

Interim Report
U.S. Department of Energy

[Novel Mass Spectrometry Mutation Screening for Contaminant Impact Analysis]

Project Number: 60218
Grant Project Officer: Cindy Kendrick
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2. **Executive Summary:**

Since it is always necessary to analyze a large number of samples to confirm the relationship between mutation and contaminant exposure, our approach is to develop novel rapid DNA detection technology so that quick analysis can become feasible in the future. The goals of this program are to develop novel DNA analysis technologies and to demonstrate these technologies can be used to address the DNA mutation due to the exposure to contaminated media. Mutations of p53 genes are found in more than 60% of animal tumors and human cancers. Analysis of mutation spectra of p53 in human cancers as well as laboratory induced animal tumors has indicated that particular carcinogens may be responsible for specific types of tumors. However, the direct linkage of each individual contaminant to specific mutation is usually not known. For genes which are highly conserve such as p53, the mutation in animal due to the environmental contaminants can have similar effects on human being. Thus, fishes from contaminated area can be used as a model system to test this hypothesis. There were six specific tasks to be pursued in the program. They include (1) Development of Laser-induced acoustic desorption for DNA detection (2) To couple laser desorption mass spectrometry with allele specific polymerase chain reaction (ASPCR) for mutation analysis (3) To improve the detection efficiency for large DNAs (4) Development of fast matrix-assisted laser desorption/ionization with fragmentation for direct DNA sequencing (5) Determination of mutations in tumor of targeted fish and (6) To achieve sample automation system for rapid DNA analysis. We have more or less successfully developed the above technologies in this program. These technologies with some effort in fabrication can be used in the future for sound risk analysis to evaluate and prioritize the waste sites for clean-up.

3. **Research Objective:**

Due to the limited budget of waste clean-up for all DOE contamination sites, it is critical to have a sound risk analysis with strong scientific basis to set priority for waste clean-up. In the past, the priority was often determined mostly by the type and quantity of pollutants and the observation of cancer rate increase. Since human cancers can be caused by various reasons in addition to environmental contamination, a rigorous study to find the relationship between specific contaminants and cancer is critically important for setting up the priority for waste clean-up. In addition, a contaminated site usually contain many different pollutants. However, it can be only a few specific pollutants are carcinogenic chemicals which are responsible for most cancers. Clean-up of small quantity of critical pollutants instead of the entire pollutant site can save significant decontamination cost.

Since a few anti-tumor genes such as p53 and *ras* genes are highly conserved among various animals and mutation of these genes have been associated with many human cancers, it is very valuable to find the relationship between specific contaminant and specific cancer. Since it is not possible to pursue any human on the relationship of cancer and specific pollutant under well defined experimental conditions, it is desirable to pursue experiments on animals such as fish and mice to find out the relationship of mutation of p53 gene and specific contaminant. It is also required that the sequence of the region of p53 gene in animal is same as human being. Mutations due to pollutant can happen at various sites and only occur at a small percentage. In order to confirm the relationship between specific pollutant and mutation, a very large number of DNA samples need to be carefully analyzed. In the past, nearly all DNA analyses were pursued by gel

electrophoresis. It is relatively slow and expensive. It is not feasible to obtain the relationship of mutations with specific contaminant with present DNA analysis technology. Thus, our approach is to develop novel new DNA technologies which can potentially achieve rapid, reliable and inexpensive DNA analysis for environmental applications.

The objective of this program is to develop innovative mass spectrometry technology to achieve fast mutation screening and to reveal the linkage between gene mutation and contaminants. Mass spectrometry has the potential to achieve very fast speed sample analysis. New innovative approaches for improving mass resolution and detection sensitivity were pursued to help to achieve rapid DNA screening. Allele specific polymerase chain reaction (ASPCR) coupled with mass spectrometry for DNA mutation detection was also pursued. This technology was applied to wildlife species such as fish for the genotoxic effect of hazardous waste to be assessed at DNA level.

4. Methods and Results:

A. Background and Methods

Aquatic environments are becoming increasingly contaminated by an alarming number of foreign chemicals, many of which are carcinogens and mutagens with the capacity to cause various types of DNA damage. The consequence of DNA structural perturbations can be innocuous because of cellular repair processes. However, those lesions not properly repaired can lead to mutation and genomic instability. Mutations which occur in germ cells and which are nonlethal can lead to heritable syndromes. Genetic mutations in somatic cells can result in malignant transformation.

Studies with various fish species have shown that neoplasms are observed in discrete geographic areas near industrial activities and large urban centers¹. Analysis of toxic organic or their metabolites in sediment or fish tissues indicates a positive statistical association between observed neoplasia and the concentration of organic pollutants²⁻⁴. In Atlantic tomcod from the Hudson River and winter flounder from Boston Harbor, activated *K-ras* oncogene, a gene consistently mutated in certain human cancers and tumors from carcinogen treated animals, was observed in hepatic neoplasms^{5,6}. These observations coupled with the findings that exposure to carcinogens can induce neoplasia of laboratory fish suggest an environmental etiology for the disease⁷.

P53 and *ras* genes are among the most important tumor-suppressing genes. Mutation of these tumor-suppressing genes were found to be strongly associated with certain cancers. For example, three *ras* genes (K, H, and N) have been identified in mammalian genome. *Ras* genes, which are present in all eukaryotes including yeasts, have been analyzed in many species, including fish, and their products show remarkable structural homology⁸. It has been extensively demonstrated that rodent cancers induced by exposure to a variety of carcinogens often contains mutations in *ras* oncogenes. With sensitive methods, *ras* gene mutations were also detected in apparently normal tissues in carcinogen exposed

animals^{9,10}. The *ras* genes isolated from tumors most commonly have a single missense mutation in codons 12, 13 or 61. These findings, along with the fact that these amino acid residues are directly involved in guanine nucleotide coordination, establish the molecular mechanism behind the origin of *ras* oncoproteins. The base changes in the mutant *ras* genes in animal models generally agree with those caused by the carcinogens in simple mutagen assays¹¹.

Ras oncogenes have been detected in a great variety of human tumors, although their incidence varies considerably with tumor type¹². Similar to the observations in animal models, the occurrence of *ras* oncogenes is primarily located in the codons for amino acid 12, 13 or 61. For example, the K-*ras* gene was preferentially found to be activated in pancreas, lung and colon at codon 12th. In lung, the majority of the mutations in K-*ras* are G to T transversions, whereas in colon tumors the majority of the mutations are G to A transitions. This difference in *ras* mutation spectrum between lung tumors and colon cancers may indicate the involvement of different mutagens in the induction of the oncogene. There is a strong association between the presence of a mutated *ras* and p53 gene in lung cancer and the smoking history of the patients, suggesting that mutagenic components in tobacco smoke may cause the mutations¹³.

Activation of K-*ras* gene has been observed in neoplasms of rainbow trout exposed to aflatoxin B1 (AFB)¹⁴ and diethylnitrosamine (DEN)¹⁵. In both studies, the mutations were essentially confined to codon 12. DNA sequencing analysis demonstrated that 9 out of 10 tumors induced by the toxin had mutated *ras* genes with a G to T transversion whereas the alkylating chemical induced tumor contained the *ras* genes with only G to A transition. These data entirely agree with those obtained from rodent experiments. The specific *ras* mutations have also been demonstrated to be relevant to genotoxicity in environmentally exposed fish population. Nucleotide sequence analysis of winter flounder tumor DNAs revealed that the mutations comprised G:C to A:T or G:C to T:A changes at 12th codon.

As a guardian of the genome, p53 plays a central role in the cellular response to DNA damage by mediating cell cycle arrest in proliferating cells until the damage is repaired or by activating an apoptotic pathway to eliminate the damaged cells. Nelson and Kastan¹⁶ demonstrated that only strand breaks, but not other DNA lesions are capable of inducing p53 accumulation. Thus, it has been suggested that p53 proteins serve as a cellular sensor of DNA strand breaks. A model by Selivanova and Wiman¹⁷ has been proposed wherein the interaction of the C-terminal of p53 protein with ssDNA ends created by strand breaks induces a conformational change in the protein. This interaction also converts p53 to a more stable form and results in its accumulation. The conformational change further causes the exposure of DNA binding domain located at the middle of the protein and leads to the binding of p53 to its responsive genes. This binding triggers the release of p53 protein from ssDNA ends. By such a mechanism, the interaction of p53 with ssDNA ends generated by DNA damage would cause transcriptional induction of p53 responsive genes that regulate one or more cell cycle checkpoints. The p21 gene has also been shown to be directly linked to p53 expression and

inhibition of cell cycle progression. The product of this gene was found by Sherr and Roberts¹⁸ to be a potent cyclin-dependent protein kinase inhibitor and was differentially expressed during cellular senescence.

If DNA damage is beyond repair capabilities, p53 triggers cell suicide by apoptosis. This mechanism provides an additional safeguard to ensure the elimination of cells with genetic damage. The role of p53 in apoptosis is distinct from its role in cell cycle regulation, as the transcriptional activity of p53 is not required for this function. In DNA damage induced apoptosis, functional p53 is required as convincingly demonstrated by Clarke et al.¹⁹.

The fact that p53 was initially incorrectly categorized as a cellular oncogene due to the use of a mutant p53 for its functional analysis clearly indicates that mutation of p53 plays a vital role in cellular malignant transformation. Since then, mutation in the p53 gene is observed to be the most common genetic alternation in human cancers. Accumulated evidence indicates that the evolutionarily conserved four regions are the most frequent sites of mutations.²⁰ Based on a recent survey of 560 p53 mutations, 87% are confined between exons 5 to 8 and the others are in exons 4 (8%) and 10(4%)²¹ In addition, the majority of p53 mutations are substitutions, particularly missense mutation of which 79% are in the conserved midregion. Among the four conserved domain (II to V), six mutation “hot spots” located at the amino residues 172, 245, 248, 249, 273 and 282 have been identified in various human tumors.²¹ The findings that the majority of p53 mutations are in the conformationally sensitive hydrophobic midregion of the protein suggests that missense mutation in these regions can alter the conformation of the protein which can further cause both a loss of tumor suppression function and a gain of oncogenic function.

Analysis of p53 mutation spectra of human cancers as well as animal model systems has revealed significant differences between the tumor types as well as their tissues of origin. These observations are suggestive of an environmental etiology for the disease. Of all p53 mutation in human tumors, the commonly observed (41%) are G:C to A:T transitions which are thought to result from spontaneous deamination of cytosine or 5'-methylcytosine, resulting in C to T replacements. These mutations occur most frequently at CpG dinucleotide. It was found that 24% p53 mutants with G:C to A:T transition occurs at CpG dinucleotide. All CpG dinucleotide throughout exon 5-8 of the p53 gene have been shown to be methylated *in vivo*²². The G:C to A:T transitions are generally considered to be associated with spontaneous mutations, although methylating chemicals can primarily induce G:C to A:T base transition.²³ Transversion mutation of G:C to T:A occurs quite frequently in hepatocellular carcinoma(HCC), particularly at specific geographic regions. In addition, the guanine residue at codon 249 is often targeted for the mutation. This type of mutation has been attributed to dietary aflatoxin B1 (AFB) exposure. Ninety five percent of Liver tumors from population of Qidong region of China was observed having G:C to T:A transversion. Most of p53 mutations in lung cancer are also G:C to A:T transversion. However, in contrast to liver cancers, the guanine residues at codons 154, 157, 248 and 273 are mutational hotspots and guanine

residue at 249 is rarely mutated in lung cancers. These types of mutations are consistent with in vitro mutational hotspots analysis of p53 sequence induced by benzopyrene and further suggest that benzopyrene present in tobacco smoke is an etiological contributing factor for human lung cancer. Solar radiation, particularly UVB part of the solar spectrum, is highly carcinogenic. Mutations, mainly CC to TT tandem mutation at dipyrimidine sites, a characteristic for UVB radiation, are often observed in p53 gene of human skin cancers. Identical type of p53 mutation was observed in normal human skin biopsies exposed to sunlight.²⁴

In order to define the structural alterations and to understand the consequence of gene mutation, a variety of methods have been developed and continuously improved²⁵. Techniques, such as fluorescence in situ hybridization (FISH) and restriction fragment length polymorphism (RFLP), for detection of large chromosomal rearrangements, insertions, and deletions were developed. In addition, a number of methods is available for detecting single-base alterations. Among all the scanning methods for point mutation, chemical mismatch cleavage (CMC) analysis is the most sensitive one. It can identify close to 100% of all mutations. In CMC, Most methods need to use gel electrophoresis and autoradiography which limit the speed of analysis. In this program, we use the novel mass spectrometry technology to analyze tumor suppressing genes in fish from contaminants controlled area .

In general, gel electrophoresis is a very time consuming step for DNA analysis. In addition, tagging radioactive materials or chemicals such as ethidium bromide to DNA is usually needed for DNA analysis. Since a large number of samples needs to be tested to identify the relationship between gene mutation and contaminants. Improvement of DNA analysis speed is critically important. A rapid alternative method for DNA analysis which is to use a time-of-flight (TOF) mass spectrometer (MS). With TOF-MS for DNA analysis, it usually takes a few seconds (not including sample preparation time) per sample for both DNA size analysis and DNA sequencing compared to hours by gel electrophoresis method. Furthermore, TOF-MS sample preparation usually does not involve the use of radiolabels or chemical tags. However, the major difficulty for using TOF-MS for DNA detection or sequencing is due to the very low vapor pressure of DNA segments at room temperature. In addition, the glycosidic bond in DNA segment is easy to break such that the production of intact DNA ions becomes a challenge. Thus, with TOF-MS for DNA size analysis and sequencing, it is necessary to achieve "soft" desorption of DNA without breakup of fragile DNA compounds and "soft" ionization without producing fragmented ions. In 1987 Hillenkamp and his co-workers²⁶ discovered that large protein molecular ions can be produced by laser desorption without much fragmentation if these biomolecules are mixed with small organic compounds that serve as matrices for strong absorption of a laser beam. This process is now called matrix-assisted laser desorption (MALD). The typical preparation technique for MALD is to dissolve biomolecular samples in solution, then prepare another solution that contains small organic compounds such as 3-hydroxypicolinic acid. These two solutions are subsequently mixed and a small amount of solution is placed on a metal plate to dry. After the sample crystallizes, the sample plate is

placed in the mass spectrometer for analysis. With the MALD process, matrix materials strongly absorb the laser energy and quickly become vaporized. Large biomolecules are carried along during the fast vaporization process. By this method, large biomolecules can be delivered into space without breakup, which is probably due to the minimal direct absorption of laser energy; thus, "soft" desorption can be achieved. Furthermore, it has also been found that biomolecule ions can be produced during the MALD process in addition to the expected neutral molecules. The production of ions is speculated to involve a proton transfer process. The process involving ionization is called matrix-assisted laser desorption and ionization (MALDI). MALDI has also been applied to DNA segments, but until recently the success has been limited to very small segments. Thus, there was a serious concern whether MALDI could be used for detecting large DNA segments. During the past few years, many research groups including Hillenkamp at University of Munster²⁷, Lubman at University of Michigan²⁸, Smith at University of Wisconsin²⁹, Chait at Rockefeller University³⁰, Williams at Arizona State University³¹, Becker at SRI International³², Levis at Wayne State University³³, Cotter at Johns Hopkins University³⁴, and Reilly at Indiana University³⁵ have made significant improvements in using MALDI and time-of-flight mass spectrometer for DNA detection. Recently, we succeeded in using MALDI to detect a double-stranded (ds) DNA with 500 base pairs by using new matrices and high ion energy for more efficient detection³⁶. These results clearly indicate that MALDI can be used for large DNA analysis. However, the resolution for long DNAs is still too poor to have application on mutation analysis. In this program, we developed a laser-induced acoustic desorption mass spectrometer to improve mass resolution.

One of the key factors causing poor resolution in the detection of large DNA molecules (>100 mer) is the very broad energy spread due to large DNA fragments having velocity similar to the small matrix molecules. Reflectron time-of-flight mass spectrometers have been used but failed to improve the resolution since many large DNA ions have limited lifetime when they are produced by MALDI.³⁷ Delayed pulsed ion extraction has been used to achieve very high resolution for small DNAs and proteins.³⁸⁻⁴⁰ However, the mass resolutions for large DNAs (>70 nucleotides) did not get significantly improved. If we take a 500 mer DNA mixed with 3-HPA which served as matrix. The desorbed kinetic energy of DNAs can be as high as a few hundred volts. With this very high kinetic energy, the short delay of relatively weak extraction voltage can not be expected to significantly improve mass resolution. Thus, in this program, we tried an innovative and very different approach. We use laser-induced acoustic desorption (LAD) so that no matrix is used and the laser fluence can be reduced to a minimum for getting better resolution. This technique gives a unique possibility for producing neutral gas phase biomolecules. The method relies on intense photoacoustical "shaking" of a surface containing adsorbed molecules, and soft protonation to obtain biomolecular ions. The intense sound wave is produced from absorption of short laser pulses directed onto a thin substrate from the side opposite to the adsorbed material. The main advantages of this approach when compared with the other laser desorption techniques are (1) There is no direct interaction of desorbed molecules with a laser beam, which could lead to photodestruction. The probability of dissociation of

a desorbed molecule is relatively low because of the absence of strong heating effects; (2) large biomolecules, such as DNA, linked to the surface at many points can be desorbed by simultaneous breaking of all adsorbate-substrate bonds. The absorption of short, intense laser pulses can lead to local heating of the surface and create thermally induced strain. Thus, photoacoustic pulses are generated and propagated in the substrate. The parameters of laser-induced acoustic pulses depend on laser pulse duration and the properties of the substrate materials. When a picosecond laser pulse is used, the photoacoustic pulses can be generated with amplitude in the range of 10^8 or 10^9 Pa. The resulting acoustic pulse formed in the laser irradiated media can propagate into the substrate and reach the opposite boundary. The acoustic pulse/boundary interaction can result in a boundary shift and eventually cause the desorption of large biomolecules from the substrate surface. An analogy may be made between the proposed laser acoustic desorption and two other well-known physical phenomena. The first is the method for cleaning a metal surface by impact. Builders commonly use the method to clean concrete, dry glue, etc., from metal surfaces. The characteristic size of particle L desorbed by impact can be estimated as: $L \sim c_{\text{sound}} t_p$, where c_{sound} is the velocity of sound in a given material and t_p is the acoustic pulse duration. With decreasing pulse duration the size of detached particles would decrease also. This is the reason that an ultrasound wave is so efficient for surface cleaning and is applied in practice in ultrasonic cleaning machines. Another example is the ultrasound shaker used by biologists to separate a specific cell from a group of cells. Operation of this device is based on microsecond acoustic pulses to separate objects of about 10 micrometers in size. To desorb large molecules by the shaking surface method, one should use an ultrasound frequency in the range of about 10^{10} Hz. Only ultrashort laser pulses are capable of generating such acoustical pulses.

In general, direct absorption of visible or ultraviolet photon by molecules usually can not cause efficient molecule desorption because the frequency of electron excitation ($\sim 10^{15} \text{ sec}^{-1}$) is very different from the frequency of adsorbed molecules ($\sim 10^{10} \text{ sec}^{-1}$). However, laser-acoustic pulses can interact with molecular-surface bonds directly without intermediate energy transfer due to the very similar frequency. Soft desorption of molecules without fragmentation is possible. For the desorption of biomolecules with molecular weights between 10^3 - 10^7 Daltons, a picosecond laser is much preferred. Although laser induced acoustic desorption is expected to have efficient desorption, the efficiency for producing ions can be low. A Pd foil which has a large amount of trapped hydrogen will be used as substrate. During the laser-induced acoustic wave desorption, the desorbed DNA can be protonated or deprotonated to form ions by the desorbed protons.

Fast DNA sequencing by MS is also very valuable when the possibility exists for more than a single base mutation. Conventional gel electrophoresis methods (including thin gel, slab gel, and capillary gel) usually require two time-consuming processes. These are (1) preparation of DNA ladder products by either Sanger's enzymatic method⁴¹ or the Maxam-Gilbert chemical degradation method⁴²; and (2) running gel to separate different sizes of DNA and detection of each different size of DNA. Either radioactive labels such as ^{32}P or ^{35}S or chemical labels such as

fluorescent dyes can be used to label the fragments. Following electrophoresis, DNA segments are detected by detecting the labels. Both radioactive methods and fluorescent dye labeling methods for DNA sequencing require the use of the time-consuming gel electrophoresis method. In this program, we developed matrix-assisted laser desorption/ionization/fragmentation (MALDIF) mass spectrometry (MS) for fast DNA sequencing specifically for mutation analysis. MS has been proposed to improve sequencing speed in the past. However, almost all the approaches are to replace the gel separation and detection process by mass spectrometry. Nevertheless, the preparation of DNA ladder products is still necessary. The overall time saved is limited by the relatively slow process of preparation of DNA ladder products. In this program, we succeeded in using MALDIF-MS for sequencing small DNA. With this approach, we need neither the preparation of DNA ladder products nor the gel electrophoresis process to separate DNA. This process needs no radioactive or dye chemicals to tag DNA⁴³. Recently, McLuckey *et al.*^{44,45} as well as McLafferty and his colleagues⁴⁶ have used electrospray mass spectrometer to obtain direct sequencing of very short oligonucleotide (< 14 nucleotides) However, this approach usually generates a large number of fragmented peaks. A mass spectrometer with ultra-high resolution (such as Fourier Transform Mass Spectrometer (FTMS)) is needed in order to identify these peaks. The very high cost of FTMS and the need of an experienced and well-trained technician can also limit the routine use of this approach for DNA sequencing. We used ultraviolet laser for MALDI with gas phase fragmentation should provide much simpler spectra for direct sequencing without the need of DNA ladders preparation. In this program, we succeeded in sequencing ss-DNA up to 35 nucleotides with ultraviolet laser desorption with matrix-assisted laser desorption/ionization/fragmentation (UV-MALDIF) for several samples using a very simple linear TOFMS.^{47,48}

B. Results:

1. Direct sequencing by UV-MALDI F with the size up to 35 nucleotides.

As mentioned above, the idea of the proposed DNA sequencing is to achieve the desorption/ionization/fragmentation (selected) all at the same time, thus saving on the labor and time required for producing DNA ladder products and gel separation can be achieved. In this program, we tried to use UV-MALDIF to achieve direct DNA sequencing by carefully adjusting the experimental conditions, which includes the use of newly discovered matrices and adequate laser fluence. In order to obtain full sequence information on DNA by MALDIF, preferred 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. Secondary fragmentation should be avoided (i.e., the further fragmentation of the primary fragment). With the approach of UV-MALDIF, the preferred bond cleavage can be established through bond weakening by the interactions between DNA and matrix molecules. 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.

A linear time-of-flight mass spectrometer typically with delayed pulse extraction was used for separation and detection. The concept of MALDIF is shown in Figs. 1 and 2. Assuming the "favored" 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

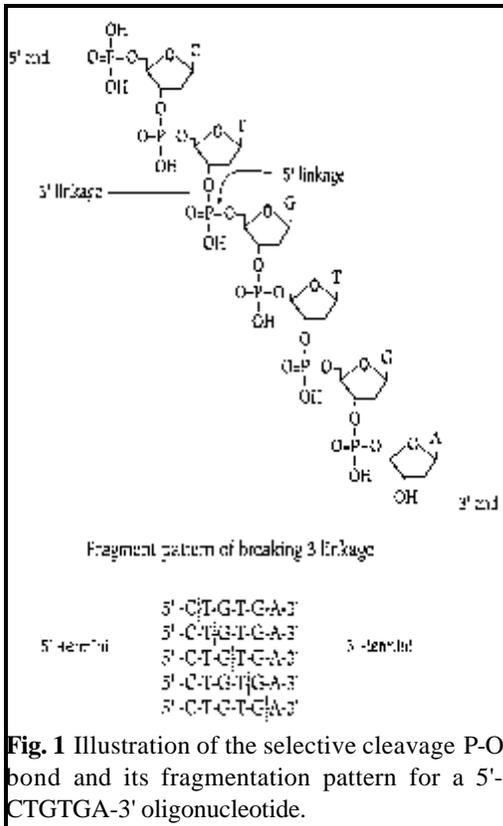


Fig. 1 Illustration of the selective cleavage P-O bond and its fragmentation pattern for a 5'-CTGTGA-3' oligonucleotide.

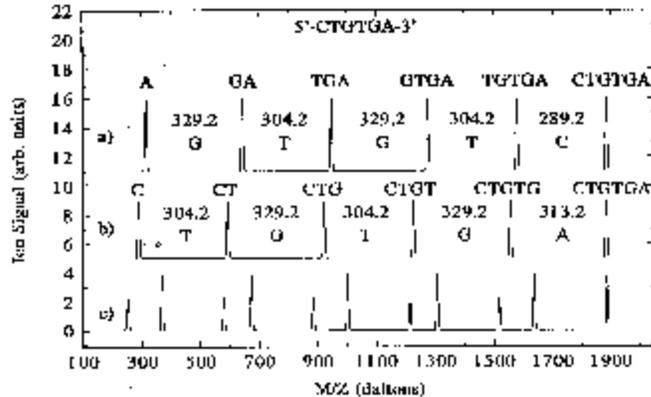


Fig. 2 The simulated spectra of the selective cleavage of P-O 3'-linkage and 5'-linkage for 5'-CTGTGA-3' oligonucleotide. Trace a) is the simulated spectrum of fragment series with 3'-termini by the 3'-cleavage. Trace b) is the simulated spectrum of fragment series with 5'-termini by the 3'-cleavage. Trace c) is the simulated spectrum of fragment series with both 5'- and 3'-termini by the 5'-cleavage.

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. It is not difficult to distinguish the 5'-termini series from the 3'-termini according to mass information in MALDIF spectra. Due to the two different P-O bonds in the DNA chain (5'- and 3'- linkage in Fig. 1), four series in principle can exist in MALDI spectra. 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 were resolved in the MALDIF experiment.

According to the sequencing strategy discussed above, two requirements for matrices have to be met in order to perform selective fragmentation. They are (1) A matrix should provide an environment to weaken the P-O bond. (2) It should provide a large cross section for DNA ionization so that the majority of fragments will be ionized and resolved. We found that the ionization (or deprotonation) of DNA is directly related to the quantity of the $\text{NH}_4^+/\text{NH}_3$ concentration in MALDI samples, and the fragmentation at P-O position is directly related to the pH value of the matrix. It is possible to adjust the ammonia concentration to provide fragmented ions for sequencing. We tested this idea by using a mixture of 2,4,6-trihydroxyacetophenone, citric acid, and NH_4OH as a matrix for the selective fragmentation of DNA.

Figure 3 presents an experimental MALDIF spectra of 35-mer oligonucleotide respectively. The match between the experimental spectrum and the simulated spectrum in these data are quite good. The full sequences information of these oligonucleotides were obtained from the experimental MALDI spectra even though a few peaks at high mass range were partially overlapped. Several samples were tested without knowing the sequence before the experiments. The results give the sequence information with 100% accuracy. Thus, we have demonstrated a DNA sequencing method using UV-MALDIF-TOF mass spectrometry. This technology is very valuable for sequencing primers for polymerase chain reaction (PCR) and DNA probes for hybridization detection.

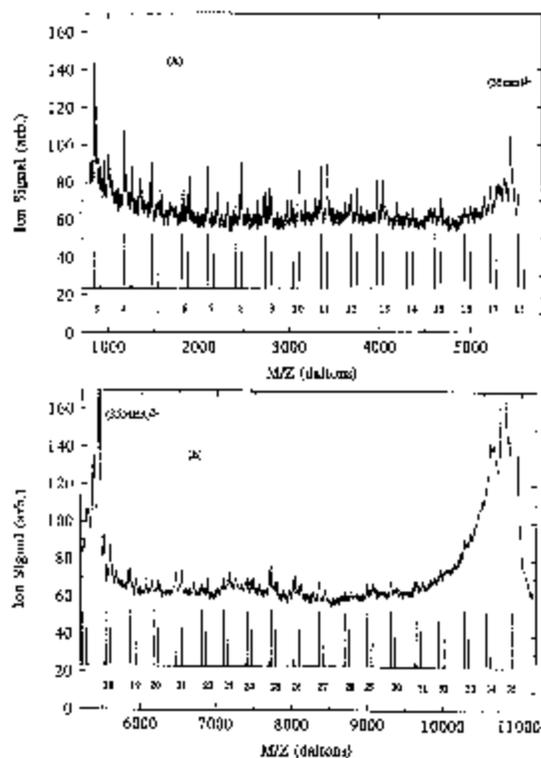


Fig. 3 The mass spectrum of experimental MALDI of a 35-mer, 5'-GCGTGATGGAATCGATGACGTGCGATGTCGTGTTT-3', oligonucleotide using the selective cleavage of 3'- and 5'-linkage method, and its simulated spectrum. The high intensity series in the simulated spectra is the 53*-series, and the low intensity series is 33*-series. A mixture of 2,4,6-trihydroxyacetophenone (1.5×10^5 moles) was used as the matrix.

2. Mass spectrometry of DNA sequencing with DNA ladders.

In addition to the direct sequencing, we also pursued DNA sequencing with Sanger's enzymatic approach and Maxam-Gilbert chemical degradation approach by mass spectrometry. We have successfully sequenced both single (>100nt) and double stranded (>200bp) DNA templates by the standard dideoxy chain termination method as well as by cycle sequencing method.⁴⁹ These results are shown in Figures 4 and 5. It clearly indicates the resolving power of mass spectrometry for sequencing applications. Maxam-Gilbert chemical sequencing is very valuable for confirmation of DNA templates with high GC ratio and secondary structures in most laboratories⁵⁰ and this method is the only method available for genomic sequencing for determination of DNA methylation profiles.⁵¹ Due to the potential to identify base modification for Maxam-Gilbert sequencing coupled with mass spectrometric detection, we also pursued chemical sequencing with mass spectrometry. The schematic for chemical cleavage mass spectrometry sequencing used to determine sequence of short synthetic

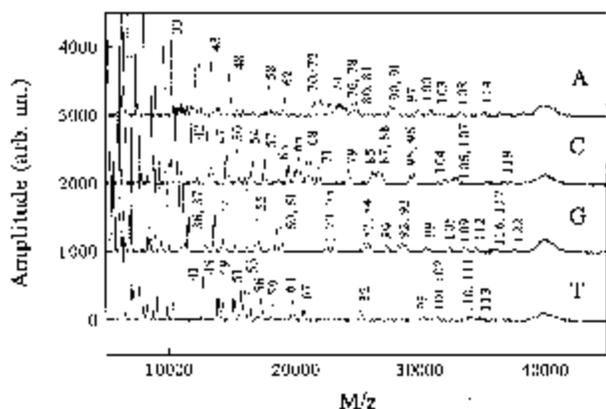


Fig. 4 MS sequencing of ss-DNA of 120 nt using Sanger's method to produce DNA ladders.

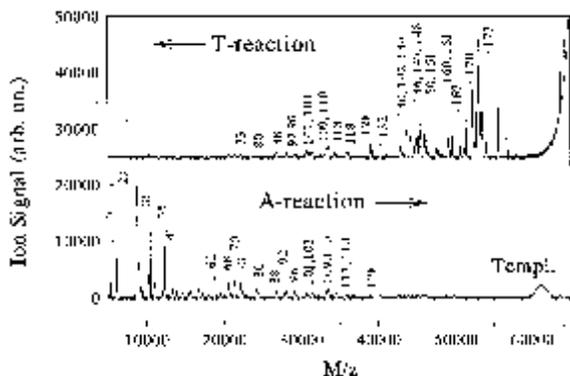


Fig. 5 Mass spectra of Sanger's sequencing ladders for a double-stranded 200-bp template. A reaction with reverse primer and T reaction with forward primer. Thus, complete sequencing can be obtained.

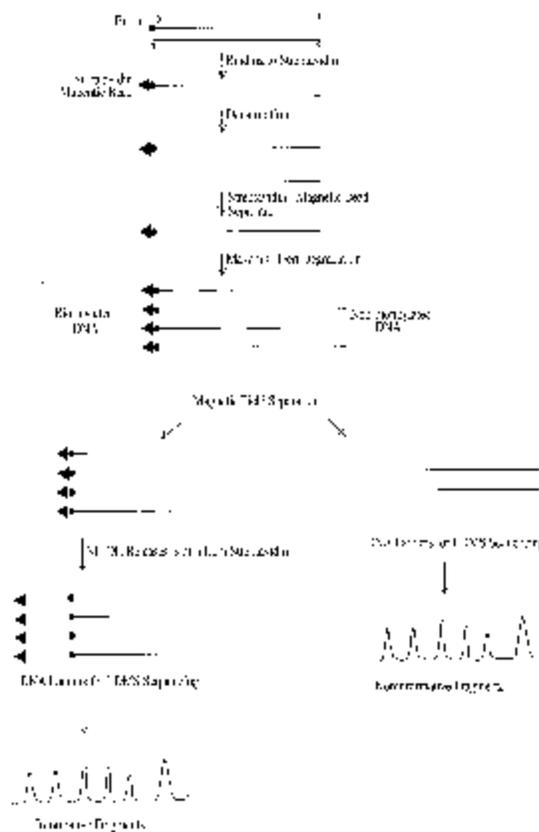


Fig. 6 Scheme for MS with Maxam-Gilbert approach to sequencing DNAs.

oligonucleotides is shown in Fig. 6. With this approach we have demonstrated the feasibility of using this method for sequences containing repeat structures. An oligonucleotide with biotin at the 5' end was subjected to all four chemical cleavage reactions as described by Maxam-Gilbert and the biotin containing fragments were captured by streptavidin coated magnetic beads. After washing off the other fragments, the biotin containing fragments were released from the beads by treatment with hot ammonia and these fragments were measured by MALDI-TOF mass spectrometry. A typical sequencing result is shown in Fig. 7. A second template (50mer) containing GAA repeat structure was analyzed

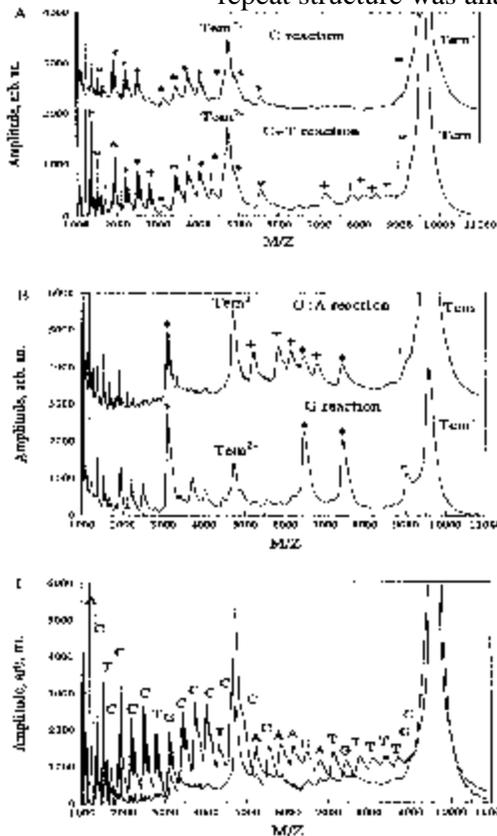


Fig. 7 (A, B) MALDI-TOF spectra of chemical cleavage reaction products captured by streptavidin magnetic beads. * and \cdot represent the determination of C and T, respectively. (C) A composite of the spectra to derive the sequence of the template.

using this method the results of this experiment are shown in figure 8. The sequence could be readily determined from the spectra without any problems comparable to the GC compression in gels⁵².

3. Development of laser induced acoustic desorption for DNA detection.

We take laser-induced acoustic wave for desorption so that matrix is no longer

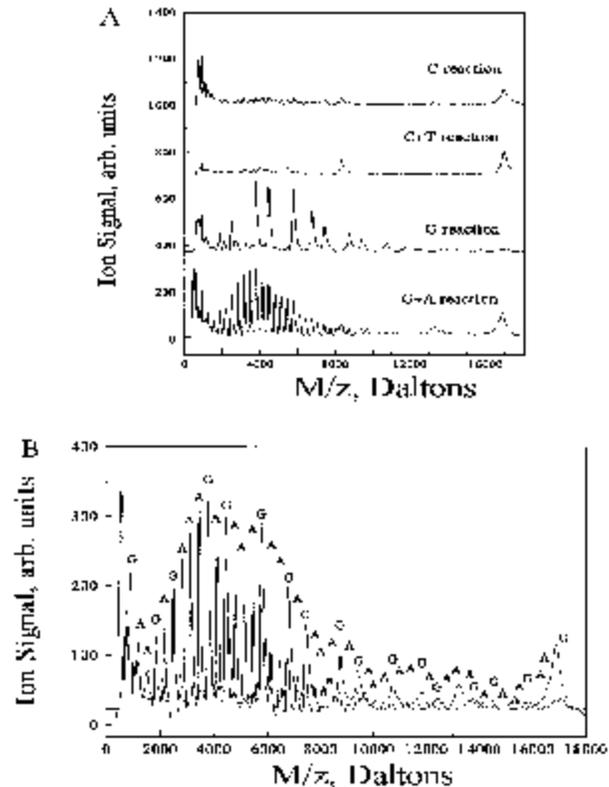


Fig. 8 Chemical cleavage sequencing of 50 mer GAA repeat. (A) Spectra from the individual sequencing reactions. (B) Composite spectrum of the template. The complete sequence 5'-AAGAAGAGAA-AGAGAAAGAA-GAGAAAGAGA-AAGAAGAGAA-AGAGAAAGAA-3' of the repeat was determined.

needed and the laser fluence can be reduced to a minimum for getting better resolution. This technique gives a unique possibility for producing neutral gas phase biomolecules. The method relies on intense photoacoustical "shaking" of a surface containing adsorbed molecules, and soft protonation to obtain biomolecular ions. The intense sound wave is produced from absorption of short laser pulses directed onto a thin substrate from the side opposite to the adsorbed material. The main advantages of this approach when compared with the other laser desorption techniques are (1) There is no direct interaction of desorbed molecules with a laser beam, which could lead to photo-destruction. The probability of dissociation of a desorbed molecule is relatively low because of the absence of strong heating effects; (2) large biomolecules, such as DNA, linked to the surface at many points can be desorbed by simultaneous breaking of all adsorbate-substrate bonds. The absorption of short, intense laser pulses can lead to local heating of the surface and create thermally induced strain. Thus, photoacoustic pulses are generated and

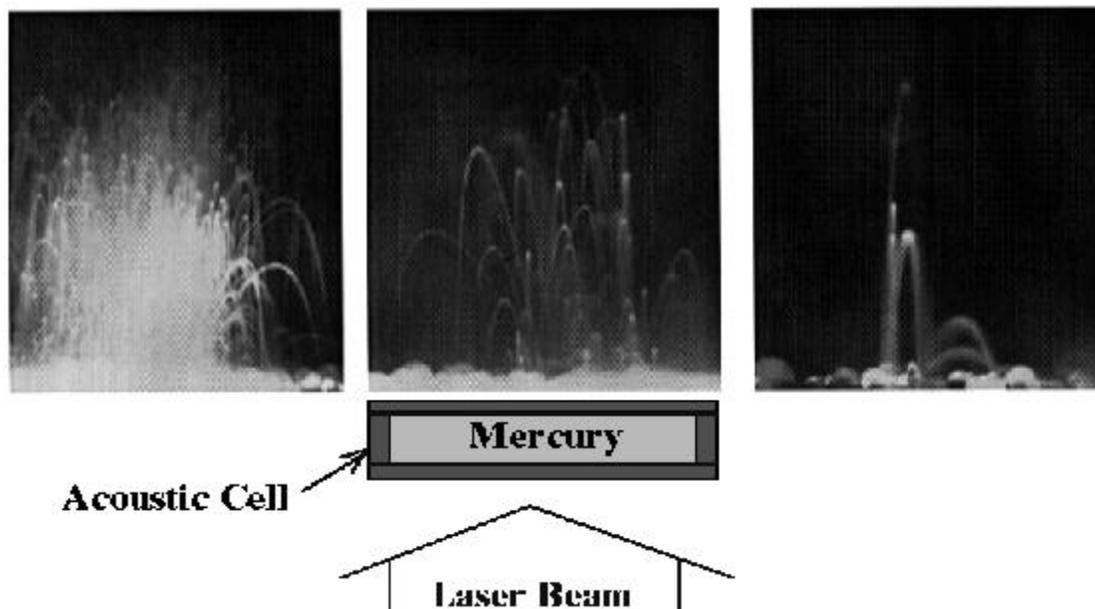


Fig. 9 Laser induced acoustic desorption of Al_2O_3 particles. The quantity of particles desorbed is a function of laser energy per pulse. The number of particles desorbed per laser shot can be as few as one.

propagated in the substrate. The parameters of laser-induced acoustic pulses depend on laser pulse duration and the properties of the substrate materials. When a picosecond laser pulse is used, the photoacoustic pulses can be generated with amplitude in the range of 10^8 or 10^9 Pa. The resulting acoustic pulse formed in the laser irradiated media can propagate into the substrate and reach the opposite boundary. The acoustic pulse/boundary interaction can result in a boundary shift and eventually cause the desorption of large biomolecules from the substrate surface. We tried to test laser induced acoustic desorption on particles with the size of a few microns. Experimental setup is shown in Fig. 9. To generate photoacoustic pulses, we used an acoustic cell formed by two sapphire plates separated by a layer of liquid mercury. The cell was irradiated by a 7ns Nd-Yag laser pulse at $1.064 \mu\text{m}$. Acoustic pulses were generated by the fast heating of the

thin surface layer of mercury near the back side of the acoustic cell. A high thermal expansion coefficient and relatively low thermal conductivity makes mercury especially efficient for generation of high amplitude acoustic pulses as compared with other metals. Since the layer of mercury blocks the laser beam, no detectable amount of laser radiation could be measured on the front surface of the cell. Only acoustic pulses are able to reach the front surface of the cell by passing

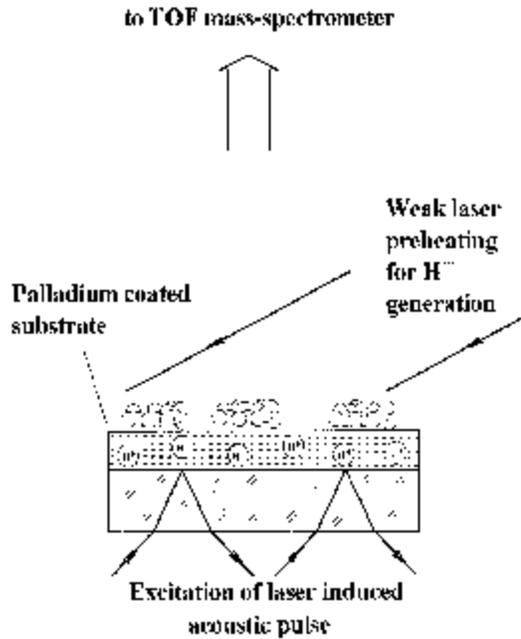


Fig. 10 Schematic for laser-induced acoustic wave desorption to produce biomolecular ions for MS detection.

through the mercury layer and the sapphire window. To observe laser-induced acoustic desorption of heavy particles, Al_2O_3 particles with an average size of $20\ \mu\text{m}$ diameter were deposited on the front surface of the cell. Fig. 9 shows the trajectories of the Al_2O_3 particles desorbed from the top surface of the acoustic cell by a laser-induced acoustic pulse. The trajectory of as low as a single particle can be observed. The trajectories of the desorbed particles were obtained by the imaging of the scattered light of a He-Ne laser beam.

Since laser induced acoustic desorption is expected to have efficient desorption of only neutral molecules, the efficiency for producing ions can be low. A Pd foil which has a large amount of trapped hydrogen can be used as substrate. During the laser-induced acoustic wave desorption, the desorbed biomolecules can be protonated or deprotonated to form ions by the attachment of the desorbed protons. The experimental schematic is shown in Fig. 10. The cell with the front surface covered by 300 nm layer of Pd was installed in the ion acceleration region of a time-of-flight mass

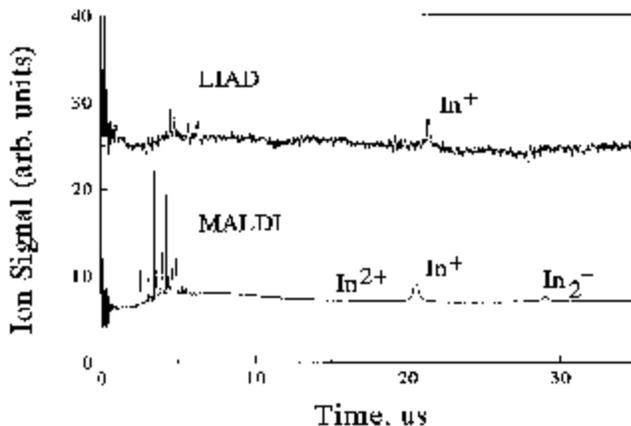


Fig. 11 (A) Mass spectrum of positive ions desorbed by optoacoustic pulse from insulin; (B) MALDI mass spectrum of positive ions from the same type sample but desorbed by a laser beam with wavelength at 266 nm. The In^+ peak represents insulin ions with the $m/e = 5800$.

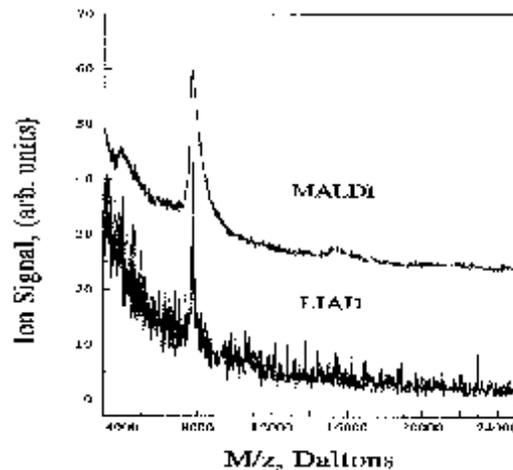


Fig. 12 MALDI and laser induced acoustic desorption of 25 mer DNA. With the same laser fluence, the resolution from LIAD is significantly better.

spectrometer to prevent any leak through of the laser beam. Furthermore, the Pd layer was saturated with hydrogen gas to enhance the possibility of protonation of biomolecules. A Nd-Yag laser was used for producing acoustic wave for desorption. A second laser such as an excimer XeCl laser with an adjustable delay can be used for desorb hydrogen to enhance protonation. However, biomolecular ion were often observed without the need of the second laser for proton desorption. Fig. 11 shows the mass spectrum of insulin using optoacoustic desorption. Similar results from MALDI is also included in Fig. 11 for comparison. The results indicate that the mass resolution by optoacoustic desorption is better than that of MALDI. There are little dimer ions produced. Similar results are also obtained for larger protein such as cytochrome c. When laser induced acoustic desorption is applied to DNA, the mass resolution is again better than that of MALDI. Results are shown in Fig.12. No dimer ions were observed.

In brief, we successfully demonstrated a new approach with optical acoustic desorption for biomolecule detection by mass spectrometry.

4. ASPCR mass spectrometry for mutation detection:

Most DNA mutations due to contaminants are point mutations. Thus, the capability to detect point mutation by mass spectrometry is in critical need. In this program, we succeeded in using MALDI for point mutation detection. The approach is to amplify the desired region of a DNA template by PCR using two primers which have their 3'-ends extend to the site of expected mutation. To test this approach, a synthetic oligo with 60 nucleotides, whose sequence is derived from human p53 mRNA from codon 144 to 163 was used as template⁵³ with two normal allele-specific primers in a PCR. The length of the primers used are 16 and 23 nucleotides. By using a normal template and normal allele-specific primer pair, a normal PCR DNA product will be produced. The size of the expected PCR products should be equal to the sum of both primers minus one. On the other hand, little PCR product will be made if the normal template is replaced with the mutant template, because the 3'-end base of normal primers results a mismatch with mutant template. Since Taq DNA polymerase lacks a 3' exonucleolytic proofreading activity, the mismatch will blocks the amplification during PCR. To test this approach, a synthetic oligo with 60 nucleotides, whose sequence is derived from human p53 mRNA from codon 144 to 163, was used as template with two normal allele-specific primers in a PCR. The length of the primers used are 16 and 23 nucleotides. The sequence of the template and the primers used are:

Primer 1 (23 mer)

5'-CAGCTGTGGGTTGATTCCACACC-3'

Template (60 bp)

5'-
CAGCTGTGGGTTGATTCCACACCCCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTAC-
3'

3'-GGGGCGGCCGTGGGC-5'

Primer 2 (16 mer)

As shown in Fig. 13, the PCR products with the expected size of 38 nucleotides could be clearly observed. On the other hand, no PCR products were detected when the normal templates was replaced with mutant templates. (See Fig. 14) The sequence of the mutant template was 5'-CAGCTGTGGGTTGATTCCACACAACCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTAC-3'. This fragment contains a point mutation at second position of codon 151. This type of mutation is found in certain human lung tumors.

Encouraged by these results, we also measured G551D point mutation⁵³ in cystic fibrosis transmembrane conductance regulator (CFTR)⁵⁴. The G551D mutation in the CFTR gene involves a G to A mutation which results in a Gly to Asp amino acid substitution. The approach is similar to the one for point mutation in the synthetic template simulating a portion of p53 gene. If the two primers based on the normal sequence matching the target DNA sequence, a normal PCR product will be produced. However, if the alternate primers that match the mutant hybridize, a PCR product specific for mutant sequencing will be produced. Thus, the mass spectrometer can be used to identify people that are homozygous normal, heterozygous or homozygous abnormal at a mutation site. A typical result is shown in Fig. 15. A heterozygous template was used. The peaks of 37 and 46 bp

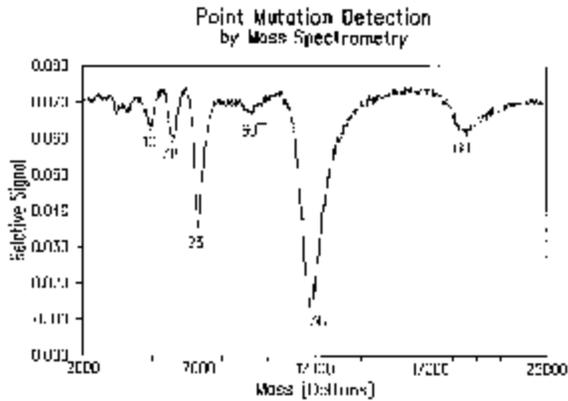


Fig. 13 Negative ion spectrum of 38 bp PCR product to demonstrate point mutations by mass spectrometry.

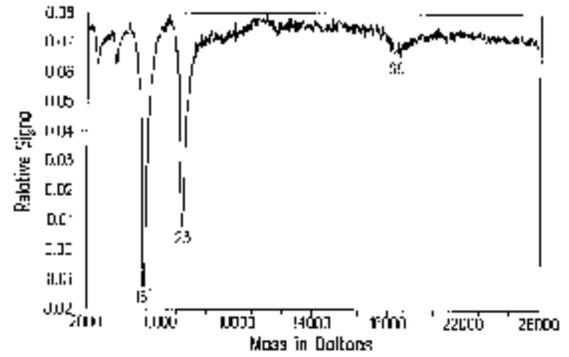


Fig. 14 Negative ion mass spectrum from PCR. No PCR products were produced due to the mismatch of primers and the template.

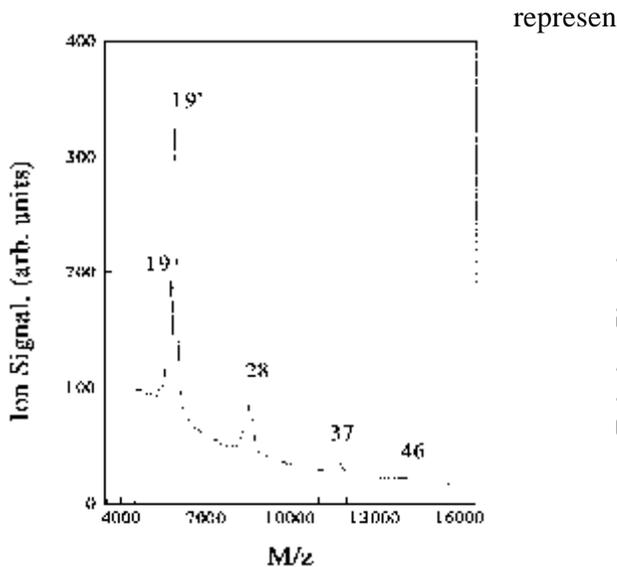


Fig. 15 Negative ion mass spectrum of PCR product in 37 bp and 46 bp (heterozygous template and four primers: two normal primers, 19 mer each, and two mutant primers, 19 and 28 mer).

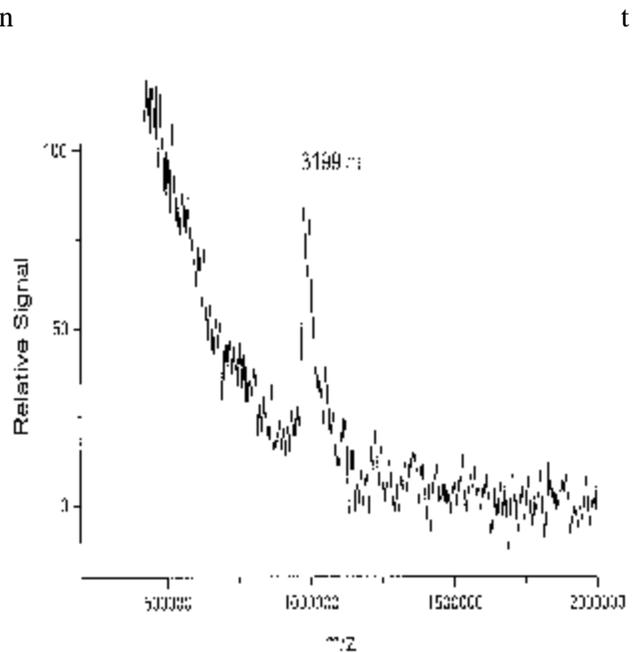


Fig. 16 Mass spectrum of a 3199 bp DNA fragment. Only a single-stranded ion was observed.

the PCR products from both normal and mutant plates. Four primers with different lengths were used in this work. In addition to G551D, we also applied this approach to p53 gene in medaka fish which has been exposed to carcinogenic contaminants. Point mutation was also observed.

5. To improve the detection efficiency for large DNAs:

In 1994, we found that picolinic acid and aminopicolinic acid^{55,56} are two good matrices for DNA. By mixing aminopicolinic acid with 3-hydroxypicolinic acid, we succeeded in detecting DNA segments as large as 500 nucleotides³⁶. This result clarified the concern about the capability of mass spectrometry for large DNA detection. The quantity of DNA in the sample was about 10 femtomole. Recently, we succeeded in detecting DNA segments up to 3199 nucleotides. (See Fig. 16) To our knowledge, these are the largest DNA fragments ever detected by mass spectrometry. These results clearly indicate that MALDI-TOF method for DNA analysis could easily complement the existing methodology, such as gel electrophoresis in resolving ambiguities.

6. Determination of mutations in tumor of targeted fish:

There are many different human cancers which are associated with the mutation in p53 anti-tumor gene. Since it is widely believed that p53 is highly conserved among different animals, our approach is to check the mutation of these tumor suppressing genes due to contaminants. If the mutation does occur to fish

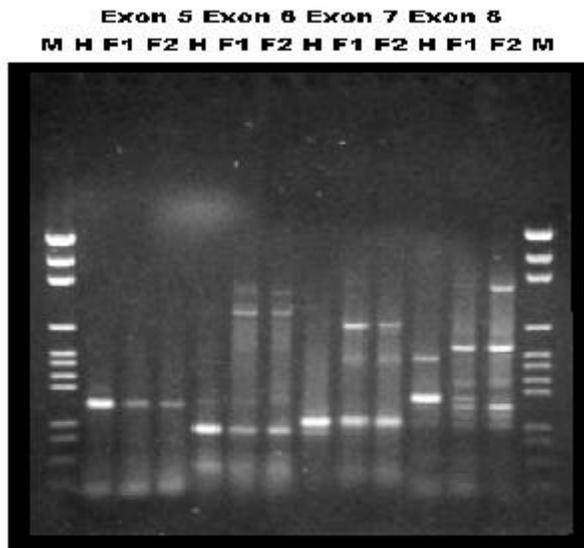


Fig. 17 Gel electrophoresis of PCR products: Primers designed from human p53 for Exons 5,6,7 and 8 were used to amplify the specific region in Human (H) and Fish (F1,F2) genomic DNAs. Single PCR product from the fish DNA samples (in Exon 5 and 6) of same size as expected from the human (H) indicates that the p53 exon of the fish is being amplified.

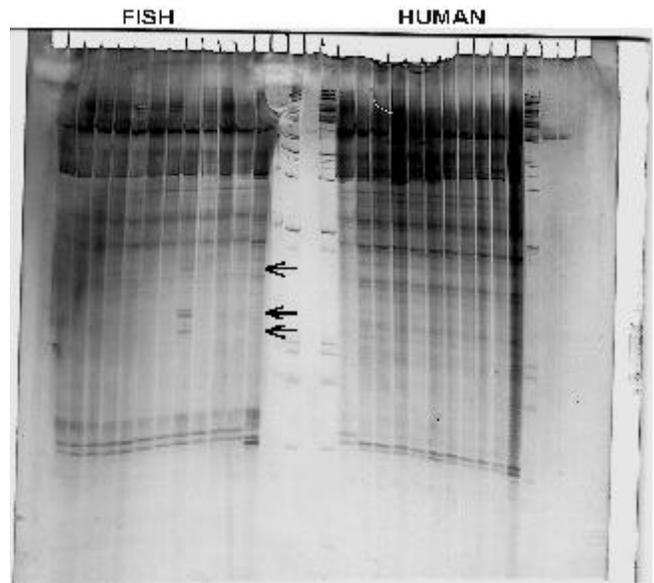


Fig. 18 P53 Exon 5 amplicons were subjected to denaturation and renaturation and treated with T4 endonuclease. The reaction products run on 6% denaturing polyacrylamide gel (sequencing gel) and the products detected by silver staining. Left panel shows 12 different fish samples and right panel shows 11 human samples. Note fragments of lower molecular weight (arrows) indicating the presence of mismatch in one of the fish DNAs.

which happens to have the same sequence as human. We can conclude that this specific contaminant is carcinogenic and should be in high priority to be cleanup. P 53 from Medaka fish has been cloned and cDNA sequence has been determined. It was found to have 42% sequence homology with human cDNA. In this work, we tried to compare exon 5~8 in p53 gene for both human and fish. Primers designed to amplify four different exons of human p53 were used to see if the fish p53 region can be amplified as well. Fig. 17 shows the results for two different fish DNA samples. The fact that only one PCR product and of the same size of human DNA definitely indicates that fish p53 corresponding to that exon is being amplified. Exon 5 and 6 were chosen for further work from these results and a panel of 12 different fish and human DNAs were used. All samples generated the expected size amplification product. That concludes no major deletion or insertion occurred in that region. These results were also confirmed by laser desorption mass spectrometry. We further checked with sequencing gel and confirmed that the sequences among these exons are same. Results are shown in Fig. 18. We also noted that there is a clear mutation for one of the fish sample (See Fig. 18).

7. To achieve sample automation system for rapid DNA analysis:

We have made the sample holder to be able to hold 100 samples a time. The sample holder's position and movement can be well controlled by the computer. The laser energy can also be controlled by the computer and the laser fluence can be measured and recorded by a laser power meter.

In summary, we have achieved most tasks stated in the proposals. 8 papers in referred journals were either published or accepted to be published. One article was published in Encyclopedia of Analytical Chemistry.

5. Relevance, Impact and Technology Transfer:

1. How does this new scientific knowledge focus on critical DOE environmental management problems?

The knowledge obtained in this program can help to develop new risk assessment technologies to determine the priorities of waste sites.

2. How will the new scientific knowledge that is generated by this project improve technologies and cleanup approaches to significantly reduce costs, schedules and risks and meet DOE compliance requirements?

The scientific knowledge obtained can be used to determine the degree of cleanup is required for each waste site so that cost and time can be significantly reduced and still meet DOE compliance.

3. To what extent does the new scientific knowledge bridge the gap of broad fundamental research that has wide-ranging applications as timeliness to meet needs-driven applied technology development?

Since the new technology can help to determine the degree of contaminant of each waste site, it bring the cutting edge of DNA technology to help the risk analysis of each waste site.

4. What is the project's impact on individuals, laboratories, department, institutions? Will results be used? If so, how will they be used, and when?

The project will have a positive impact on future DNA analysis technology which is important for sound risk analysis for laboratory, department and institutions. The technology will be used for assessment of contaminant impact on mutations which may eventually lead to cancers or tumors.

5. Are larger scale trial warranted? What difference has the project now that the project is complete, what new capacity, equipment expertise has been developed?

Yes. It can help to get better decision on the priorities of cleanup on each individual waste site.

6. How have the scientific capabilities of collaborating scientists be improved?

By more meetings and communication by internet.

7. How has this research advanced our understanding in the area?

By studying the impact on mutation by contaminant can enhance the understanding the mechanism of cancer development due to the environmental impacts.

8. What additional scientific or other hurdles must be overcome before results of this project can be successfully applied to DOE Environmental Management problems?

DNA detection sensitivity needs to be increased to be able to quickly assess the very minority of mutation due to the environmental impact. The technology needs to be simpler to be used for field analysis.

9. Have any other government agencies or private enterprises express interest in the project? Please provide contact information.

Since the project is primarily for helping risk assessment, this question doesn't apply to this project.

6. Project Productivity: We accomplish all major goals described in the projects.

7. Personnel Supported:

Chung-Hsuan Chen	Principal Investigator
Kai-Lin Lee	Co-PI
Steve L. Allman	Research Staff at ORNL
Valeri V Golovlev	Postdoctor
N. I. Taranenko	Postdoctor
N. R. Isola	Postdoctor
Richard Hurt	Postdoctor
Lauri Sammartano	Visiting Professor from St. Olaf University
John Kirk	undergraduate student
Bob Gonzales	undergraduate student

8. Publications:

A. Peer reviewed Journals:

1. V. V. Golovlev, S. L. Allman, W. R. Garrett, N. I. Taranenko and C. H. Chen, "Laser induced acoustic desorption," *International J. Mass. Spectrom. & Ion Proc.* 169, 69-78 (1997)
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4. N. I. Taranenko, S. L. Allman, V. V. Golovlev, N. V. Taranenko, N. R. Isola and C. H. Chen, "Sequencing DNA using mass spectrometry for ladder detection," *Nucleic Acid Research*, 26, 2488-2490 (1998)
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C. Proceedings:

- 1) C. H. Chen, V. V. Golovlev, N. I. Taranenko, S. L. Allman, N. R. Isola, N. T. Potter, K. Matteson and L. Y. Chang, "Laser desorption mass spectrometry for high throughput DNA analysis and its applications", *Proc. Spie-Int. Soc. Opt. Eng.* 1999, 3602, (Advances in Fluorescence Sensing Technology IV), 338-345 San Jose, California, January 24-27, 1999

C. Papers submitted:

- (1) N. R. Isola, S. L. Allman, V. V. Golovlev and C. H. Chen, "MALDI-TOF Mass Spectrometric Method for Detection of Hybridized DNA Oligomers", *Anal. Chem.* (In Press)
- (2) N. R. Isola, Z. Liu, S. L. Allman, N. I. Taranenko, Y. Kong and C. H. Chen, "Rapid MALDI-TOF Mass Spectrometric Method for Detection of Specific Polymerase Chain Reaction Products by Utilizing the 5'-3' Exonuclease Activity of *Thermus aquaticus* DNA polymerase", *Anal. Chem.* (Submitted)

10. Interactions:

A. Meeting presentations:

- (1) C. H. Chen. "Novel Mass Spectrometry Mutation Screening for Contaminant Impact Analysis" EMSP Workshop Chicago, Illinois July 27-30, 1998
- (2) C. H. Chen, "Novel Mass Spectrometry Mutation Screening for Contaminant Impact Analysis", EMSP workshop, Atlanta, GA, Apr. 24-28, 2000
- (3) C. H. Chen, N. I. Taranenko, V. V. Golovlev and S. L. Allman, "Laser Desorption Mass Spectrometry for Rapid Nucleic Acid Analysis", American Physical Society Centennial Meeting Atlanta, GA, March 20-26, 1999 (invited)
- (4) C. H. Chen, "Laser Desorption Mass Spectrometry for Microbial DNA Analysis", International Symposium on the Interface between Analytical Chemistry and Microbiology, Tregastel France, June 4-7, 2000 (invited)

B. Collaboration: Gulf coast laboratory

11. Transitions: NA

12 Patents:

The following invention disclosures were submitted.

- (1) Mass spectrometric multiplexing hybridization detection.
- (2) Laser induced acoustic desorption
- (3) High throughput method of analysis of macromolecular interactions

13. Future Work:

To develop a field use DNA technology with better detection sensitivity.

14. Literature cited:

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15. Feedback:

Since this is a long term environmental science program, the project time should not be limited to just 3 years.