Overview on ongoing Evaluation of Melting Curve Analysis (MCA) by EuroGentest

Introduction

High resolution melting curve analysis (HR MCA) is a fast and post-PCR high-throughput method to scan for sequence variations in a target gene. Single-base changes in the target amplicons are detected by their altered melting-properties which is monitored through the release of fluorescent dsDNA binding dye These altered melting properties give rise to changes in the shape of the melting curve compared to a reference sample. The plots on the right depict an example of different sequence variants identified in one amplicon using the LightScanner™ instrument and the LCGreen® Plus+ Dye (Idaho Technology). The upper curve shows normalized MCA data as fluorescent signal versus increasing temperature and the plot below depicts the difference curve. The LightScanner is a Hi-Res Melting™ system for mutation scanning using 96-well or 384-well plates and can be programmed to melt samples on an entire plate in 5 to 15 minutes (T depended http://www.idahotech.com/LightScanner/).
Preliminary data indicate that HR-MCA could be an appropriate method to pre-scan genes in order to rule out amplicons that do not have sequence variants. This would severely reduce the amount of DNA sequencing. Moreover, with the use of unlabeled probes Hi-Res Melting can also classify selected common or pathogenic sequence variants, avoiding recurrent sequencing of frequent occurring polymorphisms.

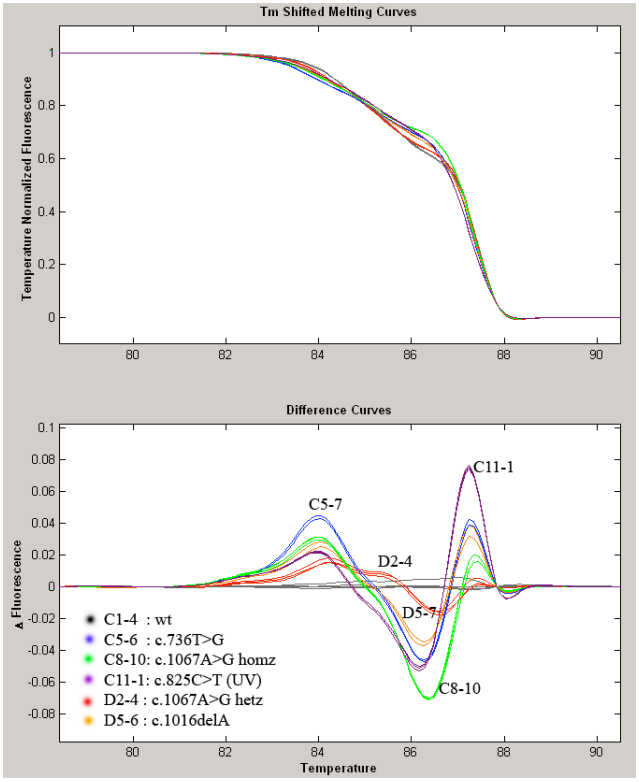

Fig. 1. Normalized melting curve results of one BRCA1 amplicon revealing different mutations (indicated). Upper plot, temperature (T) versus fluorescence (F), bottom difference curve dF/T.

Aims

The general aim of all our evaluation studies is to improve the international exchange of information on validation and implementation studies in genetic testing and to generate clear guidelines, validation files and standard operating procedures (SOPs) for genetic tests. This way we can limit the duplication of efforts and reduce the costs for the introduction of new techniques into the genetic services.
At our Unit 5 Satellite meeting on 'Innovative Techniques in Genome Diagnostics', which was held at the ESHG in Amsterdam, Deepika de Silva of Idaho Technology and Claire Taylor of The Cancer Research UK showed a nice overview of the capabilities of the HR-MCA technique. As a follow up to this meeting, Unit 5 of EuroGentest has initiated an evaluation study to test this new technique in a diagnostic setting. All evaluation experiments are carried out in fully accredited laboratories of our network of Excellence and are performed with selected clinical samples. These studies will test the sensitivity, specificity and robustness of MCA by performing scanning experiments on a large panel of clinical DNA samples with known sequence variants and by testing them in a blind experimental set up. Eventually a large panel of unknown samples will be screened in parallel with standard sequence analysis and DGGE. Moreover, we will evaluate the format in which the MC results are displayed and reported by evaluating the supplied HR-MCA LightScanner software from Idaho.

Work flow

For our study we have selected the BRCA1 gene as the target gene and developed a large panel of primers to amplify all coding regions of the gene. All amplification were optimized using the LC-green Plus^{+} mastermix and MC analysis were initiated using the 96-well format LightScanner from Idaho Technologies.
Our initial set of clinical DNA samples are derived from the LUMC Clinical Genetics Laboratory in Leiden (NL) and were extracted with the Gentra Autopure LS technology. Together the selected panel of DNA samples harbour all frequent and moderate occurring mutations and polymorphisms that are dispersed throughout the set of BRCA1 gene specific amplicons.
Our technical evaluation program encompasses 3 phases,

- In **phase 1** the technique is set up and evaluated in one laboratory. During this phase the following experimental steps were performed:

1. Development/design primers sets for target gene
2. Testing and optimizing amplification of BRCA1 gene specific amplicons using LC-green Plus+ mastermix. (if necessary redesign failing primers sets)
3. Examine detection various mutations in all amplicons using a large panel of well characterized clinical DNA samples.
4. Set up the detection for frequent occurring polymorphisms by using unlabeled probes.
5. Evaluate Lightscanner software performance and report quality/format for diagnostic use.

- **In phase 2** we will evaluate the MCA performance in a second (and third) accredited diagnostic centre by repeating the MCA studies and using the same consumables and DNA samples. This phase starts when all primer set are optimized in phase 1 and perform well in the MCA.

- Finally we will start **phase 3** during which we will perform blind studies with known samples and mutation scanning of new samples in parallel with standard sequence analysis and DGGE. Depending on the technical performance of the assay and the quality and reproducibility of the reports generated by the Lightscanner software we will initiate final validation of the technique.

Current results

At the moment 94% of the BRCA1 amplicons have been optimized and perform well in the MCA. The sizes of the amplicons range from 187bp to 422bp except for one amplicon, which is 625bp. At the moment already many amplicons have been evaluated for variant detection using different mutant DNA samples.

Results with Idaho Tech. Lightscanner (preliminary):

- **Variants analyzed by HR-MCA.**
  - 62 variants have been analyzed so far (October 1 2006)
  - 61/62 (98%)* mutations were detected (high sensitivity).

- **HR-MCA mutation detection analysis (all in duplo).**
  - Autogroup - Normal sensitivity 7 False Negative and no False Positive.
    - High sensitivity 1 False Negative* and 3 False Positive#
  - Report - Clear visual plots are generated
    - Import and export of samples and data still needs improvement (new software is already under development)
    - No general file format to generate clear diagnostic reports with selected variant plots

  * The variant sequence that was not detected was a homozygous polymorphism, which could be visualized using a mixed reaction with wt control DNA.
  # One FP was in duplo and of two FPs the duplo was reported as one wt and one FP

These preliminary HR-MCA results indicate that the performance of the mutation scanning analysis appears very promising. Al sequence variants could be detected except for one homozygous mutation. The latter issue can be solved by spiking the reaction with wt DNA. Currently more samples are being evaluated and the detection of frequent polymorphism has been set up using unlabeled probes.
All experiments have been performed with the advice and assistance of Idaho Technology and comments related to experimental analysis using the software but also to the report format are continuously being exchanged.

References/Links

1. Zhou, L., Wang, L., Palais, R., Pryor, R. and Wittwer, C. T. (2005) High-Resolution DNA Melting Analysis for Simultaneous Mutation Scanning and Genotyping in Solution. Clin Chem, in press.
2. Graham, R., Liew, M., Meadows, C., Lyon, E. and Wittwer, C. T. (2005) Distinguishing different DNA heterozygotes by high-resolution melting. Clin Chem, 51: 1295-8
3. Reed GH, Wittwer CT. (2004) Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. Clin Chem. 50:1748-54
4. Liew M, Pryor R, Palais R, Meadows C, Erali M, Lyon E, Wittwer C. (2004) Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. Clin Chem. Jul;50:1156-64.

Links http://www.idahotech.com/LightScanner/