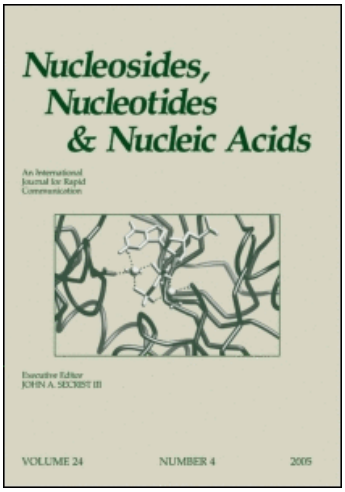

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MASS SPECTROMETRIC DNA DIAGNOSTICS

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ABSTRACT: Mass spectrometry was employed to analyze gene expression of cancer cells by RT-PCR, to determine polymorphisms within short tandem repeat regions of the human genome and to derive sequence information from diagnostic PCR products by solid-phase Sanger sequencing.

In recent years mass spectrometry (MS) has been integrated in the analytical repertoire of routine molecular biology procedures. Based on the introduction of matrix-assisted laser desorption / ionization time-of-flight (MALDI-TOF) mass spectrometry (1) approaches have been proposed for efficient verification of mutations (2, 3) and polymorphisms (4, 5) by mass spectrometry, for the detection of PCR products (6, 7, 8) and for full comparative sequence analysis (9).

Here, three formats of DNA diagnostics by mass spectrometry are presented: the direct sizing of PCR products, sufficient in many diagnostic applications, the elucidation of microsatellite repeat length by PROBE (PRimer Oligo Base Extension) and finally solid-phase Sanger sequencing for comparative sequence analysis.

Methods for rapid and specific detection of even small numbers of cancer cells in peripheral blood or healthy tissue are highly desirable for cancer diagnosis, prognosis and monitoring. One of these diseases is neuroblastoma, an extracranial solid tumor of

childhood. Approximately 15% of all cancer deaths in childhood are caused by neuroblastoma (10) and about 75% of all patients have metastatic disease at diagnosis (11). Methods have been described using the mRNA of tyrosine 3-hydroxylase (E.C. 1.14.16.2, TH) as a tissue-specific target in reverse transcription polymerase chain reaction (RT-PCR) assays (12, 13, 14). Commonly, the detection protocols include blotting of gels, hybridization with labeled probes and autoradiography.

We developed a mass spectrometric duplex RT-PCR assay which provides a high sensitive and specific detection of TH expression combined with an internal positive control for reverse transcription / amplification. The assay is based on the use of two primer pairs during PCR, one pair specific for β-actin, the other for tyrosine hydroxylase. The forward primer of each pair is biotinylated to enable immobilization on streptavidin coated solid supports. Primer binding sites are located in different exons of the respective genes to prevent amplification of genomic DNA (15) and the primers were designed to ensure the detection of known splice variants (16). Nonbiotinylated primers were used in excess to ensure optimal immobilization conditions (17). For MS analysis the PCR products were recovered from streptavidin Dynabeads using ammonium hydroxide, as described (18).

Figure 1 depicts mass spectra of duplex RT-PCRs performed with a neuroblastoma (Fig 1A) and a non-neuroblastoma reference cell line (Fig 1B). In Figure 1A, the detected mass at 17486 Da corresponds to the TH specific PCR product (calculated: 17486.4 Da) and the β-actin specific product is detected at 14493 Da (calculated: 14477.6 Da, mass error: 0.1%). Figure 1B demonstrates that no TH but only β-actin expression is detected in the reference cell line (signal at 14490 Da). The expression of β-actin, detected in both cell lines, serves as a positive control for cDNA synthesis, amplification, immobilization and PCR product recovery.

The primer oligo base extension assay (PROBE) combined with subsequent analysis of the products by mass spectrometry was originally introduced as a new method for identification of single-point mutations and small deletions / insertions in PCR amplified DNA (4, 5). It has now been applied to the analysis of nucleotide repeat units within short tandem repeat regions of the human genome (19). Figure 2 shows a schematic outline for the sizing of CA repeat units within a microsatellite region by PROBE. Amplification of the respective microsatellite region using a biotinylated primer has to be carried out prior

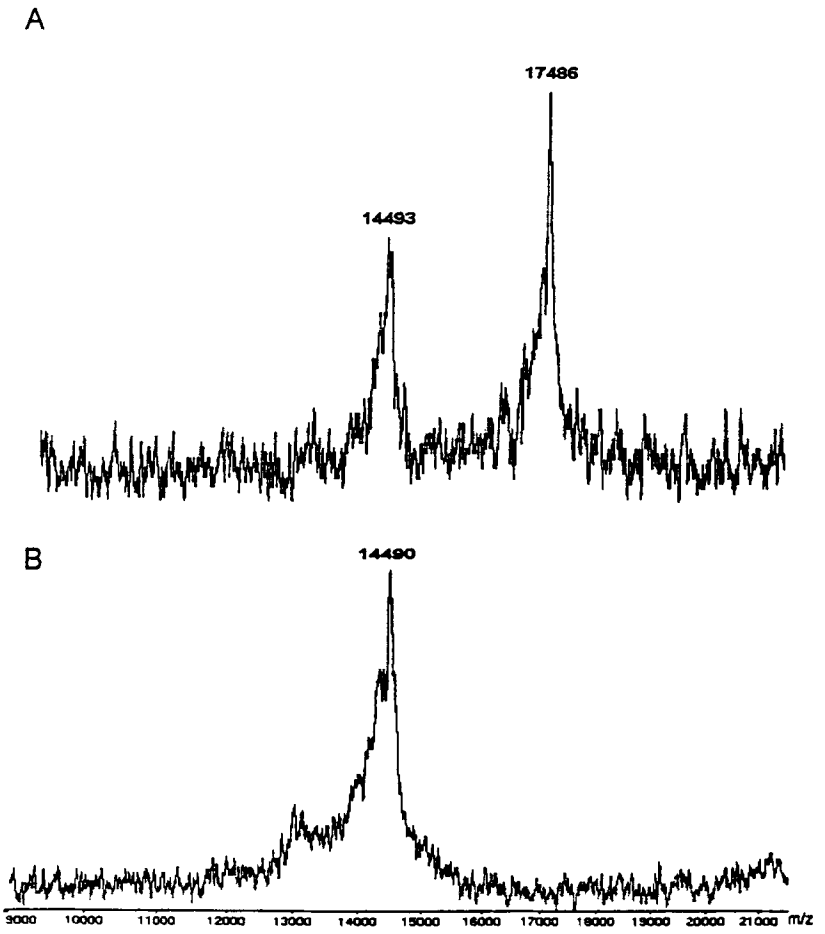

FIG. 1.: MALDI-TOF mass spectra of TH duplex RT-PCR performed with a neuroblastoma cell line (A) and a non-neuroblastoma reference cell line (B).

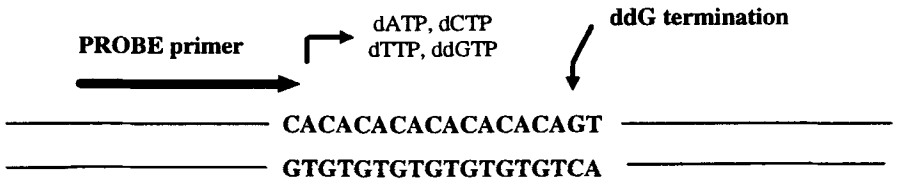

FIG. 2.: Schematic outline of a PROBE reaction for CA repeat microsatellite analysis.

to the PROBE assay. Following immobilization and denaturation, a PROBE primer is annealed flanking the 5´end of the repeat region. This primer is extended through the repeat by a DNA polymerase using a mixture of three deoxynucleotide triphosphates and at least one dideoxy-NTP.

In contrast to standard Sanger sequencing, this reaction leads to only one termination product (two in the case of heterozygosity), in which length is determined by the sequence between the primer binding site and the first possible termination event. The length, represented unambiguously by the molecular weigth signal of extension products, directly reflects the number of nucleotide repeat units when analyzed with MALDI-TOF mass spectrometry and therefore allows fast and accurate sizing of microsatellite regions for the purpose of genetic linkage analysis. Using different sets of dNTP / ddNTP-mixtures, such an assay even enables the identification of second site mutations within the repeat (19).

In the examples given in Figure 3, the PROBE format was applied to the sizing of a CA repeat microsatellite region on the short arm of chromosome 5. The status of the analyzed polymorphic microsatellite (D5S432) seems to be associated with the development of uterine cervical cancer (20).

An example of allelic homozygosity for the analyzed region is depicted in Figure 3A. The PROBE primer (calculated mass: 6458.2 Da) is fully extended to the first G following the CA repeat. The mass signal at 12792.6 Da represents this ddG terminated extension product and reflects a repeat length of 11 CA dinucleotides. A „stutter product", which is usually caused by polymerase slippage during amplification of repeat regions (21), is assigned at 12192.8 Da. The peak at 6398.8 Da represents the doubly charged molecular ion of the main extension product. In Figure 3B an example is given, where two main extension products were detected, indicating heterozygosity. The mass signals at 12798.2 Da and 13402.6 Da correspond to a repeat length of 11 CA dinucleotides for the shorter and 12 for the longer allele. As already described for the spectrum depicted in Figure 3A, a „stutter product" is assigned at 12191.3 Da and the doubly charged molecular ions are assigned at 6405.2 Da and 6705.2 Da, respectively.

The mass spectrometric analysis of DNA sequencing ladders generated by conventional Sanger sequencing still plays a major role in the efforts to establish MALDI-TOF MS as the method of choice for DNA sequence verification and mutation detection. Whereas the achievable read length is still far away from that of gel electrophoretic

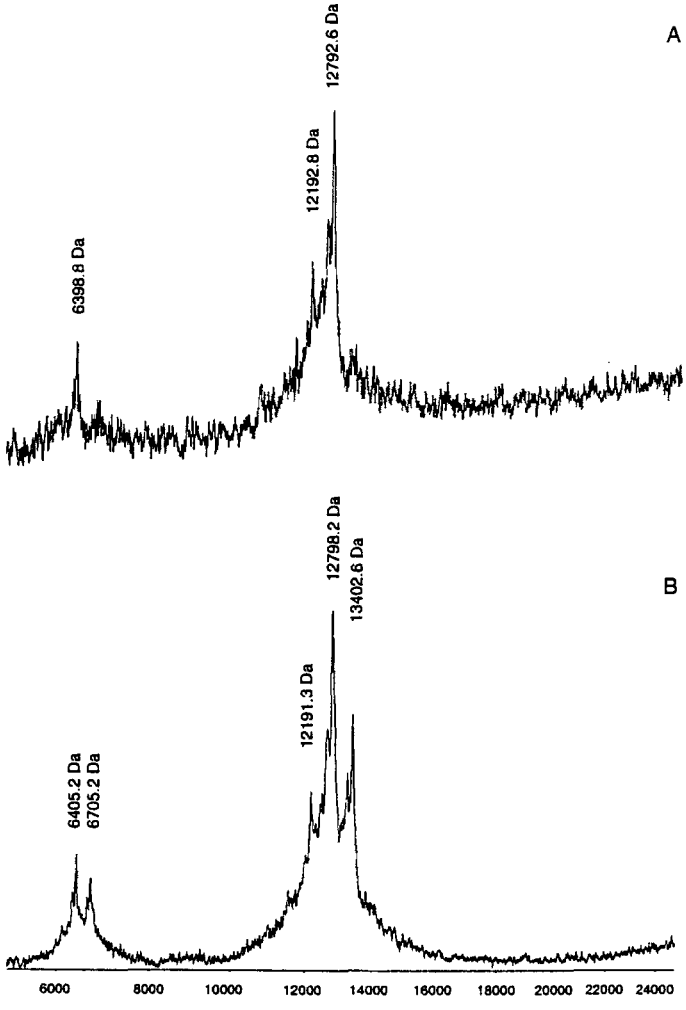

FIG. 3.: MALDI-TOF MS analysis of a PROBE reaction carried out to determine the CA repeat length in a microsatellite region. Figure 3A shows a mass signal at 12792.6 Da corresponding to a repeat length of 11 CA dinucleotides. Figure 3B shows a case of heterozygosity for the analyzed region, with mass signals at 13402.6 Da and 12798.2 Da corresponding to a repeat length of 11 and 12 CA dinucleotides.

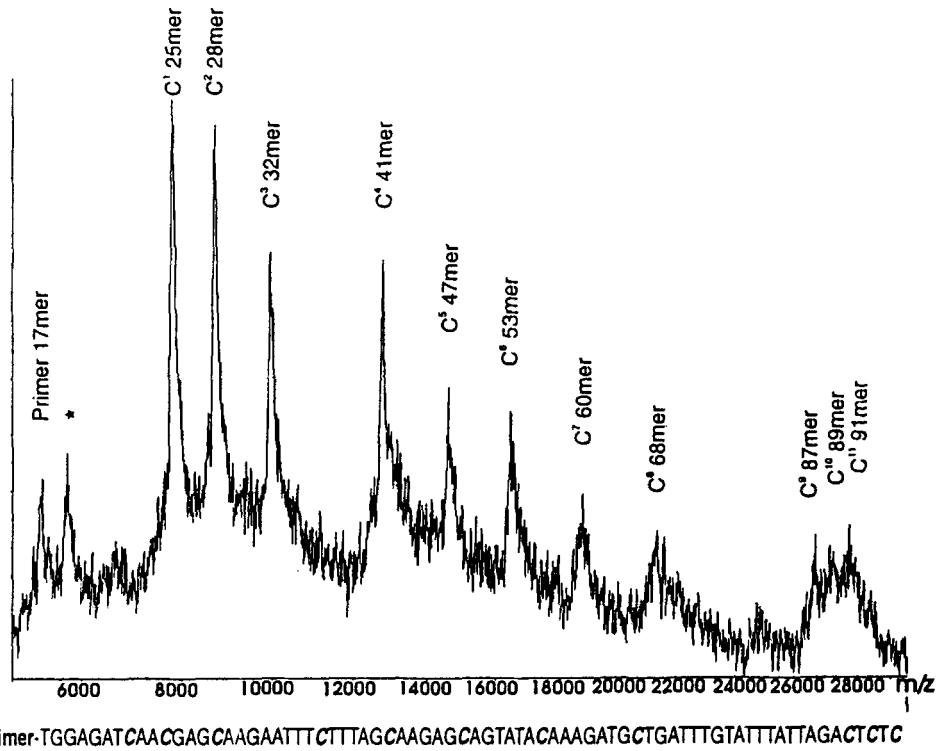

FIG. 4.: MALDI-TOF mass spectrum (raw data) of a ddC sequencing ladder generated by solid phase Sanger sequencing of an immobilized PCR product.

methods, mass spectrometry is not hampered by problems involved in gel based analyses of sequencing products, like band compressions and polymerase pauses. Using the product mass as the most informative signal, full stop events caused by secondary structures can easily be resolved (9). Therefore a mass spectrometric approach for fast and accurate DNA analysis in comparative or diagnostic DNA sequencing is of major interest.

An example how MALDI-TOF MS could be used for diagnostic sequencing is given in Figure 4. A PCR product of 207 bases was amplified from exon 11 of the human CFTR gene using a biotinylated and a non-biotinylated primer flanking the G551D mutation. Following streptavidin mediated immobilization, a Sanger ddC ladder was generated in a solid-phase sequencing reaction using an internal non-biotinylated sequencing primer, as described (22). After denaturation and precipitiation, the products were analyzed by DE

MALDI-TOF MS. The termination products are assigned up to a length of 91 bases including the 17mer primer. The effective read length of about 74 bases is sufficient for most applications in comparative DNA sequencing, since single base differences or deletions / insertions can easily be identified when a reference sequence is provided.

To achieve all the benefits of mass spectrometry's speed and accuracy, automated parallel sample processing combined with the use of miniaturized array formats is essential. Important steps towards miniaturized arrays were made recently by dispensing the products of a diagnostic assay onto a silicon chip using a nanoliter device (23). The low-volume of such samples enhances the reproducibility of crystallization and therefore significantly improves automatic data acquisition. The inception of chip-based technologies (24) amenable to arrayed biological reactions and suitable for direct use as sample holders, will lead to a complete mass spectrometric system for comparative DNA analysis (25).

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