

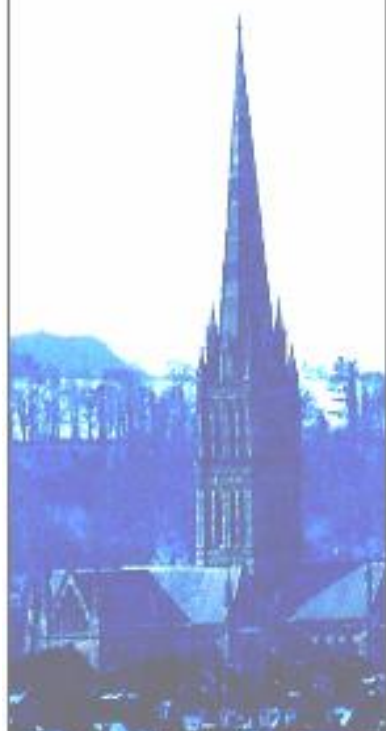


National Genetics Reference Laboratory
(Wessex)

NHS


Application
Note

Conformation Sensitive Capillary Electrophoresis



NGRL WESSEX

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1. INTRODUCTION

Conformation sensitive capillary electrophoresis (CSCE) is a simple, rapid and sensitive method for indirect mutation scanning. The method is based on the principle that homoduplex and heteroduplex DNA have different mobilities when subjected to electrophoresis (figure 1). Although this principle has been successfully applied to non-fluorescent gel based methodologies the increased resolution of capillary electrophoresis should improve sensitivity and the potential throughput is considerably greater.

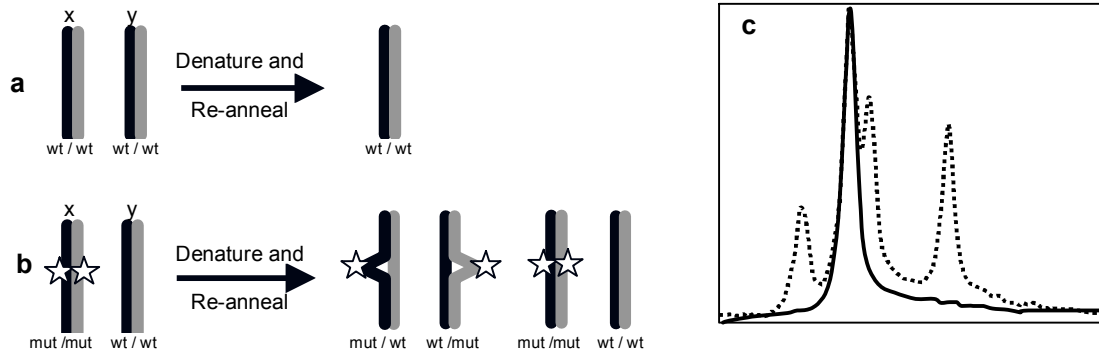


Figure 1: Heteroduplex analysis

a) Represents the analysis of a homozygous amplification product. The equivalent strands (black = sense, grey = anti-sense) of the two alleles (x and y) have identical sequence. When the mixture is denatured and allowed to re-anneal there is only one possible combination of the two complementary strands (wt/wt) and thus only one conformation. b) When a heterozygous amplification product with a single base difference in allele x (star) is denatured and allowed to re-anneal there are four possible duplex species: two heterozygous with a single base mismatch (wt/mut and mut/wt) one mutant homoduplex (mut/mut) and one wild type homoduplex (wt/wt). c) Representative CSCE trace analysis. A wild type analysis (solid line) shows only one peak whereas a mutant analysis (dotted line) shows four peaks representing the four species present in the analyte. In practice point mutations often show mobility variations with one or more of the different species remaining unresolved.

The methodology described in this document has been tested using the generic mutation detection controls produced by the National Genetics Reference Laboratory (Wessex). These controls can be used to amplify four fragments ranging from 400-450bp with an average GC content of 20%, 40%, 60% and 80% respectively. The wild type sequence has been mutated to produce every possible heteroduplex (8 in total) at three positions within each amplicon giving 48 mutant fragments in total. When analysed by CSCE a detection sensitivity of 97.9% (47/48) was obtained. Only the A:C heteroduplex closest to the primer in the 80% GC fragment could not be successfully detected. Two other mutations in the 80% GC fragment gave subtle signals but were nonetheless detectable by visual inspection. For more information on the generic mutation controls refer to document ref: NGRLW_GenericRR_1.0 available from <http://www.ngrl.co.uk/Wessex/downloads>.

This document describes CSCE on ABI 3730, 3730xl, 3130 and 3130xl instruments (also see Applied Biosystems application note). However the protocol should be adaptable to other analysis platforms if necessary: the instrument manufacturer should be consulted regarding the recommended protocol.

2. CONFORMATION SENSITIVE CAPILLARY ELECTROPHORESIS

2.1 PCR

2.1.1 Amplicon design

The aim of amplicon design is to produce a fluorescently labeled fragment that gives a single, sharp peak when analysed on a 3730 or 3130 instrument. In order to achieve optimum sensitivity amplicons should be a maximum of 500bp total length including any tags and/or stuffer sequences. In general it is advisable to impose a minimum fragment size of about 200bp in order to avoid interference from primer flare and any primer dimer peaks.

Since heteroduplexes near the end of fragments have a less significant effect on the migration of fragments under electrophoresis, the region of interest (ROI), i.e. the region intended for analysis, should be flanked by at least 50bp of unanalysed sequence (buffer zone) at either end including primer sequences. If a tagged primer system is used the length of the tag and any intervening 'stuffer' sequence can be counted as part of the buffer thus enabling the 3' end of the primer to be located closer to the ROI (see figure 2). This can be useful for example to exclude unfavorable sequence close to an exon.

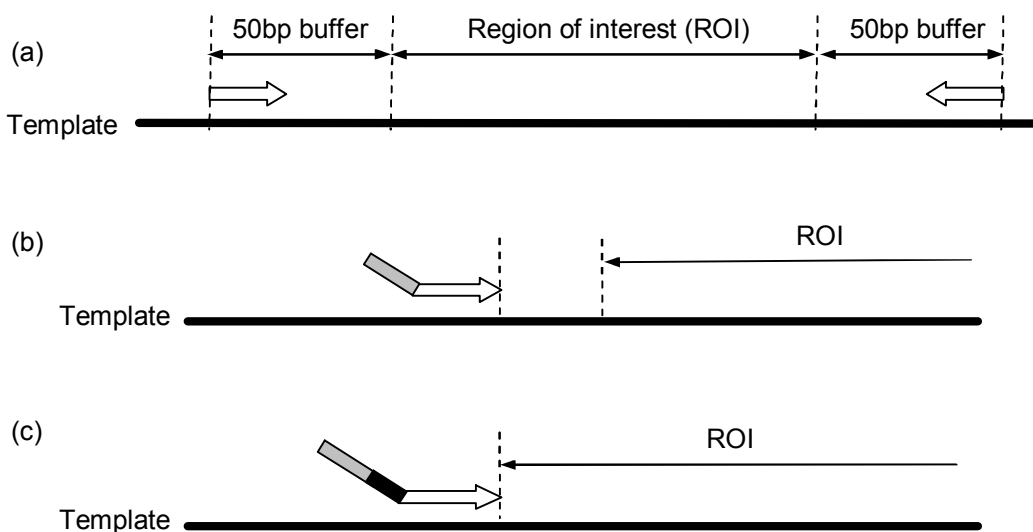


Figure 2: Defining the region of interest (lengths of primers tags and stuffers are given for illustration only)

Standard PCR using untagged primers (white arrows). If primers are 20bp long the ROI starts and ends 30bp from the 3' end of each primer.

One end of an amplicon using a tagged primer system. If the primer (white arrow) is 20bp and the tag (grey) is 18bp the ROI starts 12bp from the primer.

One end of an amplicon using a tagged primer and a stuffer. If the primer (white arrow) is 20bp, the tag (grey) is 18bp and the stuffer (black) is 12bp the ROI starts immediately adjacent to the primer. This can be useful, for example, to exclude unfavorable sequence close to an exon.

2.1.2 Difficult sequences

It has been found that, for certain sequence contexts, it is difficult to obtain a single sharp peak for a wild type fragment under the CSCE conditions described herein. This may be due to heteroduplex anomalies or the introduction of Taq errors in the PCR. In general it is best to avoid very AT rich regions (>70%) if possible and in particular sequences that contain runs of more than 8 or 9 A or T bases. Where it is not possible to design out such regions, a fragment that produces a split peak profile for the wild type can be used providing it can be shown that the peak profile is consistent between PCRs using template of varying quality. Alternatively it may be necessary to directly sequence certain difficult fragments.

2.1.3 Fluorescent labels

All fragments for analysis should be fluorescently labeled with a suitable dye for detection on the ABI 3730 or 3130 family of analysis platforms e.g. FAM, VIC and NED (Applied Biosystems dye set G5). To allow alignment of peaks for analysis a LIZ labeled size standard should be added to the loading buffer before electrophoresis (see section 2.3.3 below). The PET dye, often used for fragment analysis in this dye set (G5), is not ideal for CSCE analysis since it has two isomers that generate split peaks under partially denaturing conditions. ROX is recommended as a replacement for PET as the fourth dye by many labs performing CSCE although this laboratory has limited experience with its use.

Analyses can be multiplexed at the pre or post PCR stage with separation either by colour, size or both. A suitable minimum size separation of amplicons for multiplexing by size alone would be 40 bp (see Section 2.3.4). To keep the process simple and maximize robustness it is suggested that multiplexing PCRs is avoided unless the required throughput makes it necessary.

If fragments are to be multiplexed by colour it is recommended that a test is performed to ensure the spectral being used eliminates any 'pull-up' peaks resulting from incomplete dye separation. This is particularly important if fragments of the same size, but differently labeled, are multiplexed for analysis as any 'pull-up' may interfere with true peaks in a particular channel giving the appearance of a shifted or mutant signal. For example some small 'pull-up' peaks have been observed in the LIZ and VIC channels when ROX is used as a replacement for PET. In this case the pull-up in the LIZ channel may interfere with sizing for analysis (although it may be possible to eliminate it using a higher peak intensity threshold for the LIZ channel during analysis) and the pull-up in the VIC channel may interfere with the analysis of VIC labeled fragments.

2.2 Heteroduplex formation

Formation of heteroduplexes is a critical step in the process. Table 1 gives details of a standard heteroduplexing regime. This can be incorporated at the end of the PCR cycling program or run as a separate protocol before analysis.

Step	°C	Time		Cycle
Denature	96	10	min	
Heteroduplex formation	96	20	sec	x72 cycles decreasing temperature by 0.5°C per cycle
Hold	60	30	min	

Table 1: Heteroduplex formation regime

2.3 Preparation for capillary electrophoresis

2.3.1 Analysis window

To achieve peaks suitable for analysis PCR products require dilution before capillary electrophoresis. Under-loading (peaks too low) can reduce sensitivity as heteroduplex peaks tend to be smaller than homoduplex peaks and all peaks will be smaller for analyses where there are shifted or mutant peaks (i.e. heterozygotes). Overloading (peaks too high or off scale) may reduce sensitivity by truncating peaks and can cause pull-up peaks in other dye channels confounding their analysis. The analysis window is dependant on the instrument used for analysis, as different instruments have different sensitivities and the relative fluorescence units (RFU) used on different instruments are not comparable. The

recommended analysis window for ABI 3730 and 3730xl instruments is 1000-25000 RFU whilst for the ABI 3130 and 3130xl instruments a window of 250-5000RFU is suggested.

2.3.2 Dilution of PCR products

Since CSCE is based on differential mobility of heteroduplexed DNA, the electrophoresis is carried out under partially denaturing conditions. In order to preserve the heteroduplexes the loading buffer used is essentially water. Electrokinetic loading of products in water does not have the normal modulating effect on signal intensity that loading in formamide does i.e. much more product is loaded. Therefore to achieve a PCR product concentration that gives a peak within a suitable analysis window (see section 2.3.1) it is necessary to dilute the PCR products. This dilution also reduces the salt concentration (from the PCR buffer), which can also modulate the effectiveness of electrokinetic loading. In general the final dilution of the PCR buffer in the loading plate should be at least 1/40 to give good injection (too much salt will reduce the amount of product injected), although a strong PCR amplification will outweigh the negative effect of the salt in the loading buffer. These effects are illustrated in figure 3 below.

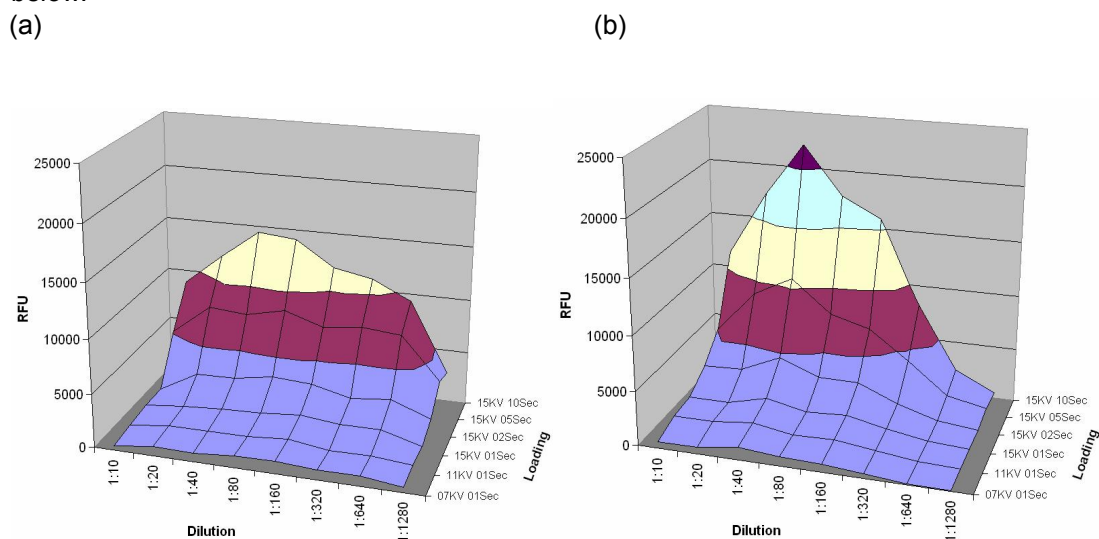


Figure 3: The effect of diluent, dilution factor and loading parameters on peak intensity.

A single PCR product was serially diluted from 1:10 to 1:1280 in (a) H₂O and (b) 0.5 mM EDTA. Eight replicates of each dilution were loaded on a 3730 using 6 different loading parameters (7KV for 1 Sec, 11KV for 1 Sec, 15KV for 1Sec, 15KV for 2Sec, 15KV for 5Sec, and 15KV for 10Sec): each different loading parameter represents a separate run lasting 1 hr 30 mins. The optimum dilution for peak intensity is 1:40. In lesser dilutions the salt content of the sample (from PCR buffer) is seen to restrict loading whilst at greater dilutions there is less product to load. As expected increased loading time and/or voltage has a positive effect on peak intensity. Products diluted in EDTA give a much better signal over time than those diluted in H₂O: By the 6th load (15KV for 10Sec) the products diluted in H₂O had lost 35% of their intensity compared to those diluted in EDTA.

The optimum dilution of PCR products will depend on the composition of the PCR (i.e. concentration of all components), the number of PCR cycles and the efficiency of amplification. The appropriate dilution for a particular PCR methodology should therefore be determined empirically but is likely to fall between 1:40 and 1:1000. However, it should be possible to determine a single dilution factor that will be suitable for most amplicons given a particular PCR methodology.

It has been found that dilution in EDTA gives better results over time as it appears to prevent degradation of the analyte (see figure 3). This is particularly important if there is a delay between the PCR amplification and electrophoresis, although it is recommended that the prepared samples are frozen at -20°C for the intervening period if an extended delay is anticipated. The concentration of EDTA in the diluent should be calculated to be equimolar to

the Mg^{++} concentration in the PCR buffer after the dilution e.g. a 1:100 dilution of a PCR that required 2mM Mg^{++} will require 20 μ M EDTA as the diluent.

In practice the dilution will usually need to be done in two stages to achieve the necessary dilution factors e.g. a dilution of 1:200 can be achieved with a primary dilution of 1:20 followed by a secondary dilution of 1:10.

2.3.3 Size standards

GeneScan LIZ500 (Applied Biosystems P/N 4322682) or GeneScan LIZ600 (Applied Biosystems P/N 4366589) size standard should be included in the secondary dilution to achieve a suitable final concentration for loading. As with PCR products the use of water as the loading medium means that it is necessary to use significantly less size standard than is normally recommended for fragment analysis. In order to determine the quantity of size standard required for each analysis on a capillary a titration of size standard concentration should be performed using a pool of fragments that is representative of the required analysis. A 2x dilution series (0.1, 0.05, 0.025, 0.012, 0.006 μ l LIZ500 per capillary) is suggested for this titration. A suitable peak height for the LIZ size standard is 1000-2000RFU for ABI 3730, 500-1000 for ABI 3130.

2.3.4 Pooling products

To increase the throughput capacity PCR products can be pooled for analysis purposes: pooling of samples before PCR is not recommended for diagnostic purposes. Different analyses within a pool can be separated by colour (i.e. fluorescent label), size or both. In general it is better to pool differently coloured products as the fate of a particular fragment can be easily distinguished from others within the same pool during analysis. It is recommended any fragments to be pooled by size should differ from others within the pool by at least 40bp. Even so, for large mobility shifts seen with some insertion or deletion mutations, it may not be possible to determine which fragment in the pool is mutated, which could necessitate sequencing more than one fragment to be sure of finding the mutation.

In practice pool sizes of up to 12 analyses per capillary have been reliably achieved (4 colours x 3 different sizes). A key issue with larger pools is maintaining signal intensity for all the fragments. For example pooling four fragments requires a minimum dilution of 1:160 to maintain a minimum PCR buffer dilution of 1:40, whereas a pool of 12 will require a minimum dilution of 1:480 to achieve the same. Therefore to achieve high level pooling strong PCR is required either to override the necessity to dilute the salt in the PCR buffer or to allow the high dilution factors required to maintain a suitable PCR buffer dilution.

2.4 Capillary electrophoresis

2.4.1 Polymer composition

CSCE is run under partially denaturing conditions. The polymer is made up from the base Conformational Analysis Polymer (CAP - Applied Biosystems P/N 4340379) by adding urea, and TTE buffer in the proportions shown below. Because of the number of capillaries on 3730 platforms, the incident angle of the read laser is such that the refractive index of the polymer must be altered by the addition of sucrose in order to achieve a usable spectral. This addition is not required for analysis on 3130 platforms.

Polymer mix (final concentrations):

5%	Conformational Analysis Polymer (CAP)
4M	Urea
7.5%	Sucrose (Not required for running on an 3130 platforms)
1x	TTE buffer

Table 3 describes the composition of 20ml of 3730 polymer. The mixture should be stirred until it is mixed thoroughly (Approx 90 minutes). The polymer should then be filtered through 5.0 µM single use syringe filter (Sartorius Minisart P/N FDP-620-110W) into a disposable plastic bottle, spun down and stored at 4°C. Under these conditions the polymer remains usable for at least 2 months.

Component	Qty	Unit
Urea (Merck 1.084787.0500)	5.5	g
Sucrose* (Merck 1.07687.0250)	1.7	g
MiliQ water	1.8	g
20x TTE National Diagnostics cat. No:EC-871	1.1	g
9% Conformational Analysis Polymer (CAP) Applied Biosystems P/N 4340379	12.7	g

Table 2: Components to make up 20ml of 3730 CSCE polymer

Weight is used rather than volume as the polymer is so viscous that is not possible to measure the volume accurately. * Sucrose should be omitted for use with an ABI 3130.

2.4.2 Instrument setup

The prepared products can be run on an ABI 3730, 3730xl, 3130 or 3130xl using 36 or 50 cm array. For the recommended dye set, G5 (FAM, VIC, NED, ROX and LIZ), a spectral should be run using the DS-33 matrix standard (Applied Biosystems P/N 4345833). The spectral calibration can be done in one of two ways:

If prepared CAP polymer (see 2.4.1 above) is used, the spectral calibration should be run using 1x TTE running buffer (National Diagnostics cat. No:EC-871P/N) with the standard spectral calibration module. The matrix standard should be prepared in the normal way using Hi-Di™ formamide (Applied Biosystems P/N 4311320) as the loading buffer. It should be noted that, when using TTE as the buffer, an artefactual red peak is sometimes seen before the calibration peaks appear, which can interfere with the calibration calculations and cause the run to fail. To resolve this problem the data delay should be adjusted in the standard spectral calibration module so that data collection starts after this peak has passed the collection window (see figure 4).

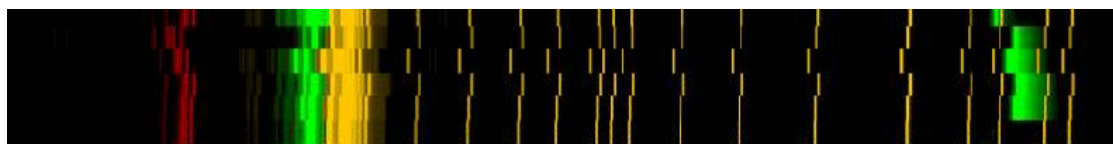


Figure 4: Red peak artefact

The red peak is shown in a standard CSCE run with a single 460bp VIC labelled fragment together with GeneScan LIZ500 size standard. This peak appears to be an artefact associated with the use of TTE buffer and can interfere with spectral calibration unless it is cut out of the data collection by adjusting the data delay in the run module.

Alternatively the spectral calibration can be run using POP7™ (Applied Biosystems P/N dependent on lot size) and TBE running buffer (Applied Biosystems P/N 402824) in exactly the standard way. The capillary should then be flushed with at least 2 water washes before replacing the POP7 with the prepared CAP polymer (see section 2.3.1). Although this spectral calibration is not carried out using the intended run polymer the colour separation is equally effective.

CSCE runs should then be performed using 1x TTE running buffer (National Diagnostics cat. No:EC-871P/N). Pre-made run modules for CSCE on approved instruments are available from ABI on request. If a 3130 series instrument is being used a module must be obtained from ABI since the pre-run current check fails as a result of using the modified polymer causing a terminal run error.

For information the modifications to the standard GeneScan module are as follows:

Run temperature: 18°C
 Sample injection: 15Kv for 5 sec (should be varied achieve suitable signal strength)
 Dye set: G5

Run time and data delay should be adjusted to suit the capillary length and the size of fragments being analysed. However details in figure 5 are provided as a guide to determining appropriate data delay and run times.

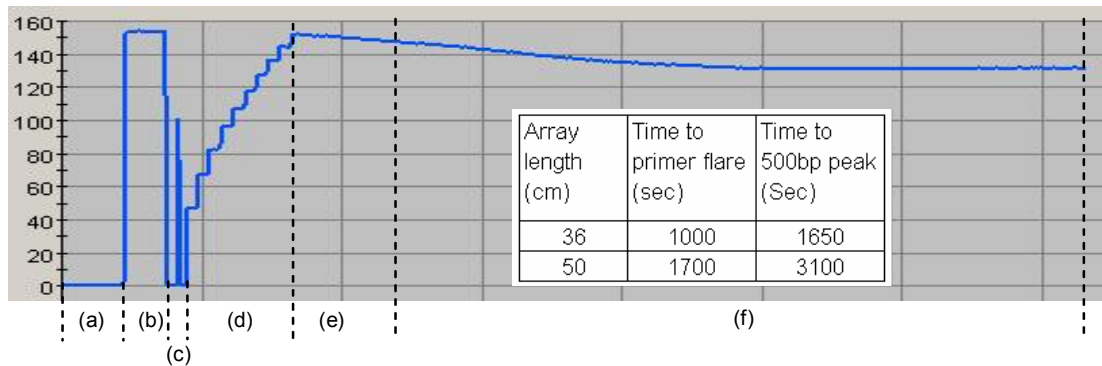


Figure 5: Components of overall instrument running time (mA trace shown)

- oven stabilising at run temperature (1st run ~20 mins, subsequent runs ~4 mins)
- pre-run (defined in module 3 mins)
- Injection window (fixed ~2 mins)
- Voltage ramp (defined by 'Voltage_No_Of_Steps' and 'Voltage_Step_Interval')
- Data delay
- Run time

Assuming a run voltage of 15KV and a data delay set to 0 sec the times for migration of the primer flare and a 500bp peak are given in the embedded table

2.4.3 Controls

For each fragment analysed the minimum requirement for controls should be a wild type and a no template control. For more stringent control a heteroduplex control, consisting of a heterozygous mutant of the fragment being analysed, should also be used. In addition, ensure adequate resolution is being achieved in each capillary, a subtle mutant can be used as a resolution control (see 2.4.4 below)

2.4.4 Array life

The life of an array is determined by the point at which resolution falls below the requirements of the methodology being used. Typically for CSCE an array should last for at least 200 runs. Loss in resolution is seen as a broadening and flattening of peak profiles that can ultimately lead to the loss of discrimination between two peaks (figure 6a). Since some mutations can give subtle shifts (i.e. a small shoulder on the wild type peak) the resolution required for CSCE is relatively high. It has been observed that different capillaries in an array can deteriorate at different rates. Therefore it is prudent to monitor the resolution of each capillary on a regular basis. This can be done by running a particular heterozygous mutant with a known peak profile through each capillary and comparing the peak profile with that observed under normal, good resolution, conditions. Most simply, a resolution control can be prepared and run in each capillary as a separate run, say once a week. Alternatively one of the dyes can be reserved for a resolution control that can be spiked into every pool at the dilution stage to provide an internal resolution control for every analysis. Since it is the subtle mutations that are likely to be lost first with a reduction in resolution, a mutant that gives a subtle shift is the most suitable choice for a resolution control (figure 6b).

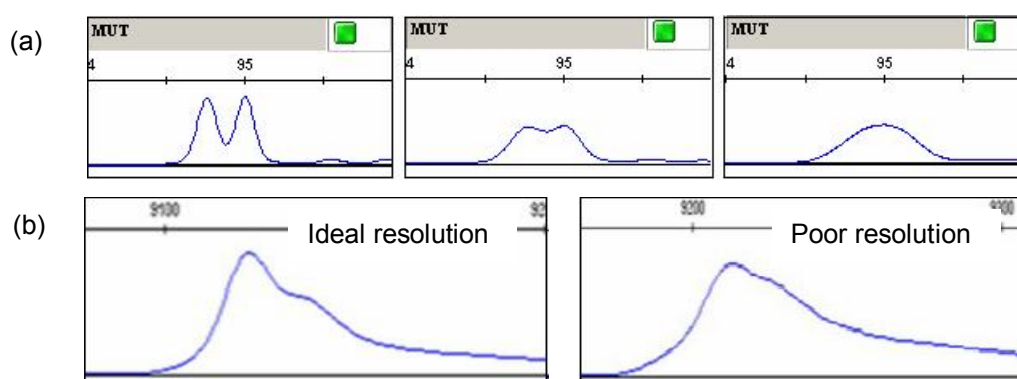


Figure 6: Resolution controls

shows the loss of resolution between two peaks with an aging capillary

Shows a heterozygous point mutation that gives a very subtle CSCE shift with ideal and poor resolution conditions. This mutation would make a suitable resolution control

2.5 Analysis

2.5.1 Software

Analysis by visual inspection should be performed using GeneMapper software. Using v3.7 of this software it is possible to define normal controls for comparison with other test traces and overlay traces if required. It is, however, not currently possible to vertically normalize peaks using Genemapper, which virtually eliminates the utility of overlaying peaks. It is possible to setup 'bins' using the 'Microsatellite Analysis Method' in order to automate the detection of peaks and highlight analyses where additional peaks are present (Details on how this can be done can be found in the GeneMapper manual or advice should be sought from Applied Biosystems technical help). However, because Genemapper has not been specifically designed for analysis of CSCE traces, the simplest and most reliable analysis is direct comparison of test traces and wild type controls by visual inspection.

A fully automated utility for analysis of CSCE traces is available as a 'plug-in' for Bionumerics, an analysis package available from Applied Maths (<http://www.applied-maths.com>). This offers:

- Direct analysis of .fsa files without any pre analysis.
- Automated trace and target identity recognition.
- Five parameters to quantitate differences between peaks. These parameters can be set by the user to give an entirely objective and automated analysis.
- LIMS compatible output.

2.5.2 Size standards

Traces should be aligned using the internal GeneScan LIZ500 (Applied Biosystems P/N 4322682) or GeneScan LIZ600 (Applied Biosystems P/N 4366589) size standard. It should be noted that since CSCE analyses are run under partially-denaturing conditions the size of peaks in the analysis do not reflect the actual size of the amplicon in base pairs (bp). Consequently the mobility of peaks in the size standard do not have a linear relationship to the actual size of the fragments and assignment of the size standard using true peak sizes will cause an error. The simplest method for assigning the size standard is to use the scan number (or a contraction of the scan number) at which the size standard peak appears e.g. if a particular size standard peak appears at 9876 scans, 98.8 could be used as the size for assignment of that peak (i.e. scans \div 100).

2.5.3 Mutation detection

Typically wild type samples give a single peak with a sharp leading edge and a somewhat more extended tail (see figure 7 to 10). The position of both test and normal peaks should be checked to ensure the correct peaks are present. The consistency of wild peaks is such that heterozygous mutants can be called for any peak that differs from the shape. Most mutations give a clear shifted peak signal comprising two or more peaks. However some are less obvious and may only be indicated by change in peak shape (see figure 8d). It is helpful to use a magnified view and / or overlaid peaks to accentuate differences between mutant and wild type peaks. It is recommended that a cautious policy with respect to analysis is adopted in the early stages of use until experience is gained.

The detection of homozygous mutations has not been extensively studied in this laboratory. Although homozygotes are certainly detectable by mobility (figure 9) it is by no means certain that all possible base changes would be detected. In addition sequence context may have an effect on the detection sensitivity.

The general view is that, for reliable detection of homozygote mutations, the analyses need to be spiked with an appropriate quantity (as close to 50% as possible) of wild type sample. It is probably most reliable to pre-amplify the wild type spike and add it after PCR amplification but before heteroduplexing. It is possible that adding the spike as template before the PCR could lead to non-ampification of one of the templates and an unreliable result. If the spike is being added post PCR it may be advantageous to label the spike with a different fluorescent dye to that used for the test samples. This would yield useful information about the ratio of spike to test sample that is being analysed and provide an assurance that both the test sample and the spike are present in each analysis.

2.5.4 Example analyses

The following figures give examples of mutant fragments analysed on an ABI 3730 as part of a validation of this technology. All amplicons are taken from the BRCA1 gene. A full range of mutation types and trace patterns has been given.

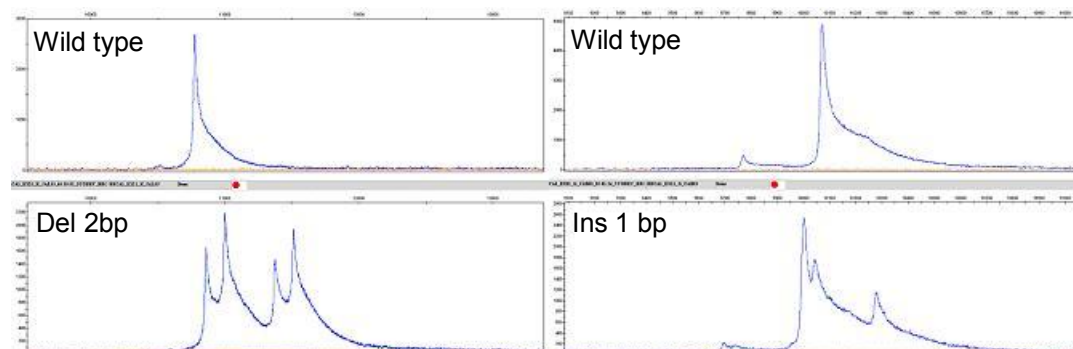


Figure 7: Insertion and deletion mutations

Typically insertions and deletion mutations give very clear indicators.

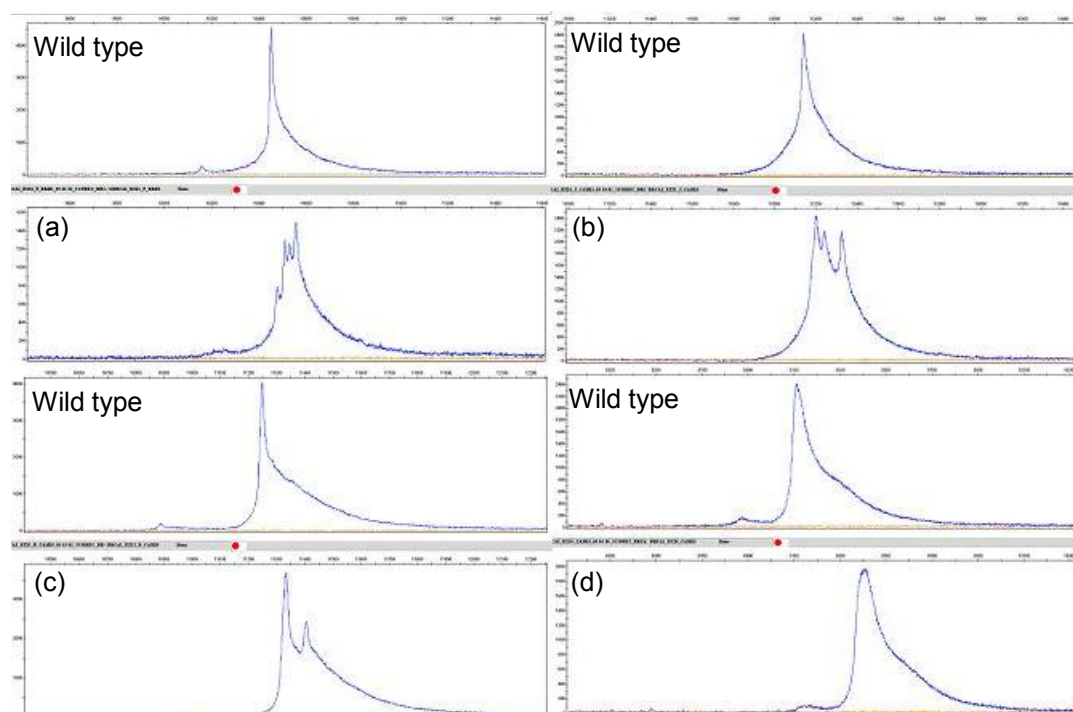


Figure 8: Point mutations

Most point heterozygous point mutations give indicators with between two and four peaks (a) to (c). The specific shape is dependant on the sequence context, the position in the amplicon and the specific base change involved. Some mutations (usually near the end of an amplicon) can give very subtle indicators (d).

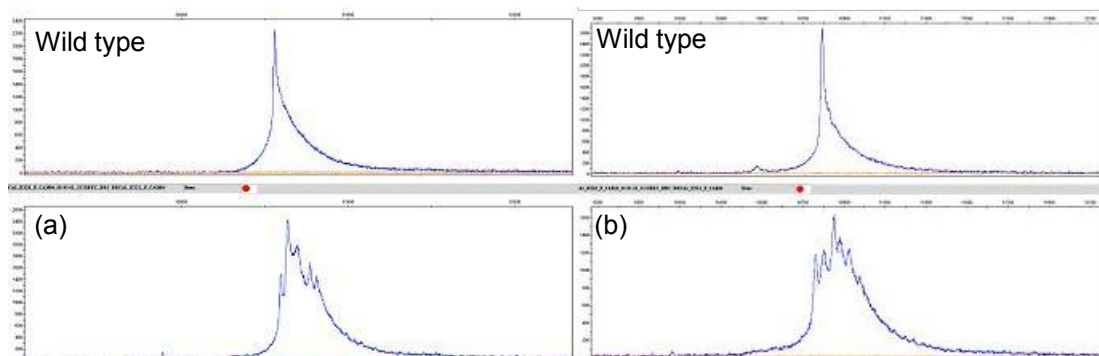


Figure 9: Compound heterozygotes

Typically compound heterozygotes give more complex spectra than single point mutations. In this example a fragment with two heterozygous point mutations (a) has five peaks (more than theoretically possible for a single mutation), whilst a fragment with the same two mutations plus one more (b) gives 6 peaks in a different pattern.

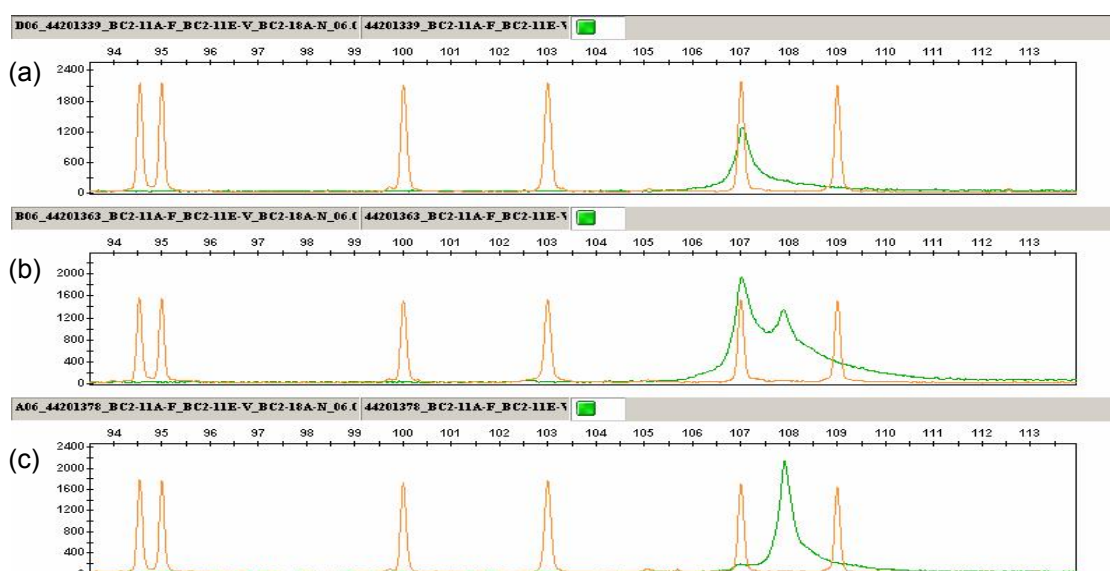


Figure 10: Homozygotes

The figure shows three analyses of a 490bp fragment that contains a common A/G polymorphism. The GeneScan LIZ500 size standard is also shown for alignment purposes. (a) A/A homozygote, (b) A/G heterozygote and (c) G/G homozygote.

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High-throughput mutation detection method to scan BRCA1 and BRCA2 based on heteroduplex analysis by capillary array electrophoresis.

Clin Chem. 2004 Feb;50(2):313-20. Epub 2003 Dec 18

Helen Davies *et al*

High throughput DNA sequence variant detection by conformation sensitive capillary electrophoresis and automated peak comparison.

Genomics 87 (2006) 427–432

Applied Biosystems Application Note

Automated Mutation Detection Using Conformation sensitive capillary Electrophoresis on the Applied Biosystems 3130 and 3730 Series Analysers

White H, Mattocks C, Potts G, Owen N.

Reference reagent report: Plasmid based generic mutation detection reference reagents; production and performance indicator field trial.

NGRL document ref: NGRL_GenericRR_1.0 2006

APPENDIX 1 EVALUATION OF CSCE USING THE GENERIC MUTATION DETECTION CONTROLS

For a detailed description of these reagents refer to:

White H, Mattocks C, Potts G, Owen N.

Reference reagent report: Plasmid based generic mutation detection
reference reagents; production and performance indicator field trial.

NGRL document ref: NGRL_GenericRR_1.0 2006

The Generic Mutation Detection controls (GMD controls) comprise four different amplicons (20%, 40%, 60% and 80% GC rich) which have been cloned into pUC 18 plasmids. At each of 3 different positions four different mutations have been introduced by site directed mutagenesis to generate all possible heteroduplexes. Tables 1 and 2 describe the constructs. The full set of GMD controls consists of 48 mutant controls and 4 wild type (WT) controls. The individual controls can be mixed with their appropriate WT control to give a representative heterozygous mutation or any level of mosaic mutation desired. Figures 1 to 4 show the results of CSCE analysis of these controls in the NGRL(W).

Table 1: Mutations introduced

Mutation	Heteroduplexes formed
A>C	C:T & G:A
A>T	T:T & A:A
G>A	A:C & G:T
G>C	C:C & G:G

Table 2: Positions of mutations in GMD controls (given in bp to nearest end of fragment)

Fragment	Length (bp)	Position 1		Position 2		Position 3	
		bp	%	bp	%	bp	%
20%	449	84	19	152	34	223	50
40%	437	78	18	146	33	215	49
60%	433	72	17	142	33	220	51
80%	424	62	15	145	34	220	52

Peak profiles obtained by CSCE for the 20% and 40% GC GMD controls.

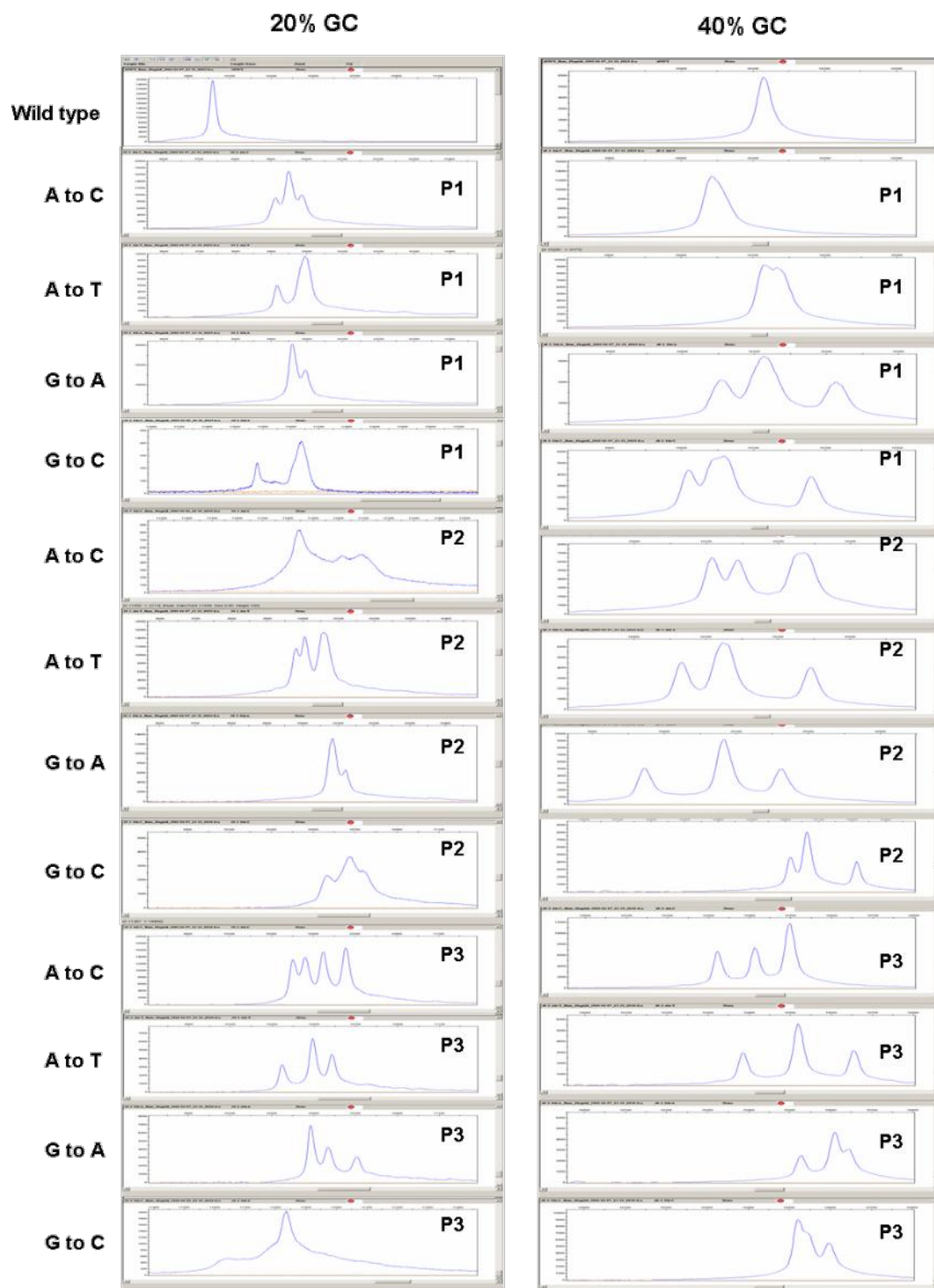
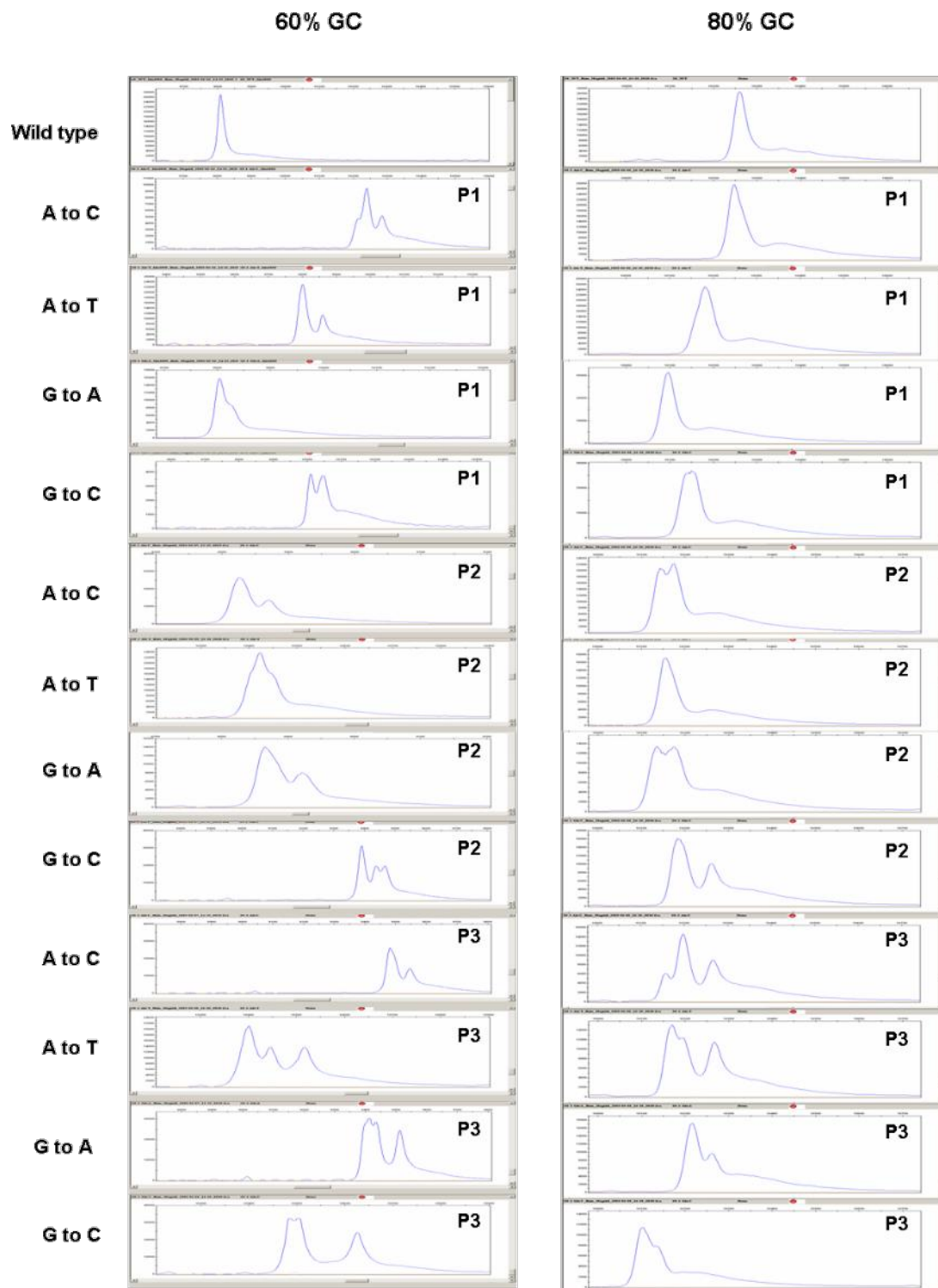


Figure 11: Peak profiles obtained by CSCE for the 60% and 80% GC GMD controls.



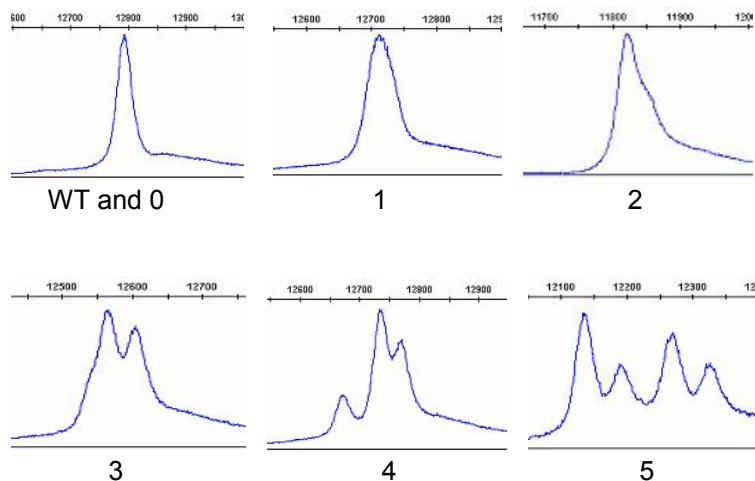


Figure 12: Scoring system for manual analysis of CSCE traces.

CSCE traces can be scored according to how clearly a mutation is detected. Here a misshaped peak (no shoulder) is given 1 point, a single peak with a shoulder 2 points etc. Note shoulders on traces with multiple peaks are not significant in this system

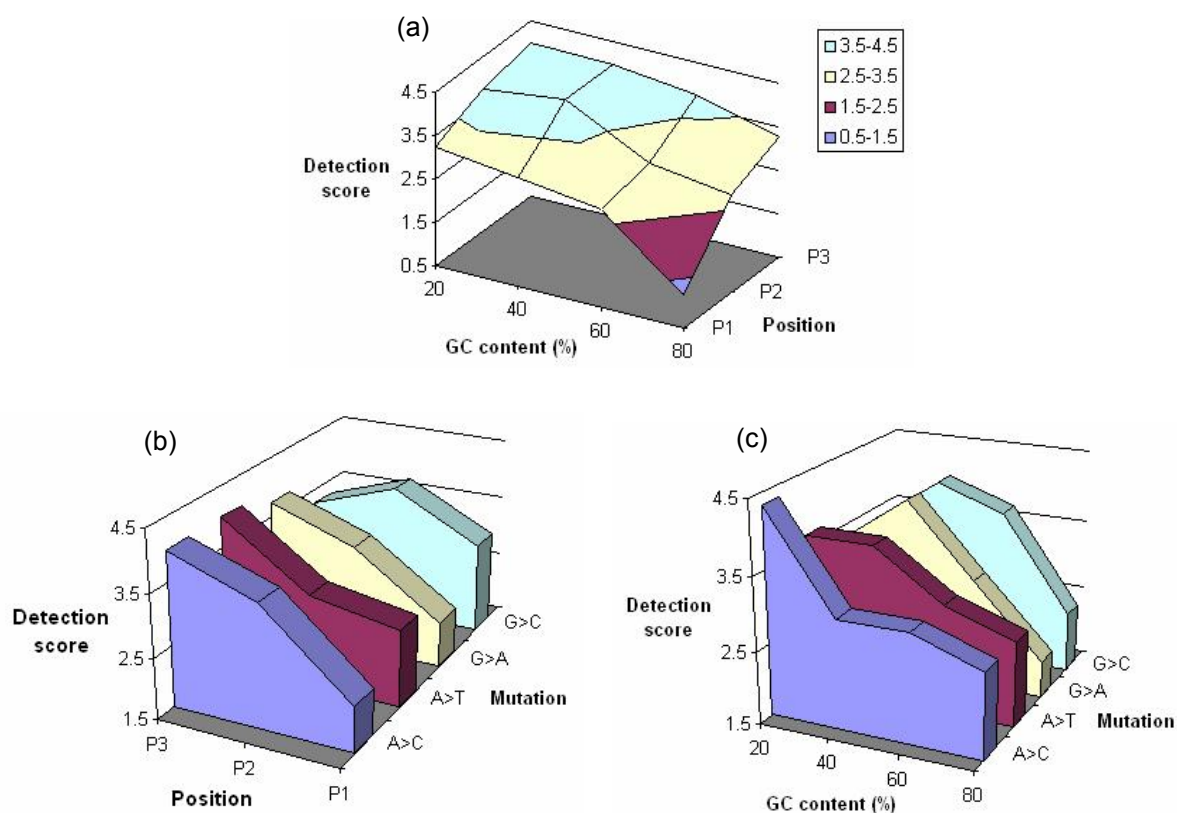
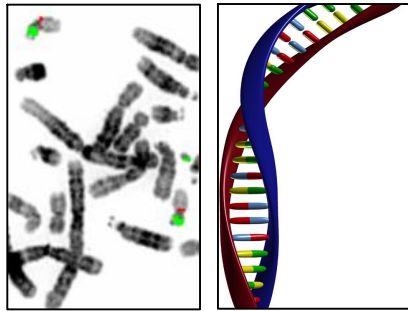


Figure 13: The effect the GC content of the amplicon (a and c), the position of the mutation (a and b) and the nature of the mutation (b and c) on the detection capability of CSCE.



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