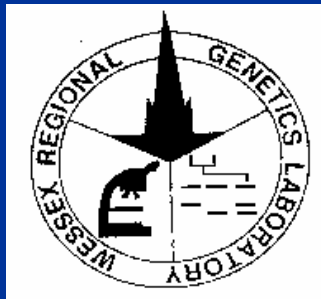


Confirmation Sensitive Capillary Electrophoresis

Chris Mattocks

National Genetics Reference Laboratory (Wessex-Salisbury)



Salisbury



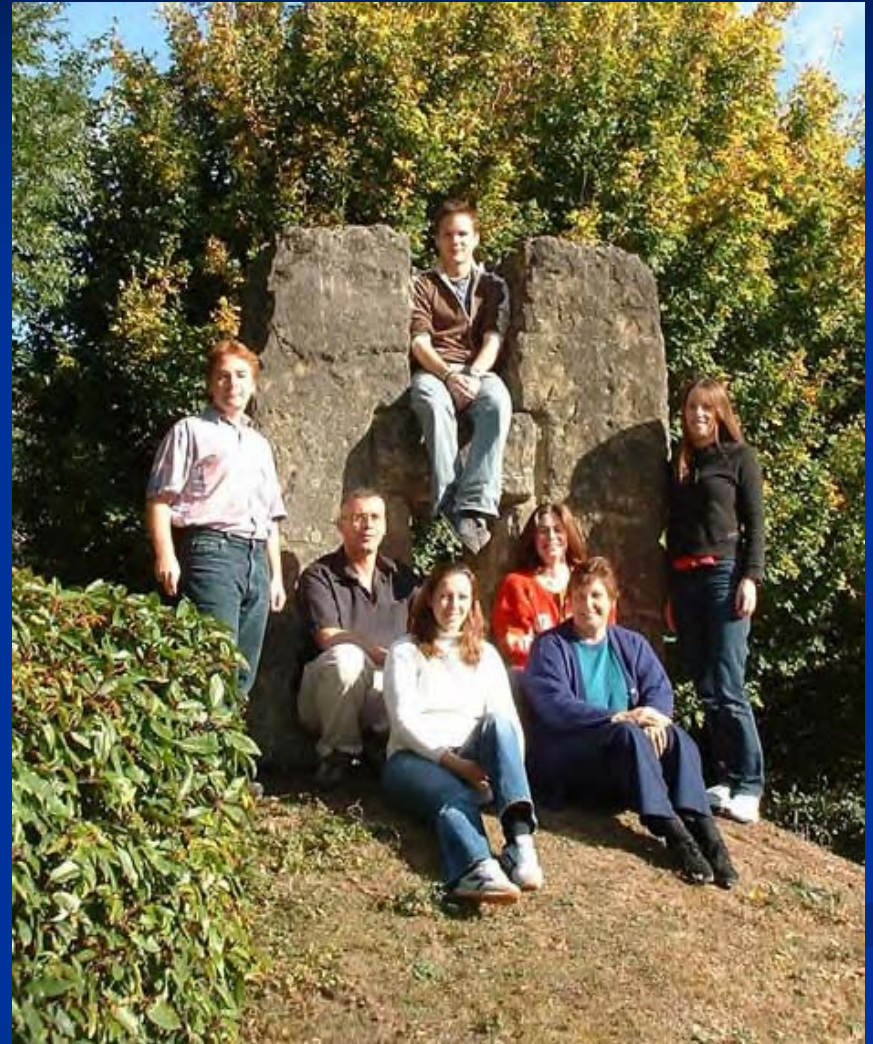
Stone henge



Salisbury Cathedral

NGRL

- Established 2002
- Aims to support and develop genetic testing within the NHS.
- Salisbury (NGRL Wessex)
 - Health technology assessment
 - Reference regents
- Manchester
 - IT focus

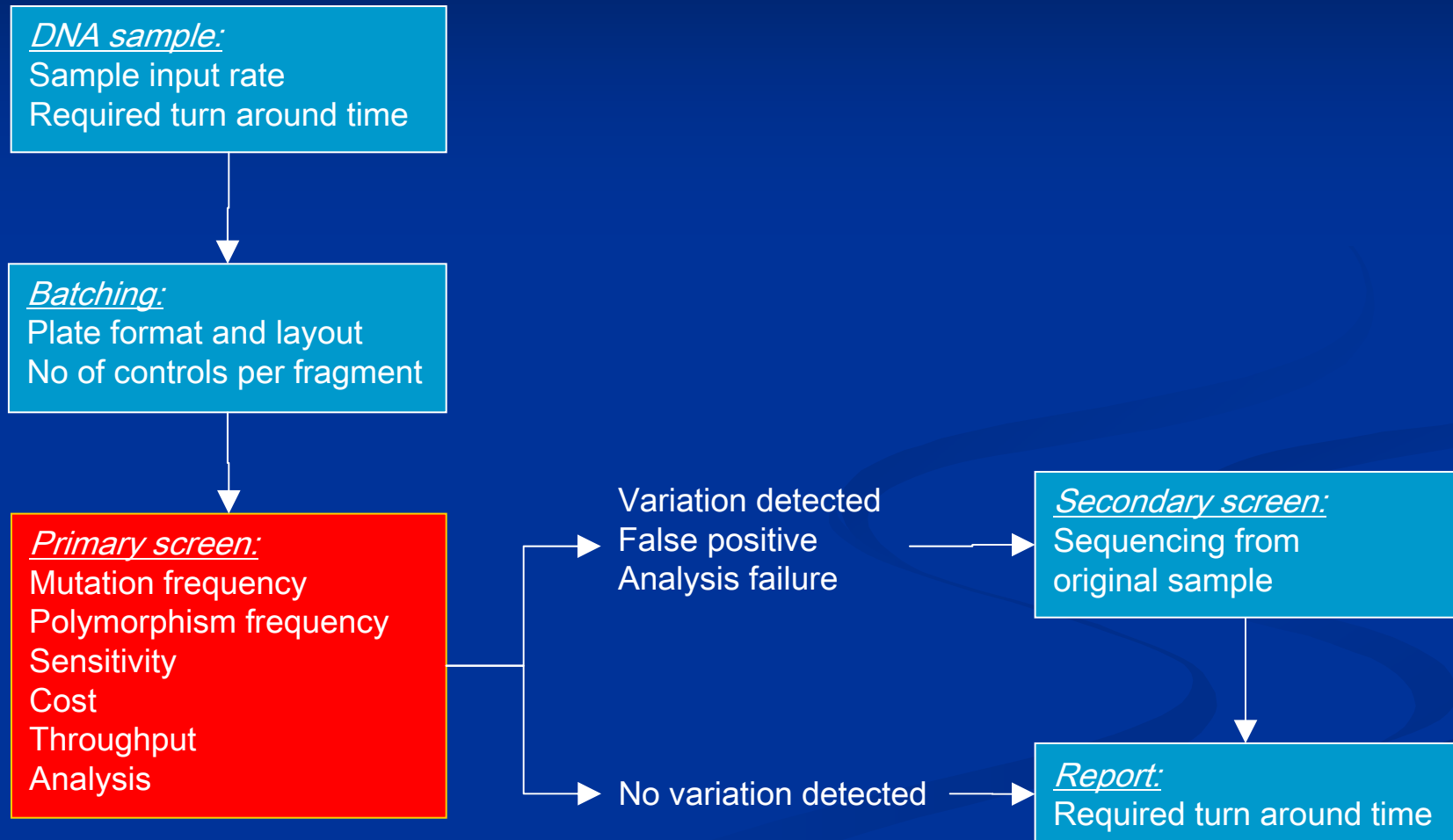


High Throughput Mutation Scanning

- Current need
 - Large cancer genes (BRCA1 & 2, hMLH1, MSH2)
 - Current turn around times
 - Backlogs
- Future need
 - New genes
 - Multi-factorial diseases
 - Phenotypically homogeneous disorders

MUTATION SCANNING

Mutation Scanning Strategy



Strategy evaluation

Model for calculating the relative value of a pre-screen strategy with respect to sequencing

Variables

Sequencing Strategy	
1 Seq orientations [primary screen]	2
2 Seq orientations [secondary screen]	1
3 False positive rate [Pseq]	0.00
4 Failure rate [Fseq]	0.05

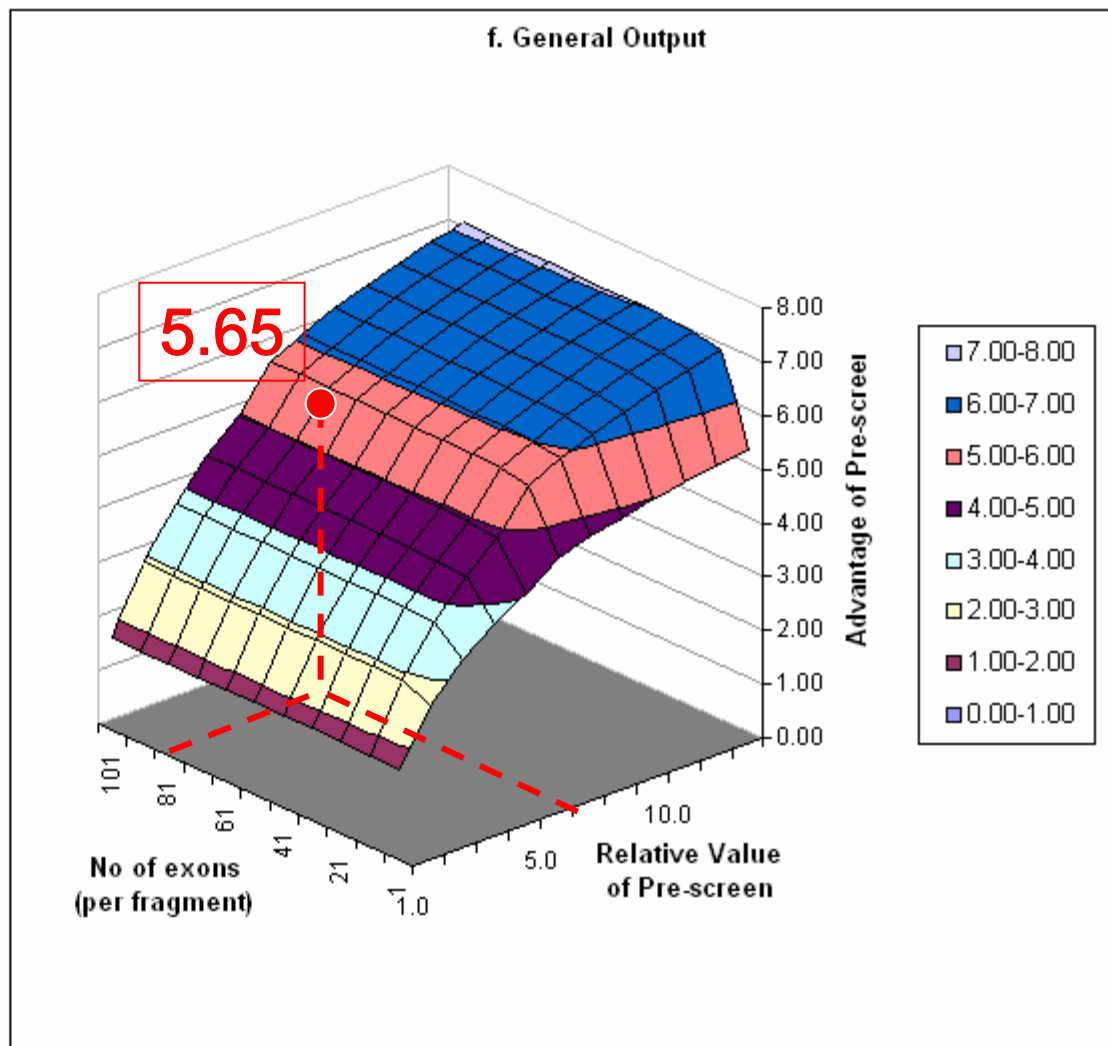
Pre-Screen Strategy	
5 Seq orientations [secondary screen]	1
6 Polymorphisms [N]	0.07
7 False positive rate [Pps]	0.05
8 Failure rate [Fps]	0.05

Test Characteristics	
9 Pick up rate (per patient)	0.15
10 No of exons	85.00
11 Relative value of prescreen [V]	6.66
12 Detection rate (per fragment)	0.0018

Absolute values	
13 Value of single pre-screen rxn	0.25
14 Batch size	1
15 Controls per fragment	0

a Advantage of Pre-screen [A]	5.65
b Value of overall screen (per patient)	52.61
c Value per mutation detected (ps)	350.702
d Value of seq equivalent (per patient)	297.45
e Value per mutation detected (seq)	1983.02

f. General Output



Pick up rate for pathogenic mutations

E.g. BRCA1 & BRCA2 screen requires investigation of 85 fragments and has a pickup rate of ~15% per patient

∴ Detection rate per fragment = $15 \div 85$

= 0.18% of fragments screened!!

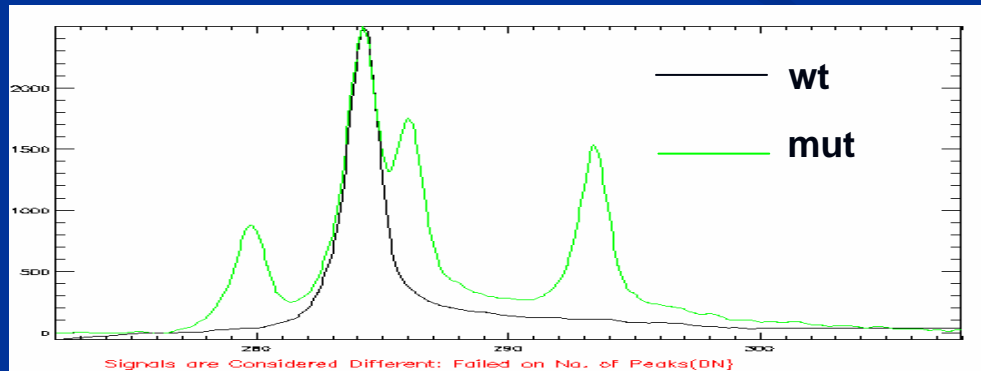
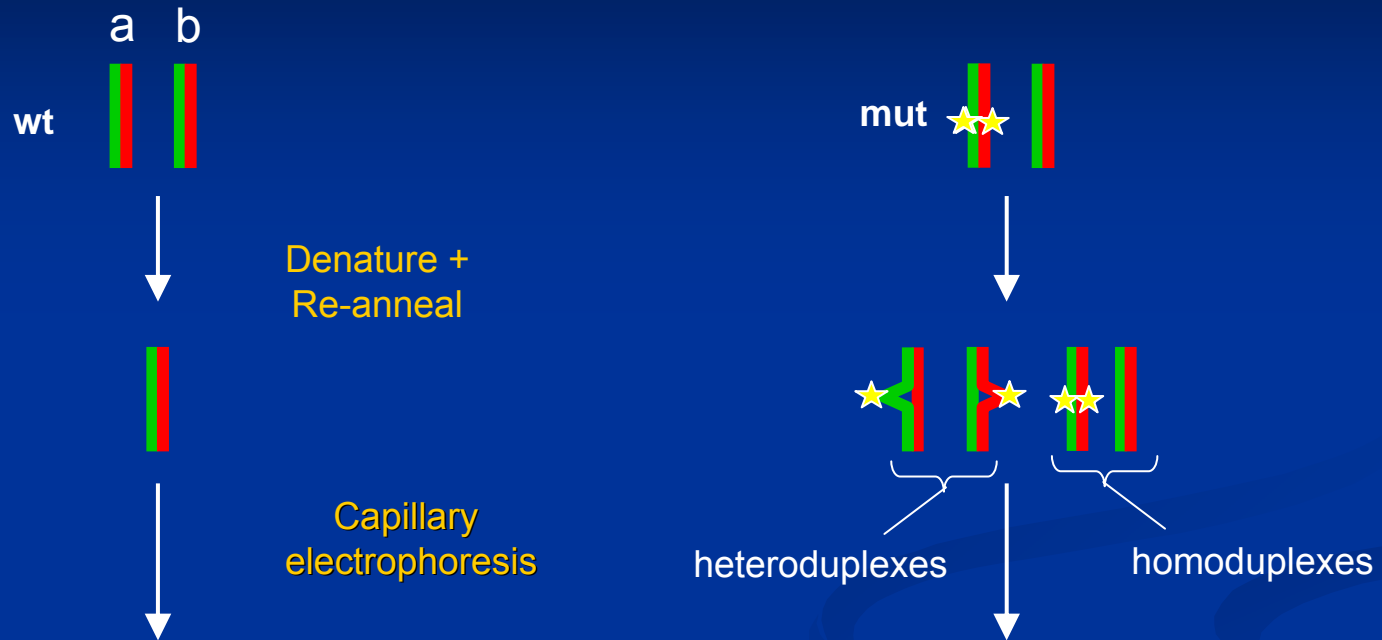
$a \div b = \text{expected pickup rate per fragment}$

Where:

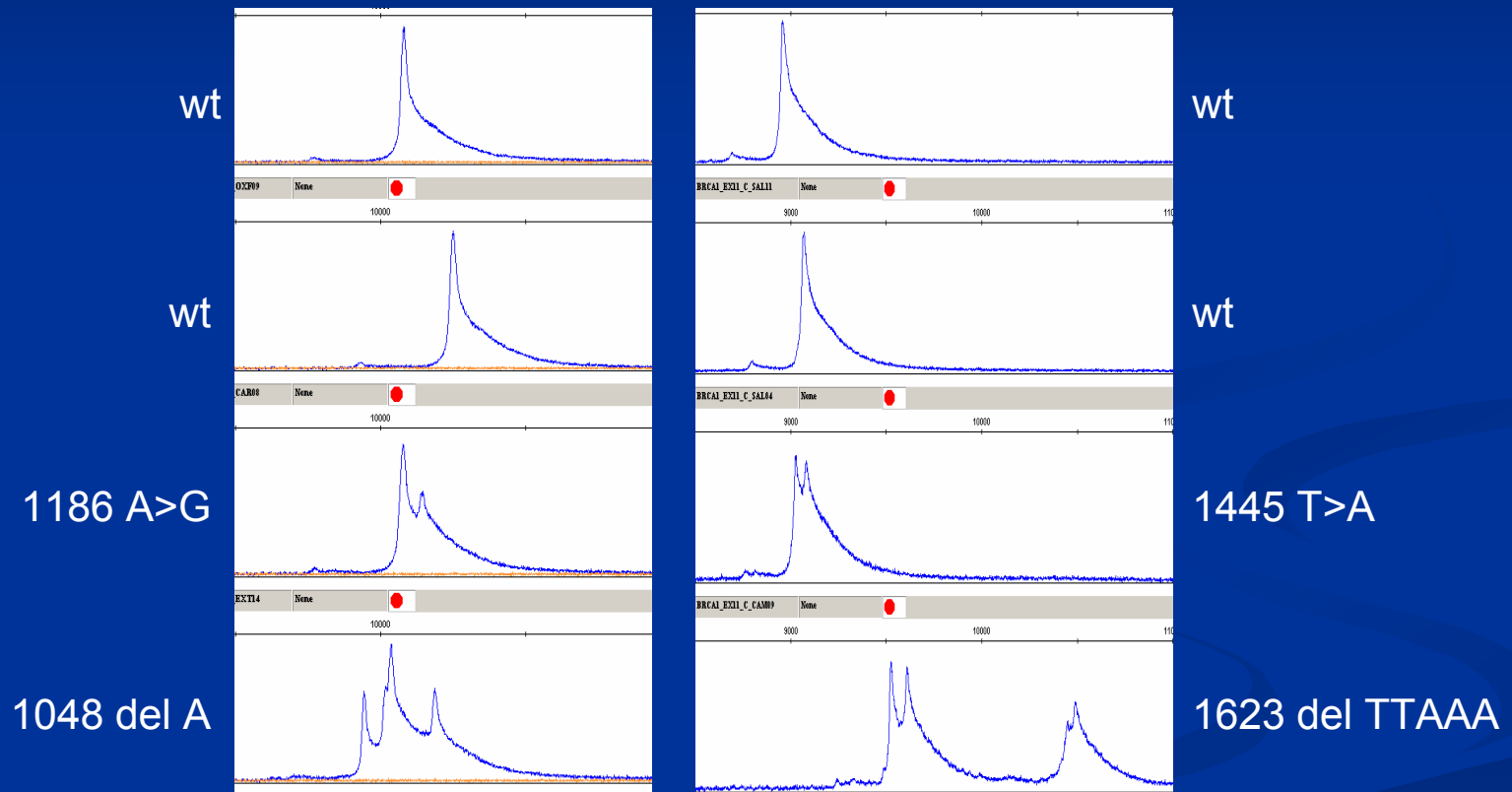
a = Expected pickup rate per patient

b = No of fragments screened per patient

CSCE



CSCE examples (BRCA1)



CSCE Validation

- Validations carried out on
 - Internally and externally extracted DNA samples
 - Retrospective 1350 analyses
 - Prospective 8460 analyses
 - 79 amplicons
 - 128 different mutations (total 348)

Type	Bp	Unique	Total
Deletions	1 to 40	19	52
Insertions	1 to 4	17	17
Point		92	277

- Statistics

Sensitivity	100%
Specificity	93%
Failure rate	4%

Generic Mutation Detection Controls

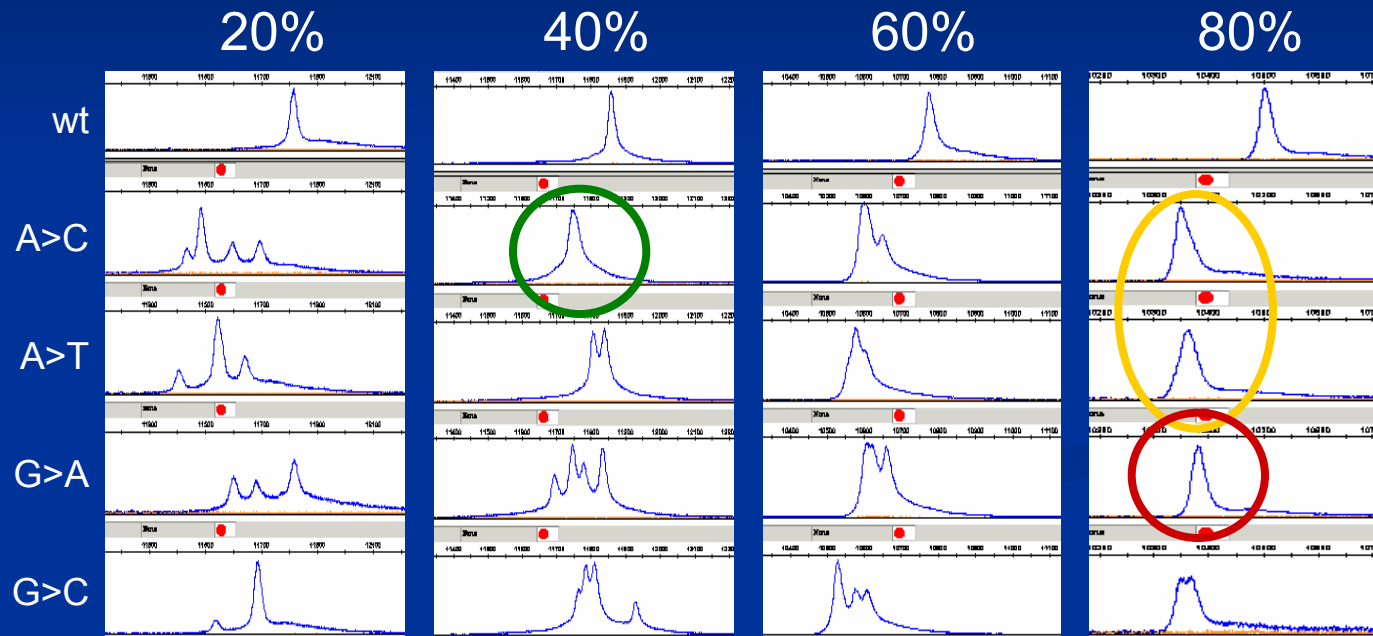
- 52 plasmid controls for systematic evaluation factors that are of general importance for all technologies including:
 - GC content of the amplicon
20%, 40%, 60% and 80%
 - Type of base substitution

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

- Location of the mutation in the fragment.

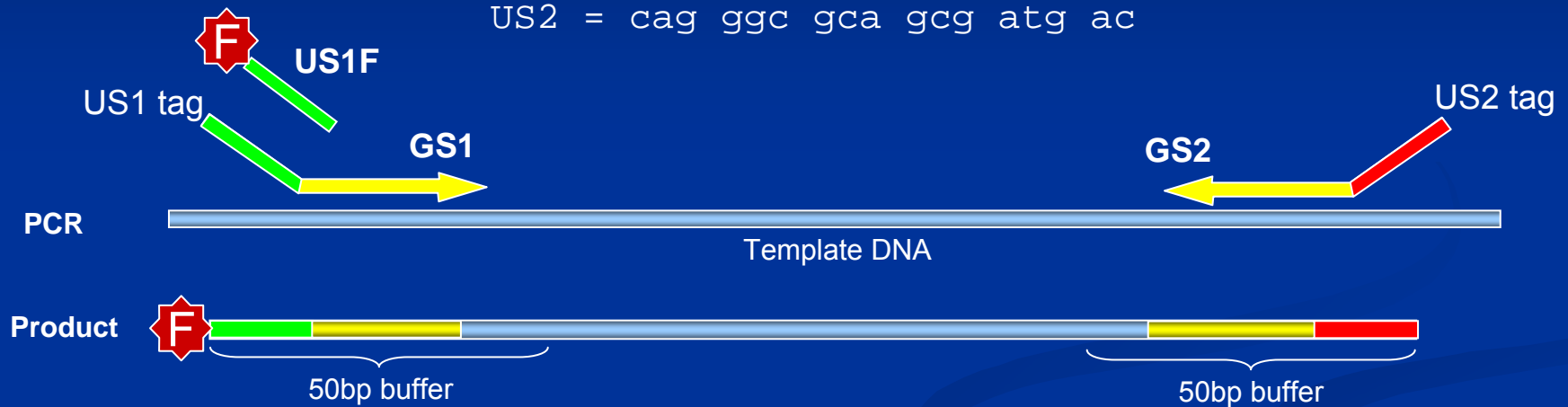


Position 1 heteroduplexes



Assay Design: Standardised primer optimisation and design specification

US1 = gta gcg cga cgg cca gt
 US2 = cag ggc gca gcg atg ac

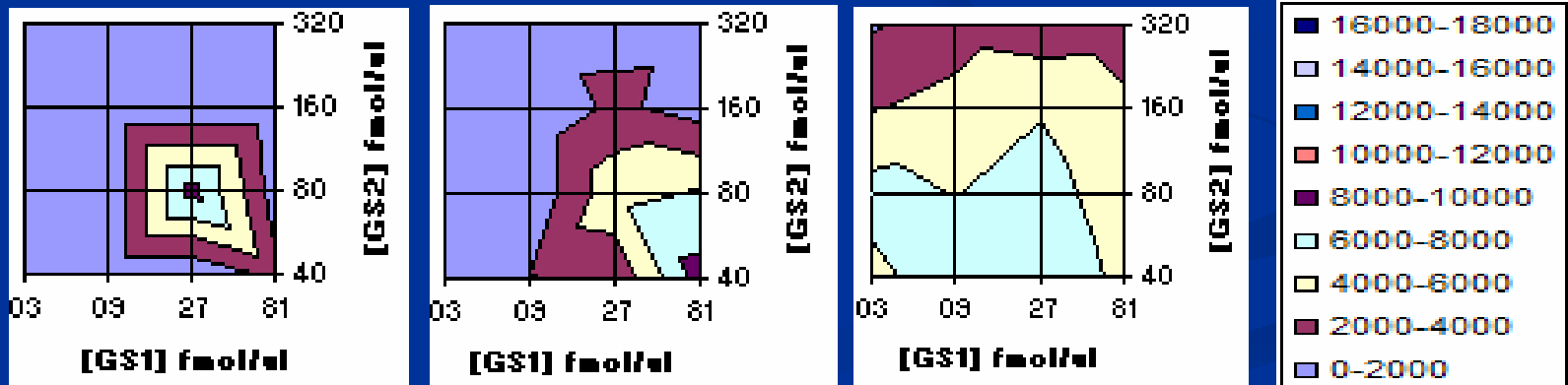


Step	°C	Time		Cycle
Taq activation	95	10	min	
Denature	95	0	sec	
GS annealing	61	30	sec	x40 cycles
Primer extension	72	30	sec	
<i>Final extension</i>	72	5	min	
<i>Hold</i>	15	∞		

Primer optimisation

Aim: clean trace with single peak within analysis window (1000 to 25000 RFU for 3730)

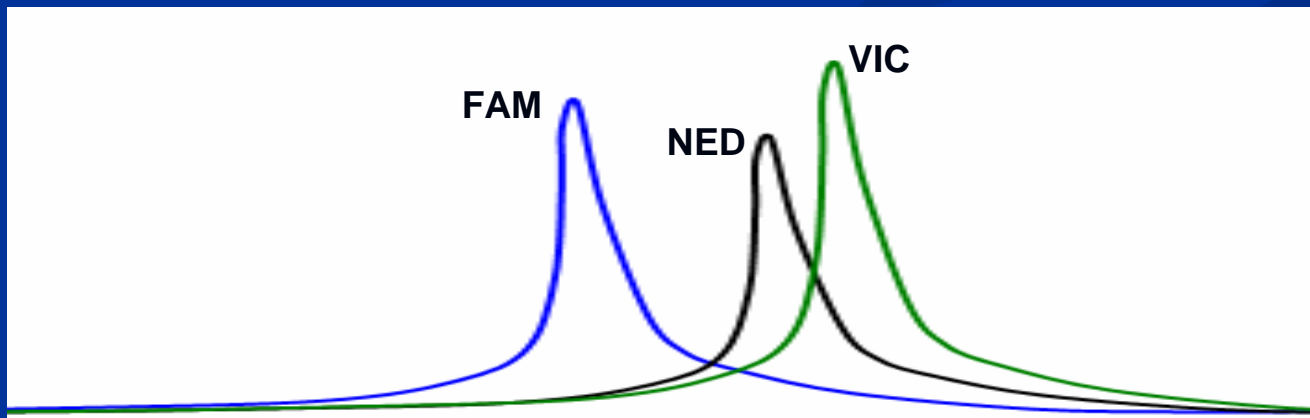
- Primary optimisation [US1F]:[GS1]:[GS2]
 - Determine fixed [US1F] (15 fmol/ μ l reaction)
 - Determine titration ranges for individual optimisations
 - [GS1] 3, 9, 27, 81 fmol/ μ l reaction
 - [GS2] 40, 80, 160, 320 fmol/ μ l reaction
- Individual fragment optimisation [GS1]:[GS2]



Dyes

- ABI fluorescent dyes

- FAM
 - VIC
 - NED
 - PET gives a split signal.
- Comparable signals but altered mobility



PCR

■ Controls

■ Per fragment

- Mutation positive DNA control
- Mutation negative DNA control
- Polymorphism control
- Water control

■ Per run

- 40.1 A>C reference control (x1)

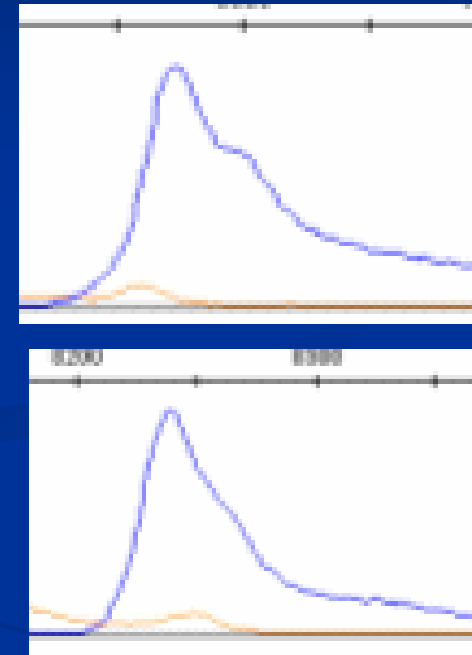
■ Per week

- 40.1 A>C reference control (every capillary)

■ Heteroduplexing

- 95°C x10 mins
- Reduce to 55 °C over 40 mins

40.1 A>C



Post PCR processing

- Dilution 1:100 to 1:2000 in H₂O(1:200)
- Mix products by colour and/or size
 - Size separation 20 to 40 bp
 - Potential up to 30 analyses per capillary
- Add size standard (0.0075µl LIZ 500 per load)
- Loading volume 10 µl
- 2 stage dilution
 - 4 products: 2 µl each + 72 µl H₂O > 1:40
 - 2 µl + 8 µl of H₂O + LIZ500 mix

Machine setup

- 3100, 3130, 3130xl, 3730, 3730xl
- Capillary 36cm or 50cm array (min 200 runs)
- Polymer
 - 5% conformational analysis polymer (AB)
 - Sucrose
 - Urea
- Running buffer 1x TTE (National Diagnostics)
- Spectral
 - For ABI dyes use dye set E5
 - Adjust data delay in spectral collection module to exclude artefactual red peak ~4000 scans



Run conditions

Run Module Editor

Run Module Description

Name: CSCE_CAP_50

Type: REGULAR

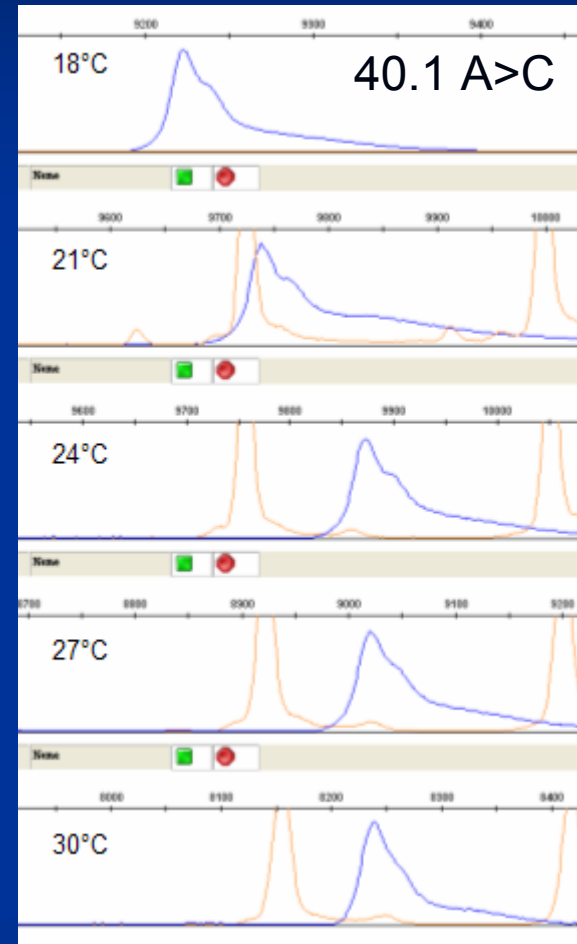
Template: GeneMapper50_POP7_Template

Description:

Run Module Settings

Name	Value	Range
Oven_Temperature	18	18...70 DegC
PreRun_Voltage	15.0	0...15 kV
PreRun_Time	180	1...1800 sec
Injection_Voltage	10.0	0...15 kV
Injection_Time	7	1...90 sec
First_ReadOut_Time	200	100...16000 ms
Second_ReadOut_Time	200	100...16000 ms
Run_Voltage	15.0	0...15 kV
Voltage_Number_Of_Steps	10	0...100 Steps
Voltage_Step_Interval	50	0...180 secs
Voltage_Tolerance	0.6	0...6.0 kV
Current_Stability	10.0	0...2000 uA
Ramp_Delay	500	1...1800 sec
Data_Delay	750	1...1800 sec
Run_Time	2600	300...14000 sec

Ok Cancel

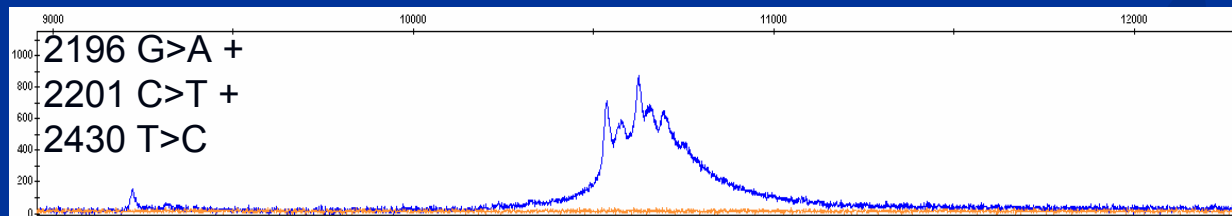
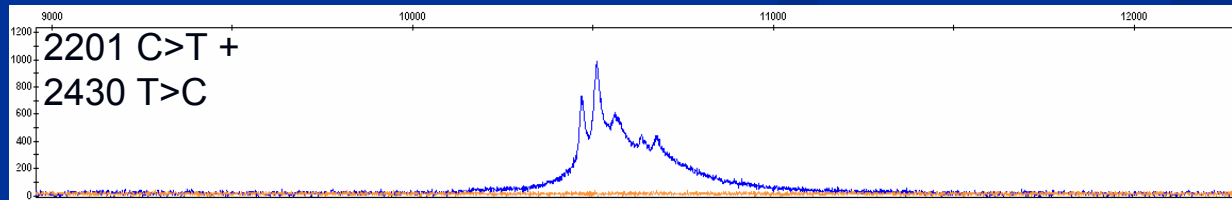
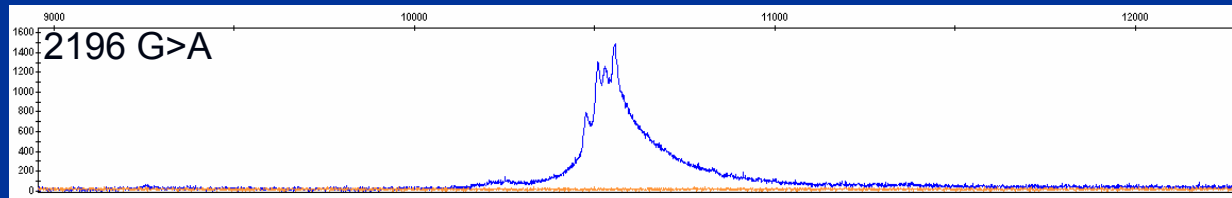
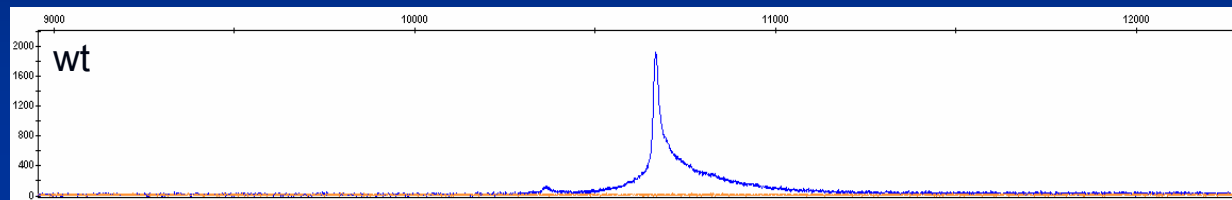


Analysis

- Analysis window
 - 3730: 1000-25000 RFU
 - 3130: 500-5000 RFU
- Manual ~30mins per 96 well plate
- Automated (Bionumerics – Applied Maths)
 - Direct analysis from instrument output
 - Trace identity recognition
 - 6 difference parameters
 - Objective analysis
 - LIMS compatible output

Special cases

Compound heterozygosity



Special cases

Homozygotes

- May give mobility shift (esp. insdels)
- Require normal spike
 - DNA before PCR
 - Amplified control after PCR but before heteroduplexing (eliminates problem of non-amplification)

Conclusions

- CSCE is:
 - Simple to perform
 - Very sensitive (approaching 100%)
 - Easy to automate
 - Easy to analyse
 - Multiplexable (size, colour)
 - High throughput

Acknowledgements

CSCE

- Helen Davies – Cancer Genome group, Sanger institute
- Dan Ward – NGRL (Wessex)
- Nick Owen - NGRL (Wessex)

Generic mutation controls

- Helen White – NGRL (Wessex)
- Gemma Potts – NGRL (Wessex)