Diagnostic Mutation Screening via homogeneous MassCLEAVE™ Method

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Discovery-RT is a comparative sequence analysis tool based on matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of nucleic acids cleaved at specific bases. Using the speed and accuracy of the system, this innovative method opens new routes for high-throughput discovery and localization of single nucleotide polymorphisms (SNPs).

SNP Discovery is focused on the determination of (unknown) single base pair substitutions and single base insertions or deletions. The automatically generated results are displayed as the summary of sequences changes in the analyzed sample set along the target sequence. In contrast, diagnostic mutation screening involves the detection of multiple sequence variation events in small regions, including long insertion and deletions. Also the accurate genotype of each single sample has to be identified. If such complex changes occur in the target sequence, the consequences for the resulting mass spectra are nearly the same as in SNP Discovery applications. Detected additional signals indicate a sequence variation. However the correct explanation of these additional signals in the context of complex and multiple base exchanges requires advanced mathematical approaches, because the number of theoretically possible combinations increases tremendously. The occurrence of missing peaks is normally not expected for this type of application.

Most of the pathogenic mutations are already ‘active‘ when only one allele is affected. Then, the wild type signal is still generated from the ‘normal‘ allele. Obtained mass spectra display additional signals reliably and reproducibly. That way MassCLEAVE™ method is able to ‘flag‘ complex sequence changes and select affected sample(s) for independent confirmation, e.g. by sequencing. In addition, the possibility to detected synonymous SNPs allows the immediate discrimination of pathogenic from non-pathogenic single base sequence changes. That will reduce the cost intensive and time consuming post-screening workload.

Technology

The MassCLEAVE™ method concept[1] relies on 4 independent base-specific cleavage reactions, which will be analyzed by MALDI-TOF MS. The genomic region of interest will be PCR amplified in two separate reactions. One reaction supports the analysis of the forward strand, whereas the second PCR aims the investigation of the reverse strand. The appropriate primer is tagged with T7 RNA polymerase promoter sequence in each PCR. The double stranded PCR product is transcribed into single stranded RNA in 4 independent reactions. Each transcript is then base specifically cleaved with RNase A. Base-specificity is achieved by selective protection of one of the native substrate bases, C and U, with modified nucleotides. These nucleotides are incorporated during transcription with a genetically engineered T7 RNA polymerase. The analysis of forward and reverse strand enables one to achieve base-specific cleavage virtually at each base. The reaction scheme is outlined in figure 1 below.

Each cleavage reaction generates a set of well-defined and unique fragments representing the target

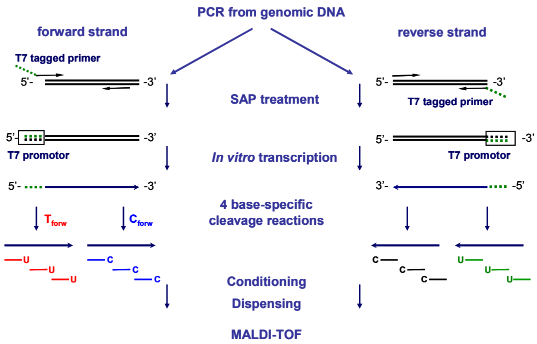

Figure 1

sequence. MALDI-TOF MS analysis of the cleavage products delivers a specific and target-identifying peak pattern in the mass spectrum. This experimental pattern is compared to an *in silico* pattern calculated from the reference sequence. Detected pattern differences, like additional and/or missing signals, indicate the presence of sequence variations in the investigated sample.

A heterozygous sequence change can generate up to 5 discriminatory observations in the 4 mass spectra by adding or removing a cleavage site as well as shift the mass of a single product by the mass difference of the exchanged nucleotide. Figure 2 shows a practical example, which illustrates the impact of sequence changes on the expected fragments and masses.

MEN2 Diagnostics - Practical Application for MassCLEAVE analysis

Multiple endocrine neoplasia, type IIA is an autosomal dominant syndrome of multiple endocrine neoplasms, including medullary thyroid carcinoma, pheochromocytoma, and parathyroid adenomas. MEN2A results from mutations in the RET protooncogene, one of the receptor tyrosine kinases, which are cell-surface molecules that transduce signals for cell growth and differentiation.

Figure 2 below shows a sequence portion of exon 10 of the **RET proto-oncogene** One of the described and characterized mutations in that region, TGC > CGC in codon 620 is indicated. This particular mutation leads to the occurrence of a new cleavage site or the removal of a cleavage site in comparison with the reference sequence when the forward strand is analyzed. The appearance in the reverse strand is associated with a mass shift of the mutation identifying signal compared to the reference pattern. The impact of the mutation on the observed fragment(s) and its mass(es) is demonstrated for each of the 4 performed cleavage reactions.

Note: For all graph illustrations in this document, the reference sequence spectrum is indicated in blue, and the spectrum affected by SNP is indicated in red.

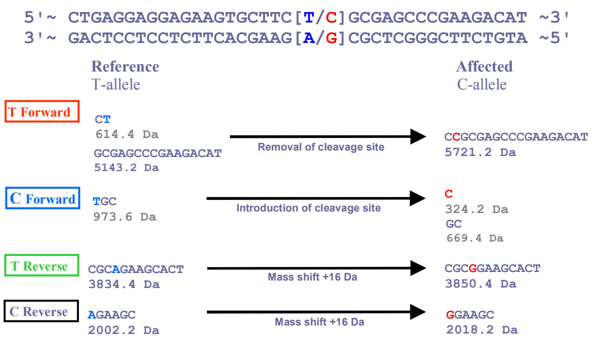

Figure 2

Forward Reactions

T-specific forward reaction. Cleavage occurs after every T in this reaction. The present T to C mutation on the forward strand removes a cleavage site. In result two fragments will be combined to one new, single fragment with higher mass. This fragment is observed as a new signal in the spectrum of a mutation affected sample, as illustrated in figure 3.

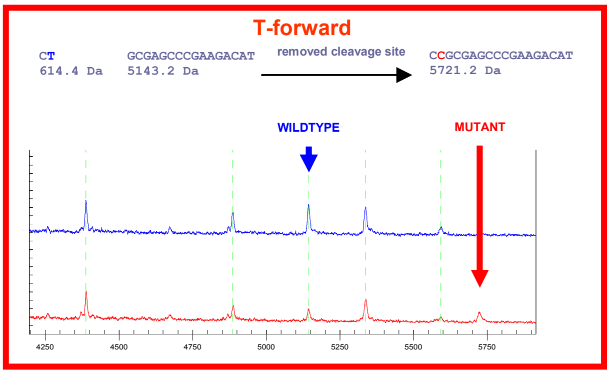

Figure 3

C-specific forward reaction: Cleavage occurs after every C in this reaction. The T to C mutation on the forward strand adds a cleavage site, which cuts a single fragment into two fragments of lower mass. The lower mass fragments generated in the mutation neighborhood are below mass cut off and non-informative. There is no detectable pattern change in the acquired mass spectra between normal and affected sample (figure 4).

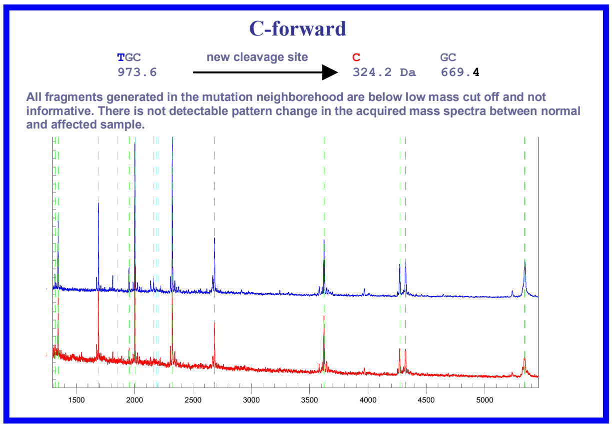

Figure 4

Note: The T to C mutation on the forward strand corresponds to an A to G mutation on the reverse strand

T-specific reverse reaction. For the example Mutation at cd620, the spectrum shows one new signal. This observation of an +16 Da mass shift suggest an A to G transition (figure 5).

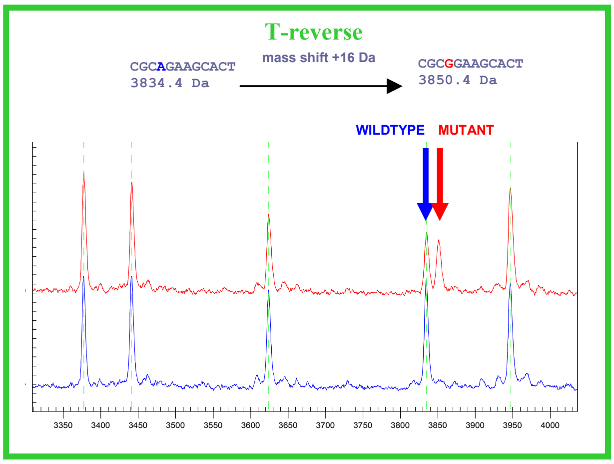

Figure 5

C-specific reverse reaction: The A to G change on the reverse strand generates a new single signal that indicates a fragment with higher mass. This expected pattern change between wild type and mutant sequence is again observed in the corresponding spectra (figure 6).

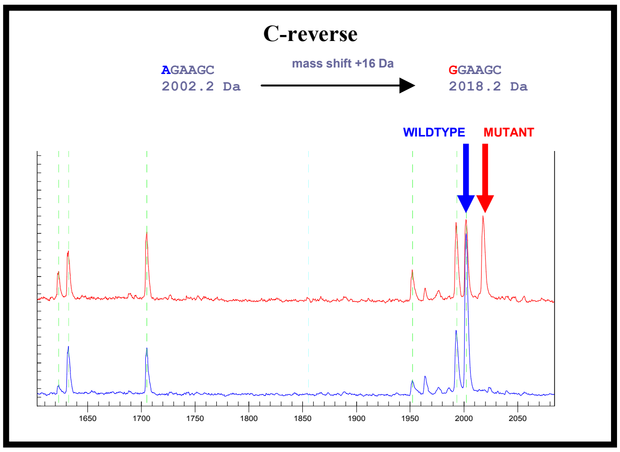

Figure 6

Summary:

The observations from the four cleavage reactions are compiled. Results of the 4 cleavage reactions and the corresponding spectra confirm each other. That allows a solid conclusion on the presence or absence of the investigated T to C exchange at codon 620 in exon 10 of the human RET proto-oncogene.

Conclusion:

SNP discovery combines automated SNP identification and localization with the speed and accuracy of the MassARRAY™ system. Currently the system can process up to 3 million base pairs per day. Even with a limited sample set, SNP Discovery is able to detect known and previously unknown SNPs. SNP Discovery provides the following benefits.

- Reliable detection of low frequent variations
- High accuracy and reproducibility
- detection of sequence changes at bases immediately adjacent to the PCR primer sites
- Scalable throughput on an established MassARRAY™ system

References

[1] Sanssens et al.: High-throughput MALDI-TOF discovery of genomic sequence polymorphisms. Genome Res. 42 (2004), 339-346

[2] Ehrich, Corell: SEQUENOM Application Notes; SNP Discovery Using the MassARRAY™ System

[3] Storm, N., Darnhofer-Demar, B., van den Boom, D., and Rodi, C.P. (2002) MALDI-TOF Mass Spectrometry-Based SNP Genotyping. Methods in Molecular Biology. 212:241-262.

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