# Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry as a Rapid Screening Method to Detect Mutations Causing Tay–Sachs Disease

**Jannavi R. Srinivasan[1], Yan-hui Liu[1†] Patrick J. Venta[2], David Siemieniak[3], Anthony A. Killeen[4], Yongdong Zhu[1] and David M. Lubman[1*]**

[1]Department of Chemistry, University of Michigan, Ann Arbor, MI 48109, USA
[2]College of Veterinary Medicine, Michigan State University, East Lansing, MI 68824, USA
[3]Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI 48109, USA
[4]Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, USA

SPONSOR REFEREE: Professor Robert J. Cotter, The Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Baltimore, MD 21205, USA

**Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has been used as a rapid method for the detection of human genetic polymorphisms. In particular, the mutations in the human HEXA gene that cause the infantile Tay–Sachs disease have been studied using MALDI-MS to demonstrate the feasibility of this technique for use in clinical and diagnostic analysis. The protocols involved in this approach include, polymerase chain reaction for the amplification of the mutation site from buccal cell DNA, followed by restriction enzyme digestion of the amplified regions of the template cells. The products of amplification and digestion were studied using MALDI-MS. MALDI-MS experiments are shown to provide essentially the same information as obtained from gel electrophoresis but orders of magnitude faster. © 1997 by John Wiley & Sons, Ltd.**

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The identification of genes and specific mutations associated with different genetic disorders is an important problem in clinical diagnosis. One such genetic disorder that has received much attention is Tay–Sachs disease (TSD). This genetically inherited disorder has a relatively high incidence in specific populations, in particular, in the descendants of Ashkenazi Jews[1,2] and French-Canadians.[3] It is an autosomal recessive lysosomal-storage disorder that results from a deficiency of the α-subunit of hexosaminase A (HEXA). In the absence of this enzyme, unhydrolyzed $G_{M2}$ ganglioside accumulates in the neuronal tissues resulting in progressive neurologic degeneration. For infants who are homozygous recessive for Tay–Sachs alleles, death usually occurs by the age of three.

Voluntary screening programs based on biochemical assays for Tay–Sachs heterozygous carriers are available. These tests measure the HEXA activity in the serum and tissues of patients. However, results from such tests are not accurate due to many factors that could result in an increase[4,5] or a decrease[6] in the apparent percentage of HEXA activity, thus making it an unreliable indicator of the carrier state. Also the presence of benign or 'variant' mutations can cause HEXA enzyme deficiency, giving rise to false positive results in the enzyme assays. An alternative to the enzyme analysis that is more specific, is a DNA-based screening test for carriers of TSD. This involves methods to identify the specific mutation in the HEXA gene of patients. Many different mutations have been identified in the HEXA gene, but three are responsible for most of the mutations in the Ashkenazi Jewish population. Studies have shown that 70% of the patients have a four base-pair insertion mutation in exon 11.[7] A splice-junction mutation at the 5' end of intron 12 of the gene, which was the first identified mutation, accounts for 20–30% of the cases of infantile TSD.[8] The last major mutation identified to date that is responsible for adult onset $G_{M2}$ gangliosidosis, involves a G to A nucleotide substitution in exon 7 and is a rare mutation, found only in 3% of the patient population.[9]

The presence or absence of a mutation site in DNA analysis can be confirmed by the combination of polymerase chain reaction (PCR) and restriction length fragment polymorphism (RFLP). The amplification of small regions of the gene for detection of polymorphic sites has been made possible by the introduction of PCR.[10] The amplified DNA can be detected by various methods[11–13] most commonly, gel electrophoresis. In addition, the presence of a mutation can be recognized using RFLP if a mutation occurs at a restriction site or results in a change in distance between two restriction sites.

The most common detection method used to study PCR products and their restriction enzyme digests is gel electrophoresis. The main drawback of the gel electrophoretic method is the time required for separation of the amplified and digested products. Another disadvantage of the gel method is that detection of low

†Current address: Scherring-Plough Research Institute, Kenilworth, NJ 07033, USA
*Correspondence to: D. M. Lubman
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**Table 1. Primer sequence and PCR amplification conditions**

| Amplifying gene | Primers' sequence | Denaturation | Annealing | Extension | Final extension | # of cycles | Final product |
|---|---|---|---|---|---|---|---|
| Tay–Sachs Exon 11 | 5 – AGA TTC AGC CAG ACA CAA TCA T | 94 °C for 1 min | 58 °C for 1 min | 72 °C for 1 min | 72 °C for 10 min | 35 | 195 199 |
| | 5 – ACT TTA GAT CTT CAA ATG CCA GGG GTT C | | | | | | |
| Tay–Sachs Intron 12 | 5 – TCT AAG GGA GAA CTC CTG CT | 94 °C for 1 min | 59 °C for 1 min | 72 °C for 1 min | 72 °C for 10 min | 35 | 120 |
| | 5 –TGG AGA GGC TTG TAT GTG G | | | | | | |

**Table 2. Restriction endonuclease digestion of PCR amplified Tay–Sachs genes**

| PCR product | Restriction enzyme | Fragment length bp | Recognition sequence | Incubation conditions |
|---|---|---|---|---|
| Tay–Sachs Exon II | Hae III | $84,56,43,12^{normal}$ $84,56,47,43,12^{mut}$ | 5'-GG/CC-3' | 37 °C for 4 h |
| Tay–Sachs Intron 12 | Dde I | $100,20^{normal}$ $64,36,20^{mut}$ | 5'-C/T(G,A)AG,-3' | 37 °C for 4 h |

molecular weight products ($\leq$ 40 bp, for example, from restriction enzymatic digestion) is difficult without the incorporation of radioactive labeling. With the routine ethidium bromide staining method for DNA detection by gel electrophoresis, low molecular weight samples have reduced number of intercalation sites for the dye. As a result, the bands on the gel are not easily detected. New rapid methods are required that can quickly provide the same information obtained from the gel methods.

A technique capable of rapid analysis is MALDI-MS. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was originally introduced in 1988 by Karas and Hillenkamp[14] for analyzing proteins. MALDI typically uses a small matrix molecule that absorbs photons of a given energy to eject labile molecules into the gas phase intact or with minimum fragmentation. Since its introduction, this method has undergone a number of modifications to accommodate analyses of molecules as complex as DNA. Oligonucleotides up to 100 bases long were detected using MALDI after the advent of 3-hydroxypicolinic acid (3-HPA) as a DNA specific matrix.[15] Using 3-HPA, alone or in combination with picolinic acid (PA), detection of double stranded DNA up to 622 bp in complicated digest mixtures has been reported from this lab.[16] Chen and co-workers used 3-HPA to detect DNA of 500 bp in length produced via PCR.[17] Other developments in MALDI analysis of DNA include on-probe[18] and off-probe[19–21] purification procedures to reduce adduct formation from $K^+$, $Na^+$ and $Fe^{2+}$ cations. The use of nafion and nitrocellulose membranes as active substrates improves MALDI-MS detection of DNA in terms of reproducibility and sensitivity as shown by Liu *et al.*[22] Further, improvements to mass spectral resolution using pulsed delayed extraction methods[23–25] have become the recent focus of many labs.

In this report we demonstrate the feasibility of

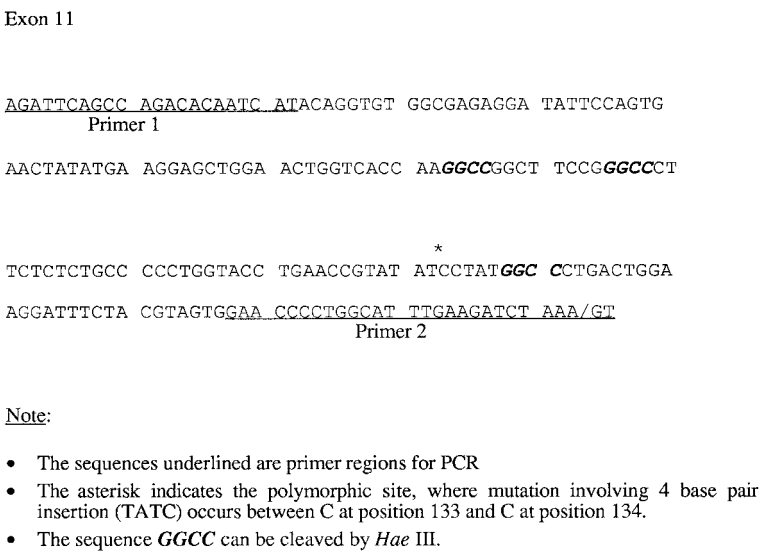


**Figure 1.** Sequence of the amplified Exon 11 region of the Tay–Sachs gene showing the 4 bp polymorphic site.

 

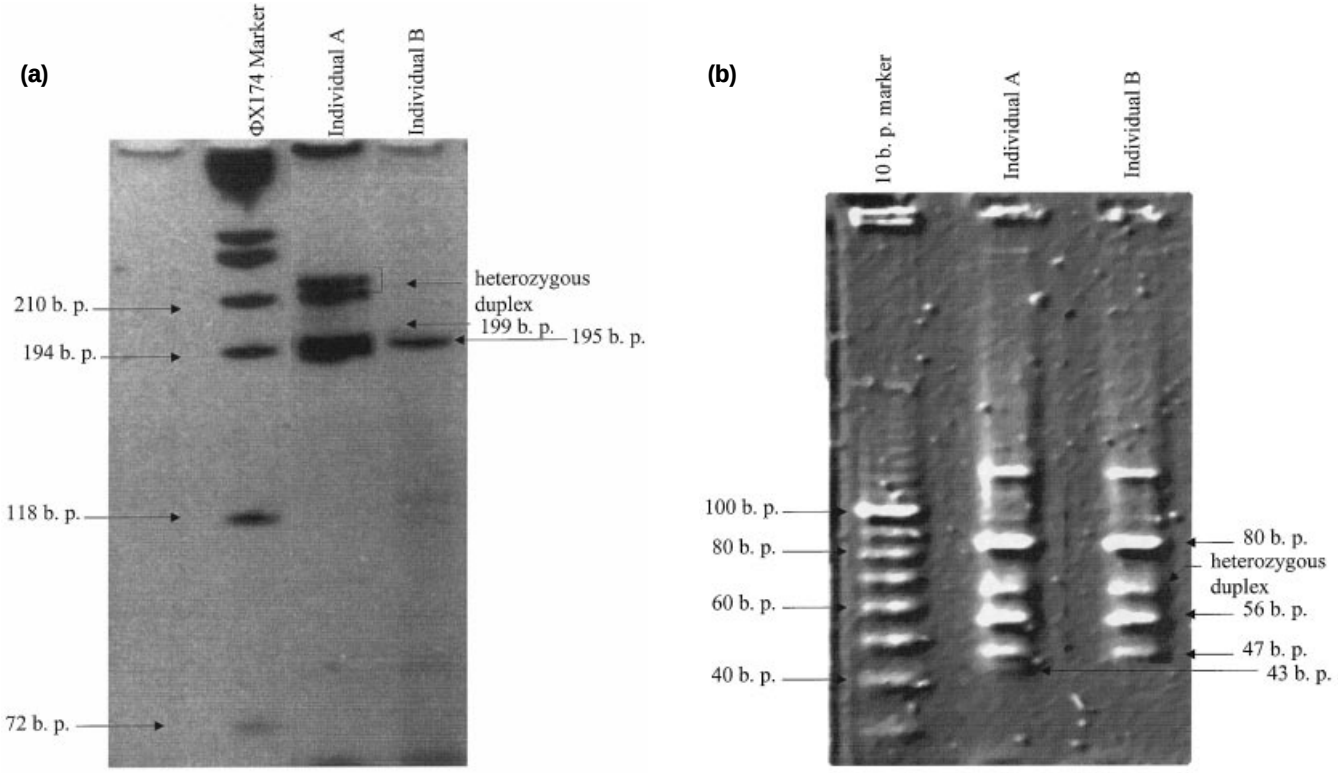


**Figure 2.** (a) 8% Polyacrylamide gel electrophoresis of PCR amplified Exon 11 of Tay–Sachs gene. Lane 1: $\phi \times 174$ marker ladder; Lane 2: Individual A, heterozygous state; Lane 3: Individual B, normal. The ']' marks two heteroduplex bands characteristic of the heterozygous state for the 4 bp insertion mutation. (b) Gel electrophoresis results of Hae III restriction digests of Exon 11 of Tay–Sachs gene. Lane 1: 10 bp marker ladder; Lane 2: Individual A, characterized by 43/47 bp bands; Lane 3: Individual B, normal sample (only 43 bp band).

MALDI-MS in combination with on-probe purification by nitrocellulose membranes as a cost-effective and rapid technique to detect human genetic variants for Tay–Sachs disease. Both the four base-pair insertion mutation in the exon 11 of the α-chain of the Tay–Sachs gene as well as the splice-junction mutation at the 5' end of the intron 12 were studied. Samples from various donors, taken using the buccal cell sample method were amplified and enzymatically digested followed by MALDI-MS analysis. The mass spectral results obtained were compared with the corresponding data from gel electrophoresis demonstrating the capabilities of the MALDI technique for clinical diagnosis. We have thus demonstrated the use of MALDI-MS to detect both low and high molecular weight products with high sensitivity and accuracy.

## EXPERIMENTAL

### Extraction of DNA from buccal cells

The use of buccal cell DNA from the inner cheek for PCR amplification has been reported earlier[22] and will be briefly discussed here. For our experiments, buccal epithelial cells were extracted by twirling a sterile cytology brush for 30 seconds on the inner cheek of the mouth. The DNA was then extracted by lysing the cells with 400 μL of 50 mM NaOH in a microcentrifuge tube. After heating the mixture at 95 °C for 5 min, the DNA solution was neutralized by adding 40 μL of 1M Tris and the stock solution was stored at 4 °C for further use in PCR analysis.

An alternative method of extracting DNA from buccal cells was also used. In this procedure we used the purification kit provided by Gentra Systems (Puregene Inc.) which ensures the removal of protein impurities from the final DNA solution. Comparison of the PCR results from the two extraction methods showed no difference, except that with NaOH extraction, the sample could not be stored for more than six months at 4 °C. DNA from cell lines of infantile Tay–Sachs patients (NA03461, NA03575) were obtained from Coriell Institute for Medical Research (Camden, NJ, USA).

### Polymerase chain reaction

The primers for PCR amplification of the mutation site were synthesized at the DNA synthesis core facility at University of Michigan. A typical 100 μL reaction was set up and the protocol was followed as shown in Table 1. All of the amplification reactions were performed in a MJ Research thermocycler (M. J. Research, Watertown, MA, USA) for 35 cycles.

### Restriction enzymatic digests

The restriction enzymes and the conditions for digestion are shown in Table 2. The enzymes were added directly to the completed PCR reaction mixture along with the respective buffers and incubated for 4 hours at 37 °C.

### Purification of DNA analyses

The commercially available purification kit from Qiagen (Qiagen Inc., Chatsworth, CA, USA) was used to purify the PCR products. The manufacturer's protocols

 

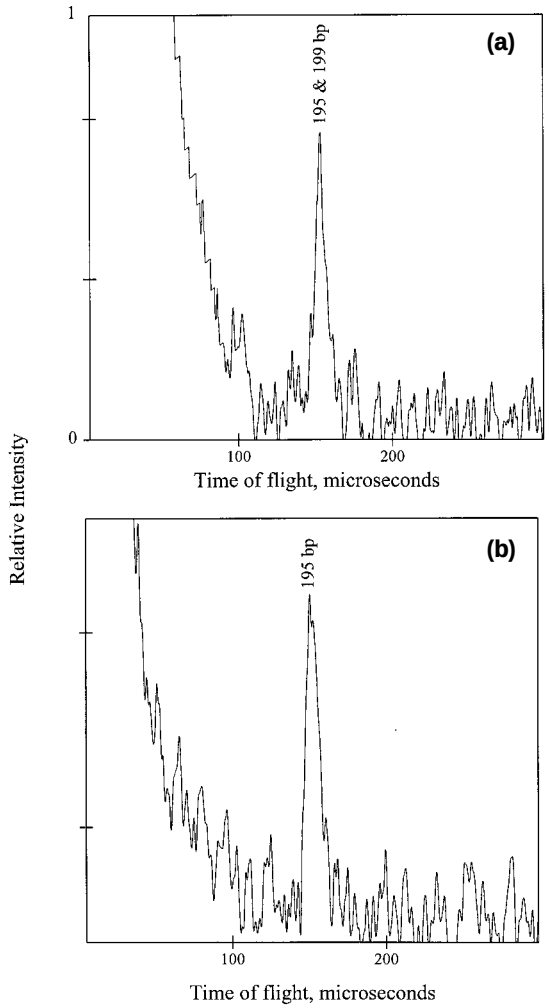


**Figure 3.** Negative ion MALDI-MS spectra of PCR amplified Exon 11 of Tay–Sachs gene of: (a) Individual A (195/199 bp) and (b) Individual B (195 bp). NC film substrate was used.

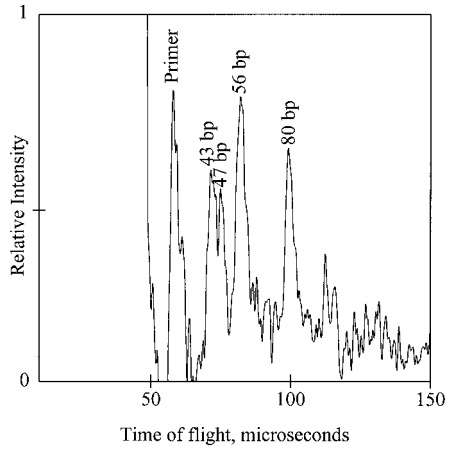


**Figure 4.** Negative ion MALDI mass spectrum of *Hae* III digested Exon 11 of Tay–Sachs gene of heterozygous Individual A, characterized by presence of 43 bp and 47 bp.

were followed for selectively purifying DNA ≥ 100 bp. The final eluent was dried in a SpeedVac and the DNA sample was resuspended in 10 μL of deionized water. 1.5–2 μL of this DNA solution was used for MALDI-MS analysis. For purification of the RFLP products < 100 bp, the digested DNA samples were precipitated

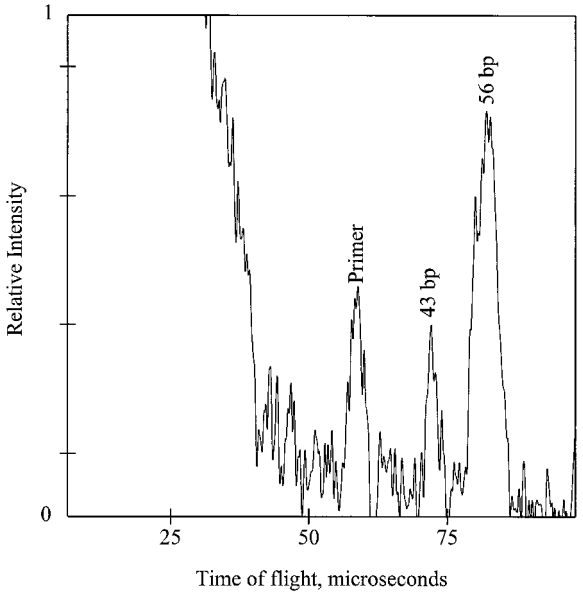


**Figure 5.** Negative ion MALDI mass spectrum of *Hae* III digested Exon 11 of Tay–Sachs gene of Individual B shown as normal (only 43 bp).

with the addition of 0.1 volume of 3M sodium acetate and 2 volumes of ice cold ethanol by incubating at –75 °C overnight. This extended incubation period was required to retrieve the smaller DNA fragments. The precipitated DNA was allowed to form a pellet by spinning the samples at maximum speed (~ 14 000 rpm) for 30 min at 4 °C (Eppendorf). The DNA pellets were then washed with 70% ethanol and resuspended in 10 μL of deionized water for further MALDI-MS studies.

## MALDI-MS analysis

The linear time-of-flight mass spectrometer (R. M. Jordan Co., Grass Valley, CA, USA) used for the MALDI analysis has been described previously.[25] Briefly, 355 nm radiation obtained from a Nd:YAG laser (Spectra Physics, Mountainview, CA, USA) was used for desorption/ionization of the DNA samples. The voltage settings of the ion optics used in the mass spectrometer to detect negative ions were: –15 kV for the repeller and –10 kV for the extractor. The detector was a triple microchannel plate detector with a CuBe conversion dynode capable of post acceleration up to +15 kV for the MALDI-produced ions. Ion signals were recorded on a LeCroy 9350M digital oscilloscope and subsequently transferred to an IBM PC for data processing. An average of 50–100 mass spectra were recorded for each of the results presented here.

The matrices used for MALDI analysis, 3-HPA and PA, were obtained from Aldrich Chemicals and used without any further purification. A mixture of 3-HPA/PA at a molar ratio of 4:1 in 36% acetonitrile and water was used as the matrix. Nitrocellulose membrane, as an on-probe ion-exchange membrane (Immobilon-NC Pure, Millipore, Bedford, MA, USA) was found to be essential in obtaining the reported mass spectra. 2 μL (at a concentration of ~16 mg/ml) of nitrocellulose in acetone was applied to the stainless steel probe tip to produce a thin film. 1.5–2 μL of the DNA sample was spotted on the nitrocellulose film and allowed to air dry.

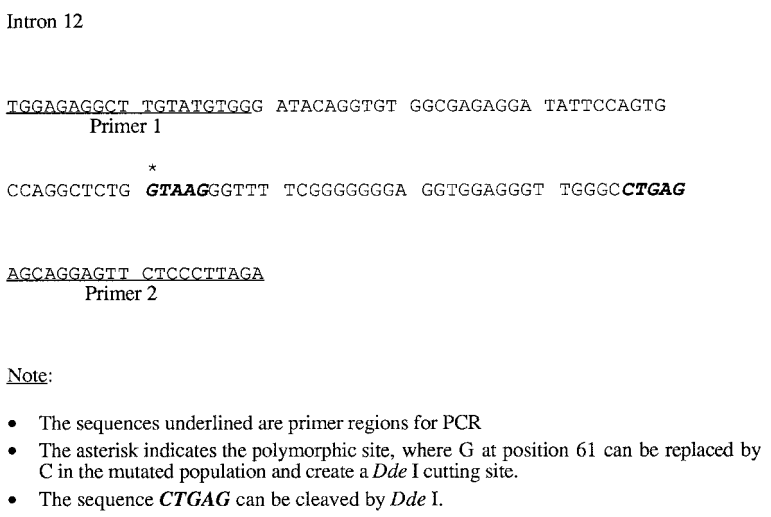


**Figure 6.** Sequence of the amplified Intron 12 region in the Tay–Sachs gene showing the splice junction mutation site available from RFLP studies by *Dde* I.

2–3 μL of HPA/PA matrix was then applied on the dried sample.

## RESULTS AND DISCUSSION

The DNA obtained from buccal cell samples of randomly selected individuals were analyzed for two of the three known HEXA mutations among the Ashkenazi Jewish population via polymerase chain amplification followed by restriction-enzyme digestion. Both the amplified as well as the digested products were detected by polyacrylamide gel electrophoresis as well as MALDI-MS analysis. The mutations in the gene could be easily detected since each creates a characteristic cut-site for the restriction endonuclease used.

The most common of the three mutations, a 4 bp insertion in the Exon 11 of the Tay–Sachs gene, was studied in two different individuals. Fig. 1 shows the sequence of the amplified region and the restriction sites in the Exon 11. In individual A the mutation was identified in the heterozygous state after PCR amplification which gave rise to two bands on the gel (195 bp and 199 bp) (Fig. 2(a)). Individual B tested normal based on the occurrence of only the 195 bp band (Fig. 2(a)). When the PCR product was separated by polyacrylamide gel electrophoresis (8%) but not agarose gels, the heterozygote sample always contained two extra bands that migrated more slowly. These bands are heteroduplexes resulting from annealing of DNA strands from the amplified normal and mutant alleles.[27] When these strands anneal, the 4 bp insertion forms a 'bubble' that slows the migration of the duplex in polyacrylamide gels. Since either strand can anneal with its counterpart in the normal amplified product, two types of heteroduplexes are formed that migrate at different rates.

In comparison, Fig. 3 is the negative ion MALDI mass spectrum of PCR amplified Exon 11 of the Tay–Sachs gene for the two cases. As always, the double stranded samples from PCR were detected as their respective single strands. The heteroduplexes observed on the gel were hence absent in the final mass spectrum. The expected amplification product of 195 bp was clearly observed in both but could not be discerned from the 199 bp product in Individual A (Fig. 3(a)). The low resolution of the mass spectrometer at the high mass end, did not allow the detection of the 4-bp insertion as detected by gel electrophoresis (Fig. 2(a)).

The restriction enzyme, Hae III provides three cutting sites (5'-GG/CC-3') in the amplified exon 11 sequence. The fragments generated are listed in Table 2. In the presence of the 4-bp insertion, a 47 bp fragment was seen in addition to the 43 bp fragment for the heterozygote sample (Individual A) when separated by gel electrophoresis (Fig. 2(b)). The negative ion MALDI mass spectra of the Hae III digested Exon 11 gene from Individual A is shown in Fig. 4. All the generated fragments from the enzymatic digests were obtained in a single spectrum with relative ease. The peak in the TOF mass spectrum arriving at 53 μs corresponds to a primer (20 mer). The absence of the 12 bp fragment from digestion and the second primer is attributed to purification procedures. An important feature of the MALDI mass spectrum shown in Fig. 4 is the intensity of the individual peaks which is directly related to the concentration of the fragments generated by the digestion. The 56 bp and 80 bp fragments are twice as concentrated as the 43 bp and the 47 bp fragments which are generated from a single strand of the heterozygote duplex. This feature is also depicted in the intensity of the bands observed on the gel (Fig. 2(b)). The negative ion MALDI mass spectrum after enzymatically digesting DNA from Individual B, clearly showed the absence of the 4 bp insertion mutation. Fig. 5 represents the average of 50 laser shots in the MALDI-MS recorded from the sample of Individual B.

The strategy for detection of the splice junction mutation on the intron 12 of the Tay–Sachs gene was to amplify a 120 bp fragment flanking the mutation site. In case of the unmutated Intron 12 gene, there is only one cutting site for the restriction enzyme, Dde I (5'-C/T(G,A)AG-3'). Subjecting the PCR product to enzymatic digestion with Dde I will produce fragments of 100 bp and 20 bp. When a point mutation occurs, the G-C base substitution at the 5' end of the intron 12 creates a new Dde I site. In this case, restriction enzyme

 

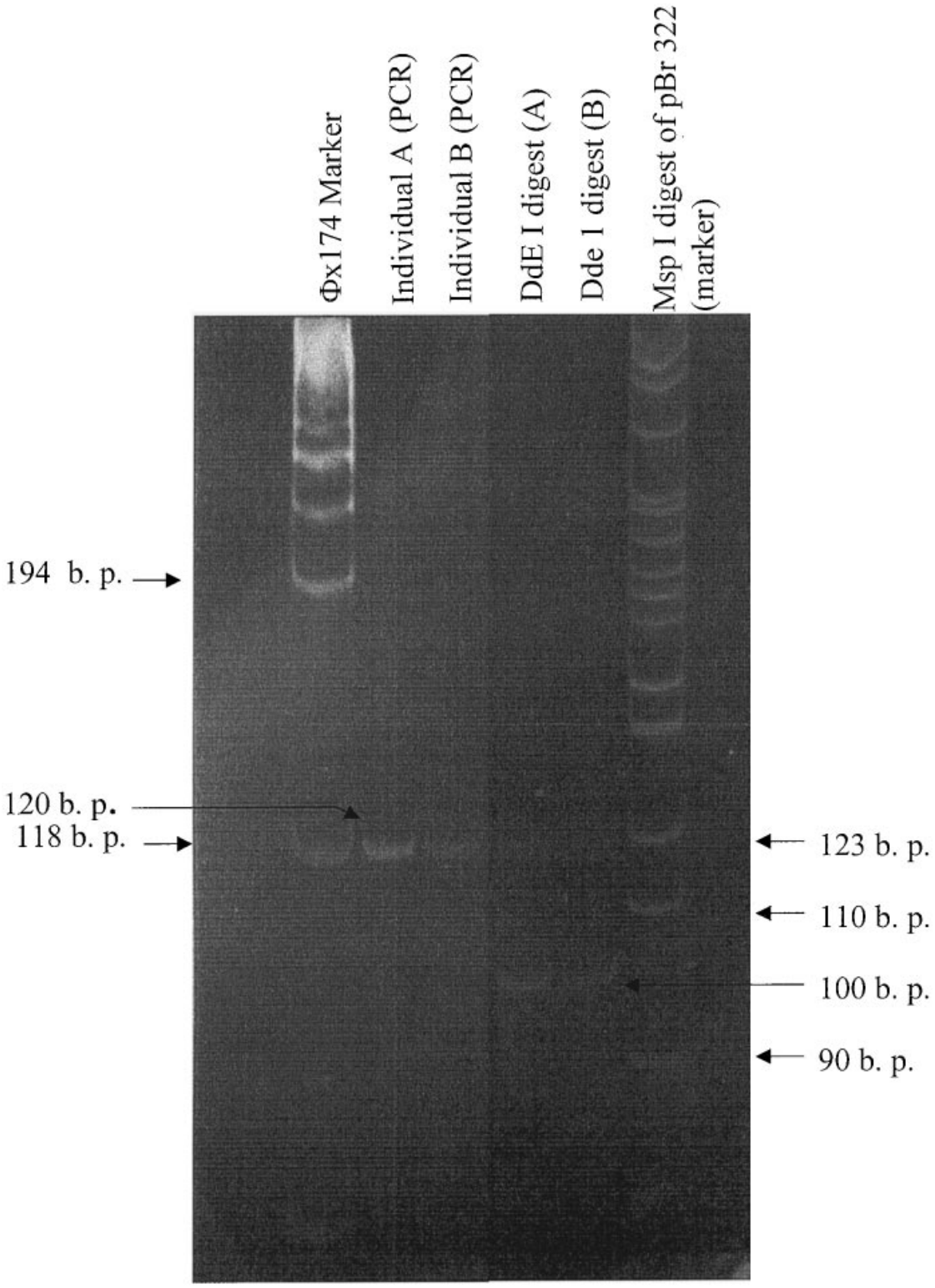


**Figure 7.** 8% Poly acrylamide gel electrophoresis of Intron 12 of Tay–Sachs gene, Lane 1: $\phi \times 174$ Marker, Lane 2: PCR product of Individual A, Lane 3: PCR product of Individual B, Lane 4: *Dde* I digest of Individual A (normal), Lane 5: *Dde* I digest of Individual B (normal), Lane 6: Msp I digest of pBr 322, marker.

digest products include DNA fragments of 64 bp, 36 bp and 20 bp. Fig. 6 shows the sequence of the amplified region and the cut sites from Dde I in the Intron 12. The fragments generated are listed in Table 2. Two individuals were randomly selected for this study and both were observed to be normal. The cell lines of an infantile TSD sample obtained from Coriell Institute for Medical Research on amplification followed by restriction enzymatic digestion with Dde I, was shown to be normal using both gel electrophoresis as well as MALDI-MS detection. Figure 7 is the gel electrophoresis results obtained for this study. The MALDI-MS of a representative PCR sample is shown in Fig. 8. The purified 120 bp PCR product was observed at 120 μs. This spectrum was obtained as the average of 50–100 laser shots. The MALDI-MS results (Fig. 9) of the

 

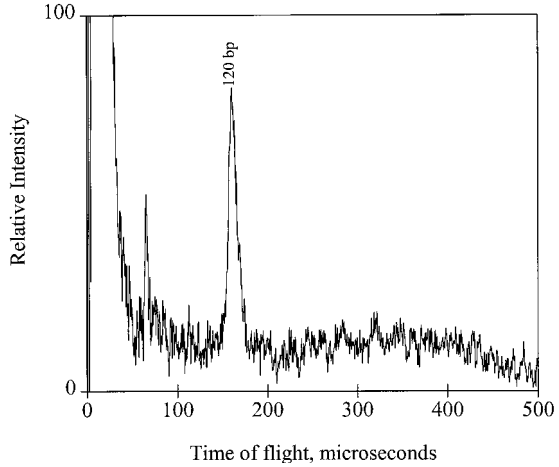


**Figure 8.** Negative ion MALDI mass spectrum of PCR amplified Intron 12 of Tay–Sachs gene (120 bp).

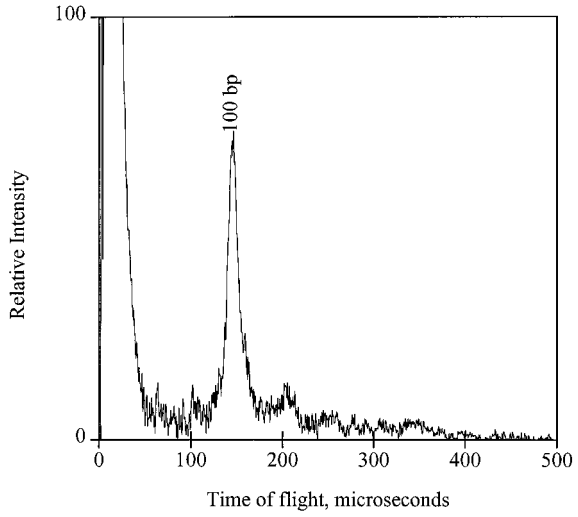


**Figure 9.** Negative ion MALDI mass spectrum of restriction enzyme digest of Intron 12 of Tay–Sachs gene by Dde I, demonstrating DNA is from a normal sample.

RFLP study indicated an unmutated gene due to the presence of the 100 bp fragment. The absence of the small digestion fragment of 20 bp and the primers from the PCR process may be lost during sample purification.

The MALDI-MS approach to study DNA samples for screening purposes in genetically inherited disorders such as the Tay–Sachs disease seems viable in clinical labs for its speed and accuracy. Enzyme based assay programs to prevent the disorder are designed more to distinguish carriers from non-carriers. MALDI based DNA testing could be a useful adjunct to the enzyme-based assays. The most important benefit of this technique would be the ability to identify carriers of specific Tay–Sachs mutations, who are otherwise indistinguishable based on enzyme assay alone. Such a rapid and accurate technique could play a significant role in prenatal diagnosis.

## CONCLUSIONS

The results presented demonstrate the capabilities of MALDI-MS as a clinical diagnostic in analysis of genetic disorders amplified by PCR and RFLP. The replacement of gel electrophoresis by the MALDI method can speed up the time required by current protocols followed in molecular biological labs. Use of alternative specimens from DNA analysis such as buccal epithelial cells instead of blood add to the cost-effectiveness of the MALDI-MS based method. The MALDI-MS technique has been demonstrated to be sufficiently sensitive to detect both the PCR products as well as their enzymatic digests with relative ease.

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