/PA (9:1). Laser wavelength was 337 nm, laser fluence was 50 mJ/cm^2.
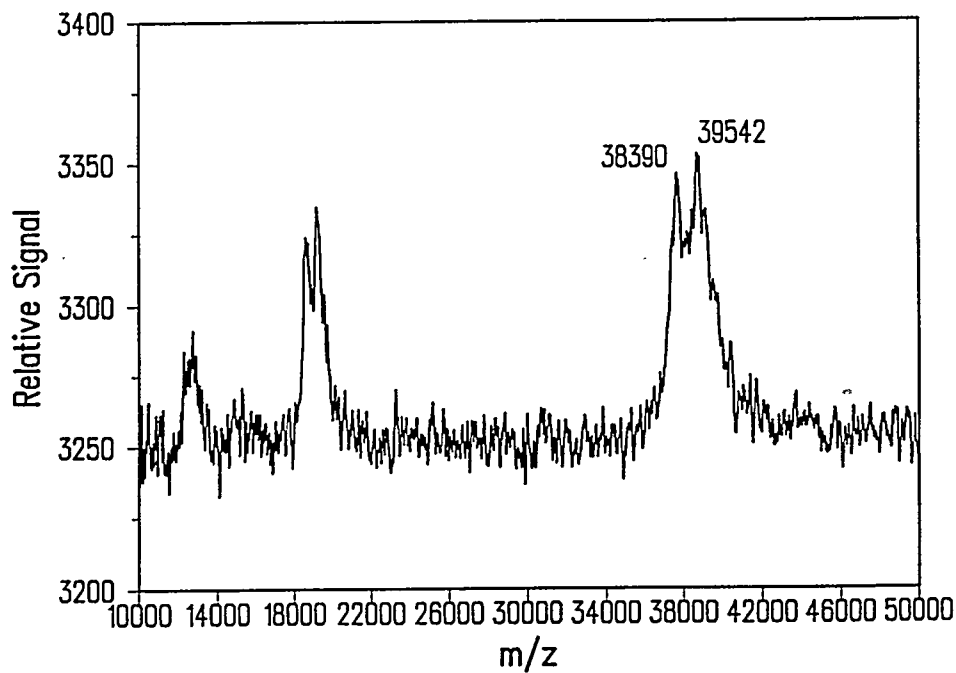

Figure 9. Experimental results on PCR products of 128 bp and 132 bp.