Inter-laboratory Diagnostic Validation of Conformation Sensitive Capillary Electrophoresis.

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Department of Health

EuroGentest — Genetic Testing in Europe — Network for test development harmonization, validation and standardization of services

National Genetics Reference Laboratory — Wessex

Applied Biosystems

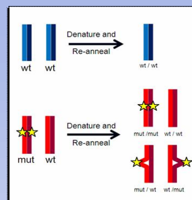
Introduction

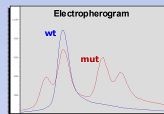
Much of the work carried out by molecular diagnostic laboratories is mutation scanning in probands referred with a particular disease phenotype. Indirect methods are of interest to diagnostic laboratories as they have the potential for significant time and cost savings when compared with a direct sequencing approach. However, thorough validation is required to demonstrate suitable analytical accuracy for diagnostic use. Conformation sensitive capillary electrophoresis (CSCE) is a simple, high throughput method based on the principle that homoduplex and heteroduplex DNA have different electrophoretic motilities under partially denaturing conditions. In this study we have performed a comprehensive optimisation and validation of CSCE for diagnostic detection of heterozygous mutations.

Study setup

This work was jointly funded by the participating Laboratories, EuroGentest and Applied Biosystems (AB Europe). The study was coordinated by The National Genetics reference Laboratory - NGRL (Wessex) - and carried out in three diagnostic centres in Salisbury, Leuven and Nijmegen. The work was divided into two core packages:

1. **Optimisation** - To determine optimal conditions for CSCE.
2. **Validation** – To determine analytical accuracy of CSCE.





Principle of CSCE

Validation constraints

PCRs

- All PCR products were designed to be ≤500bp
- Region of interest was considered to start and 50bp from either end of the product. Regions of interest >500bp were therefore covered with fragments overlapping by ≥50bp.

Electrophoresis

- All electrophoresis was performed on AB 3130xl and 3730 instruments using 50cm capillary arrays.
- Electrophoresis conditions were based on the method developed at the Sanger Institute[1]. Polymer was a CAP (Applied Biosystems) base with urea, sucrose (Merck) and TTE (National Diagnostics).

Analysis

All traces were scored by:

- Manual analysis GeneMapper v3.7 (Applied Biosystems) based on the number of peaks present – see below
- Automated heteroduplex analysis in Bionumerics v5.10 software (Applied Maths). This compares peak shape with a wild type control using 5 parameters: SRMS, MAXDIFF, DFH3 and DFH4 examine the primary peak shape whilst SECPK looks for secondary peaks.

Manual scoring schema

Optimisation

Samples and methods

- Generic mutation detection controls [2] (GMD controls) developed by NGRL (Wessex) were used. These are 52 artificial PCR templates comprising 4 fragments of different GC content (20%, 40% 60% and 80%) each with one of 4 mutations (together representing all possible heteroduplexes), introduced at one of 3 positions within the fragment (Total 4wt +52mut controls).
- Samples were amplified and diluted into 96 well plates ready for analysis by NGRL (Wessex) and distributed to participating labs.

Experimental schedule

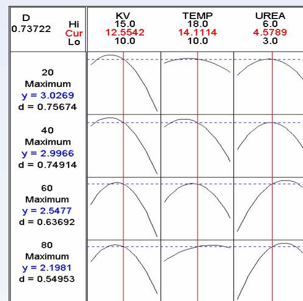
Ref	Description	Analyses
O1	To determine **loading parameters** (time, KV) that gave peaks in analysis window (3130xl:500-7000RFU, 3730:1000-30,000RFU)	►3130xl: 6 runs comprising 16wt analyses ►3730: 6 runs comprising 48wt analyses
O2	To determine if **sequential loading** of electrophoresis runs has a detrimental effect on resolution	►3130xl: 6 runs comprising 16wt analyses ►3730: 6 runs comprising 48wt analyses
O3	**Core optimisation**: Full factorial experiment to determine optimal urea content in polymer, run temperature and voltage	►18 plates of analyses per lab, each comprising all GMD controls randomised and blinded
O4	Serial dilution of heterozygous mutations with wt to determine **lower limit of detection** for mutation load	►10 plates of analyses, each comprising all GMD controls randomised and blinded (3730 only)

Results

The experimental design enabled examination of the response both overall and in various subgroups including: by laboratory, by mutation type, by position of the mutation within the fragment and by GC content of the fragment. The diagram (below) represents the averaged manual scores with given levels of the three variables according to the GC content of the fragment. The setup shown was optimal, taking into account both analytical requirements and technical constraints.

Key findings

- ►There was no significant variation between laboratories.
- ►Key factors affecting resolution of mutants were GC content and position.
- ►Optimal conditions for 20-40% GC content fragments are shown (right)
- ►It may be desirable to use different conditions for 80% GC fragments although shorter fragments may suffice
- ►Mutation type was not well correlated to resolution of mutants.
- ►All mutants were detectable using 4.6M urea, 12.5KV at 14°C



D= overall desirability of current configuration (0-1)
d= sub-group desirability of current configuration
y= expected ave. score with current configuration

Validation

Samples and Methods

- 359 samples collected from the 3 participating laboratories previously characterised as carrying ≥1 BRCA1 or BRCA2 variation which was originally detected by a method other than CSCE
- Samples were normalised at 20ng/µl and randomly distributed across 10x96 well plates at NGRL(Wessex)
- PCR, pre-loading sample preparation, electrophoresis and analysis were all performed in participating labs
- Samples analysed multiple times for different fragments - analyses not carrying mutations used as wild type controls
- 79 different fragments analysed: ≥2 different variations/fragment. 30 mutations located in two overlapping fragments

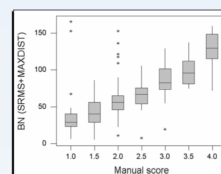
Experimental schedule

Ref	Description	Analyses
V1	**Core validation (Sequentially loaded):** to determine analytical accuracy of CSCE	►358 randomised and blinded wt analyses ►444 randomised and blinded mut analyses ►158 wt reference control analyses (2 per fragment)
V1R	**Partial re-run of V1 plates (Sequentially loaded):** to control for observed loss in resolution in V1	Re-run plates 06-10 of V1 (Salisbury 3730 only)
V2	**Re-run Core validation (Gapped loading)**	New dilution of V1 plates - 4hr gap between plates (3730 only)

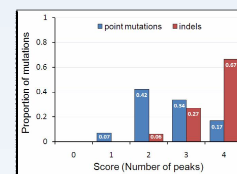
Results

Key findings

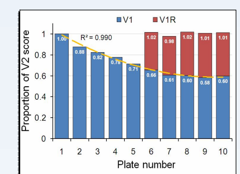
- ►All variations were detected using both analysis methods.
- ►There was good correlation between manual scores and Bionumerics parameters SRMS and MAXDIST.
- ►93% of point variants and 100% of insertion / deletion variants gave ≥2 distinct peaks (95% overall). 2% of point variants gave only a subtle peak shape change.
- ►Compound variants consistently gave >4 peaks and were easily distinguishable from the individual variations.
- ►Significant loss in resolution was observed initially when running plates sequentially (V1). This was not observed with a limited re-run of the same plates (V1R) or with 4hr gaps between plates (V2)



Correlation between manual and automated analysis

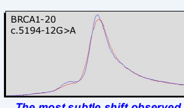


Manual scores by variation type

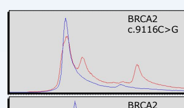


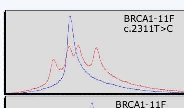
Loss in resolution

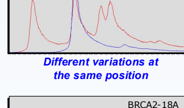
Examples



The most subtle shift observed







Different variations at the same position

BRCA1-11F c.2077G>A c.2082C>T

BRCA1-11F c.2077G>A c.2082C>T c.2311T>C

Compound variations

BRCA2-18A c.8243G>A

BRCA2-18B c.8243G>A

Same variation in different fragments

Conclusions

- ► **Sensitivity and specificity** of CSCE, within the constraints of the current validation, are suitable for most applications of diagnostic mutation scanning for heterozygous mutations (sensitivity >99% with 95% confidence – see right).
- ► **Optimal run conditions** for most purposes are: CAP polymer with 4.6M urea with electrophoresis at 12.5KV and 14°C. Regions of interest with higher GC content may require higher urea content in the polymer and/or shorter fragments for analysis.
- ► **Peak intensity** is a critical consideration for design of fragments for CSCE analysis. Optimisation needs to be more precise for the 3130 compared to the 3730 as the analysable range is considerably smaller (3130xl:500-7000RFU, 3730:1000-30,000RFU).
- ► **Equivalent sensitivity** was achieved using manual analysis by visual inspection in GeneMapper (AB) and automated analysis in Bionumerics (Applied Maths).
- ► **Automated analysis** is preferable as it is faster and more objective. Suitable cut offs for Bionmerics parameters are: SRMS=3.4 and MAXDIST=4.4
- ► **Manual analysis** requires care as some point mutations may be indicated by subtle peak shape changes only (2%). Some frame shift mutations have a primary peak virtually indistinguishable from the wt and very dispersed secondary peaks. A two phase approach to analysis is recommended: an overall view to detect large shits followed by detailed examination of the primary peak in close-up to detect subtle shifts.
- ► **Resolution** if multiple runs are performed sequentially. This should be monitored running a resolution control with known peak shape (preferably a subtle shift) with every run (preferably in each capillary) and will also serve to define suitable capillary life. The loss in resolution requires further investigation but the fact that it is not seen on secondary loading suggests it may result from component in the sample that is depleted during a first load.
- ► **A full report** of all this work is in preparation and will be available from NGRL and Eurogentest websites and submitted for peer reviewed publication.

Variations	Sensitivity (95% CI)$
All	**99.3 - 100**
Point	98.9 - 100
Ins /Del	97.5 - 100
Compound	82.9 - 100

Analysis	Specificity (95% CI)$
Manual	92.4 - 96.6
Bionumerics*	89.0 - 94.0

$Confidence intervals by exact method
*SRMS = 3.4
MAXDIFF = 4.4
SECPK, DFH3 & DFH4 not used

References

1. Davies H, Dicks E, Stephens P, Cox C, Teague J, Greenman C, Bignell G, O'meara S, Edkins S, Parker A, Stevens C, Menzies A, Blow M, Bottomley B, Dronsfield M, Futreal PA, Stratton MR, Wooster R. High throughput DNA sequence variant detection by conformation sensitive capillary electrophoresis and automated peak comparison. **Genomics. 2006 Mar;87(3):427-32. Epub 2006 Jan 9.**

2. Plasmid based generic mutation detection reference reagents; production and performance indicator field trial - available for download at http://www.ngrl.org.uk/Wessex/downloads.htm