Confirmation Sensitive Capillary Electrophoresis

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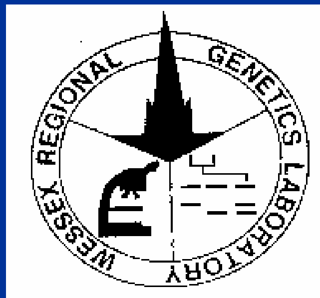


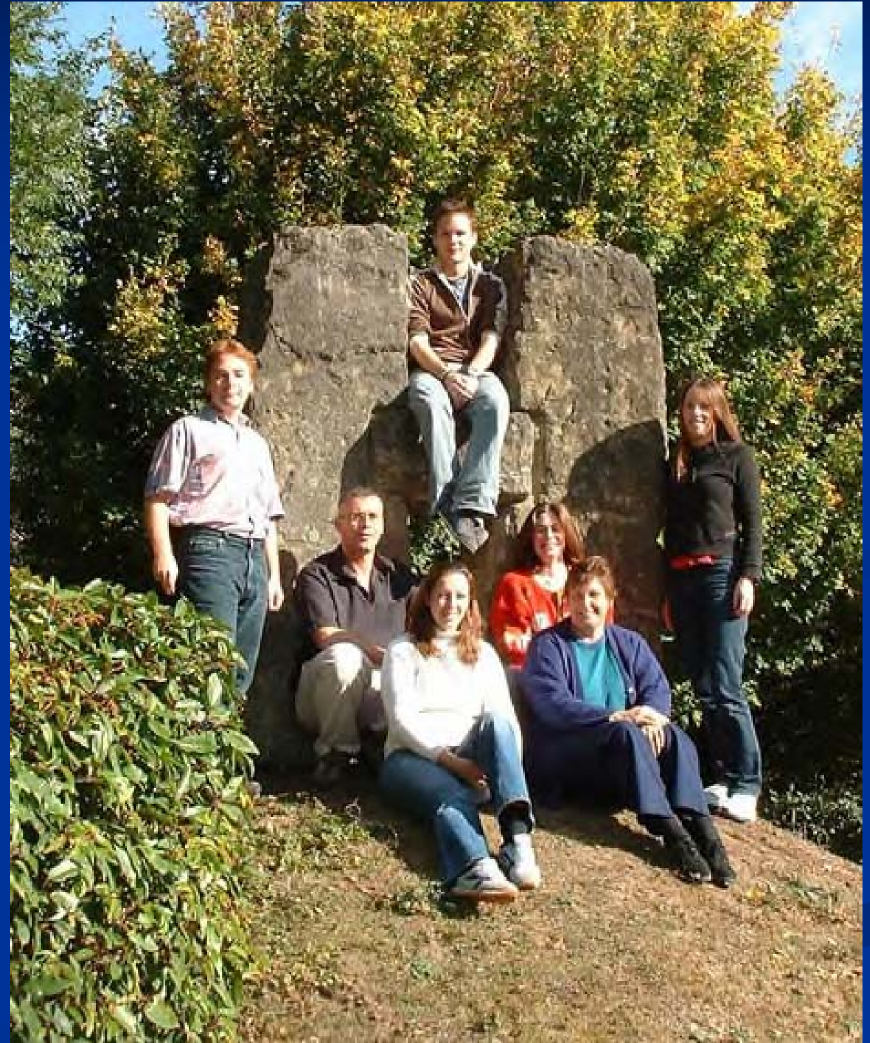
Salisbury

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

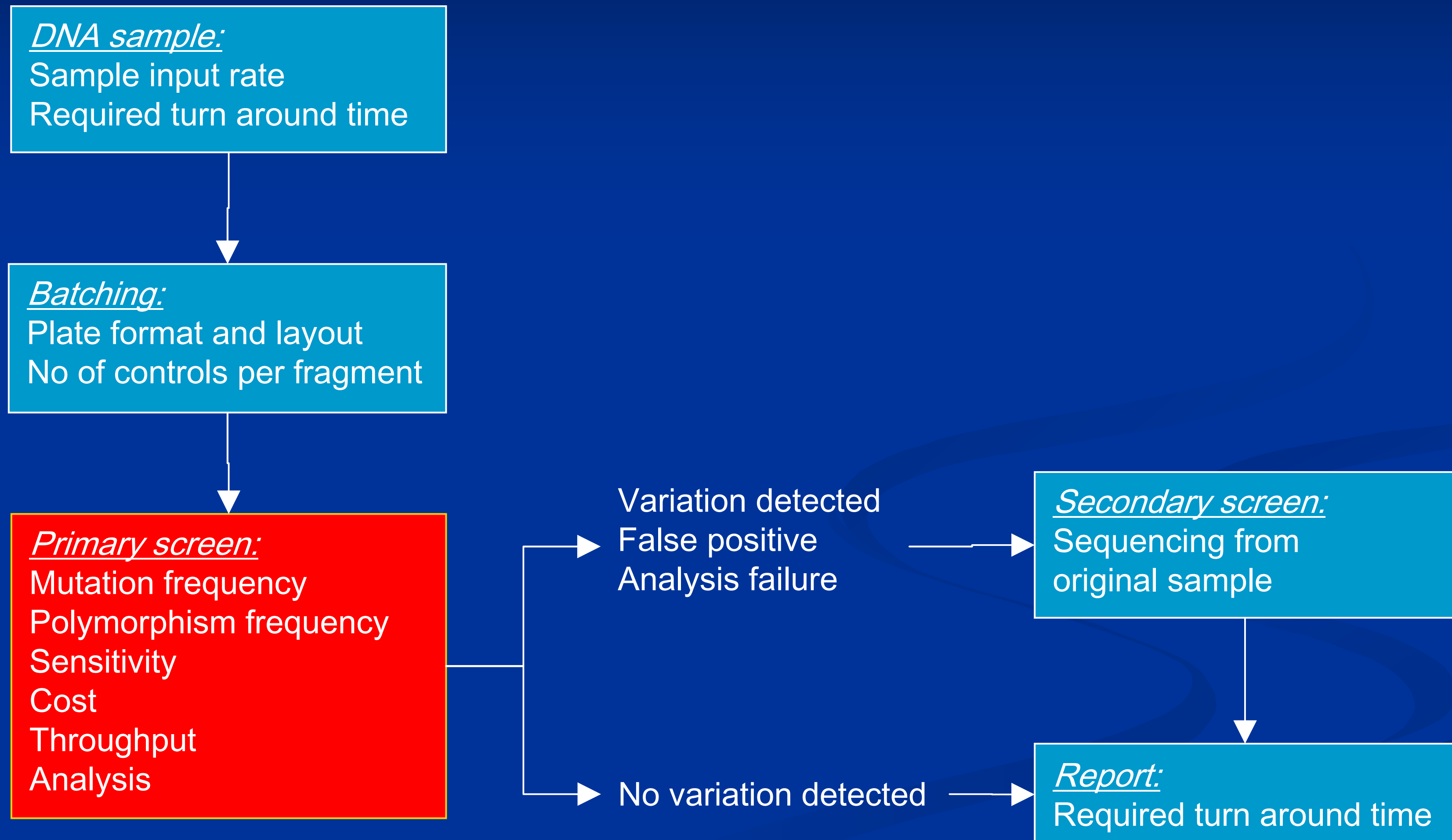


High Throughput Mutation Scanning

- 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

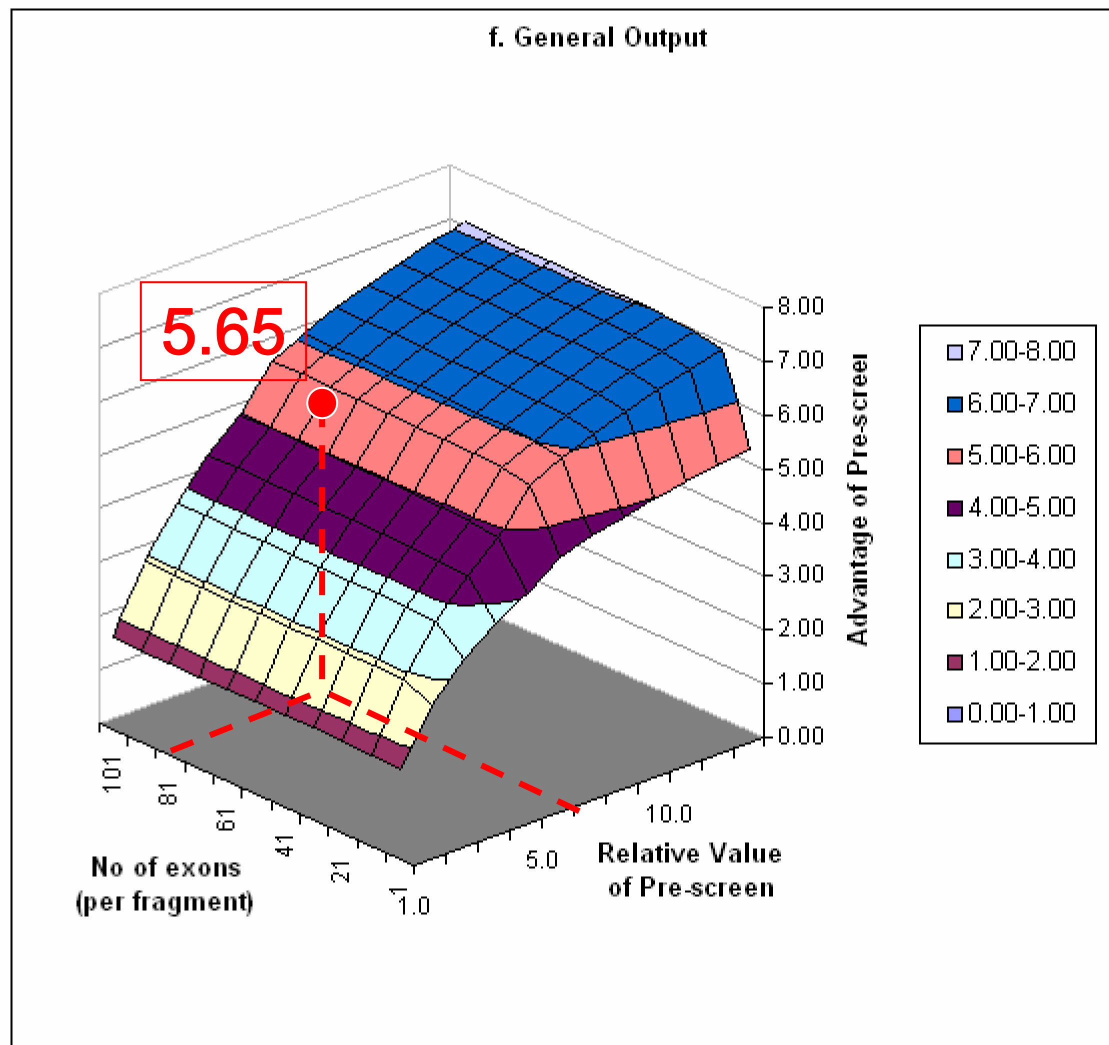
	Sequencing Strategy	
1	Seq orientations [primary screen]	2
2	Seq orientations [secondary screen]	1
3	False positive rate [Pseq]	0.00
4	Failure rate [Fseq]	0.05

	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



www.ngrl.co.uk/wessex

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

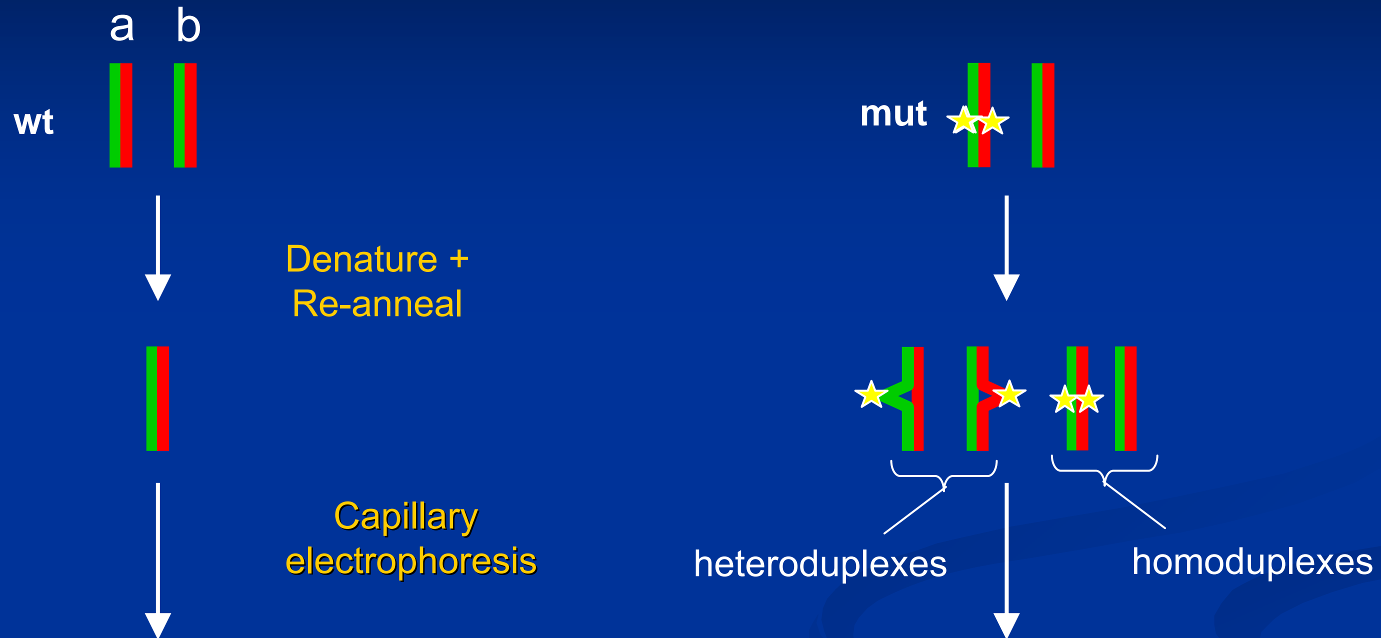
$\therefore$ Detection rate per fragment = 15 $\div$ 85

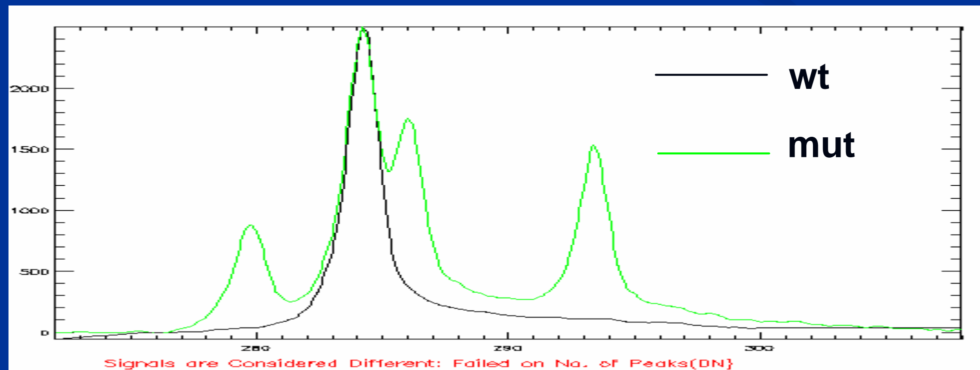
= 0.18% of fragments screened!!

a $\div$ b = expected pickup rate per fragment

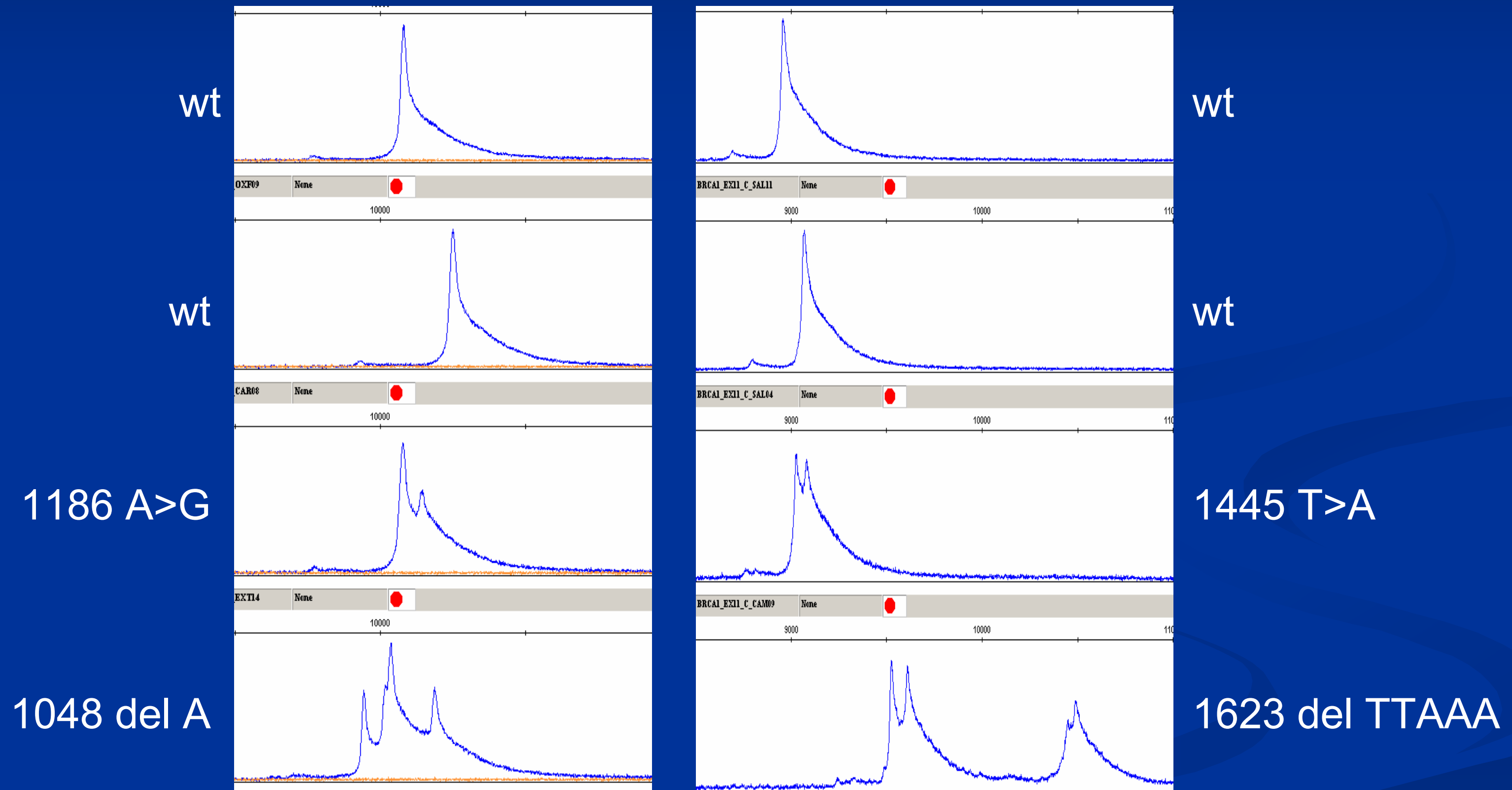
Where:
a = Expected pickup rate per patient
b = No of fragments screened per patient

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

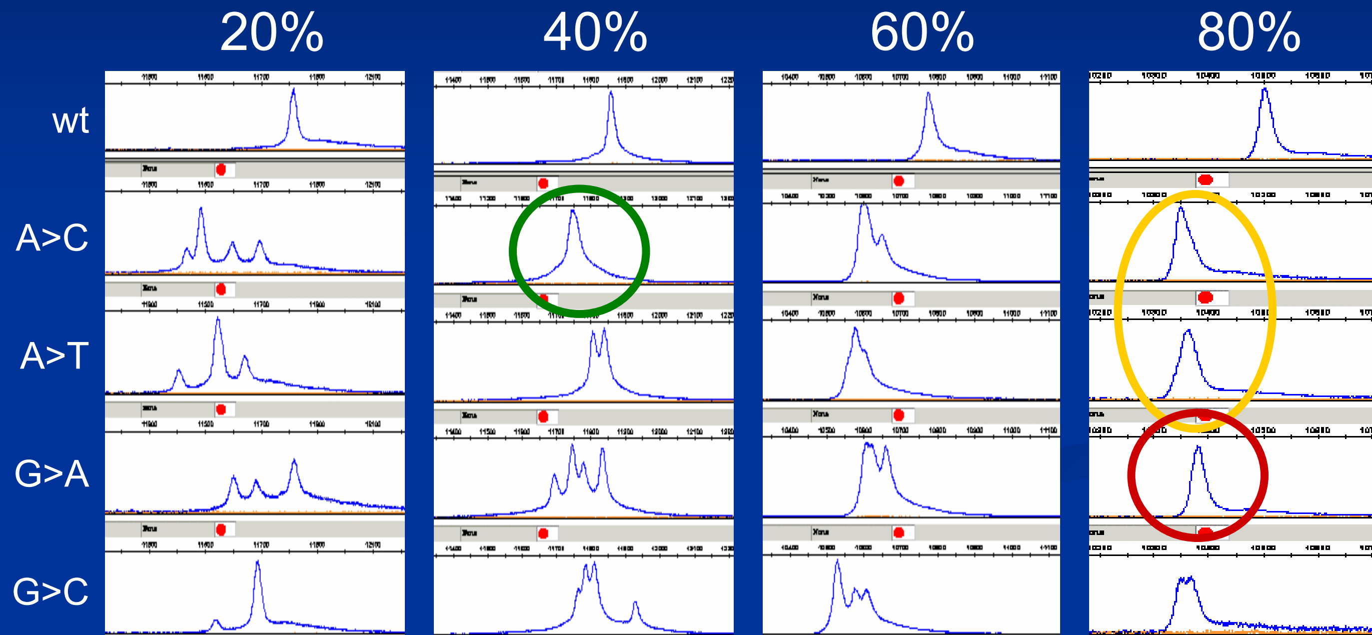
Mutation	Heteroduplexes formed
A>C	C:T & G:A
A>T	T:T & A:A
G>A	A:C & T:G
G>C	C:C & G:G

  - Location of the mutation in the fragment.

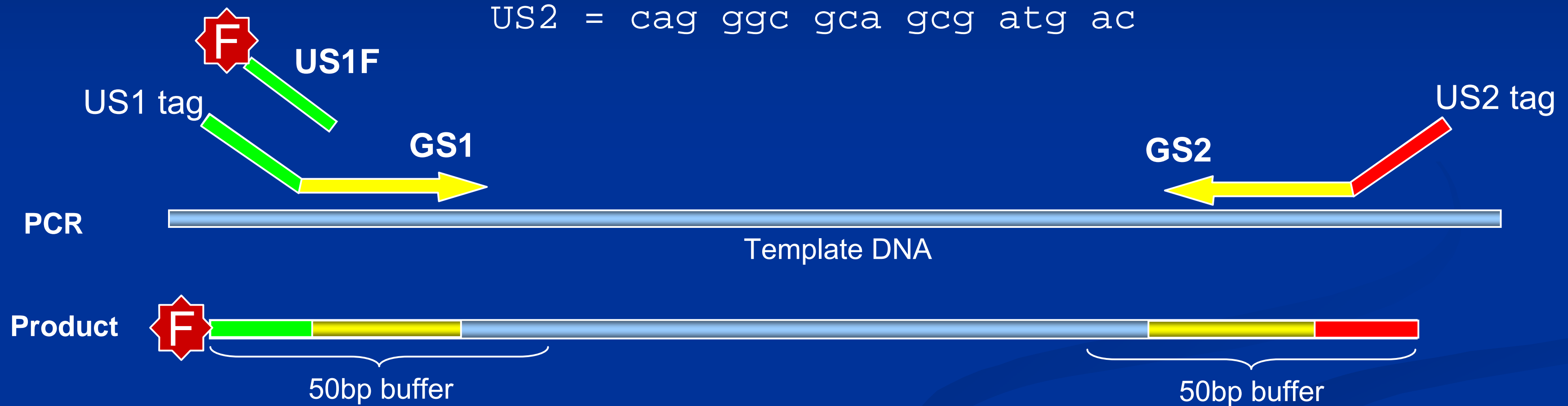
CSCE validation using generic controls

	POSITION 1				POSITION 2				POSITION 3			
	A>C	A>T	G>A	G>C	A>C	A>T	G>A	G>C	A>C	A>T	G>A	G>C
20% GC	1	1	1	1	1	1	1	1	1	1	1	1
40%GC	1	1	1	1	1	1	1	1	1	1	1	1
60% GC	1	1	1	1	1	1	1	1	1	1	1	1
80% GC	0.5	0.5	0	1	1	1	1	1	1	1	1	1

Position 1 heteroduplexes



Assay Design: Standardised primer optimisation and design specification



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	
Final extension	72	5	min	
Hold	15	∞		

Primer optimisation

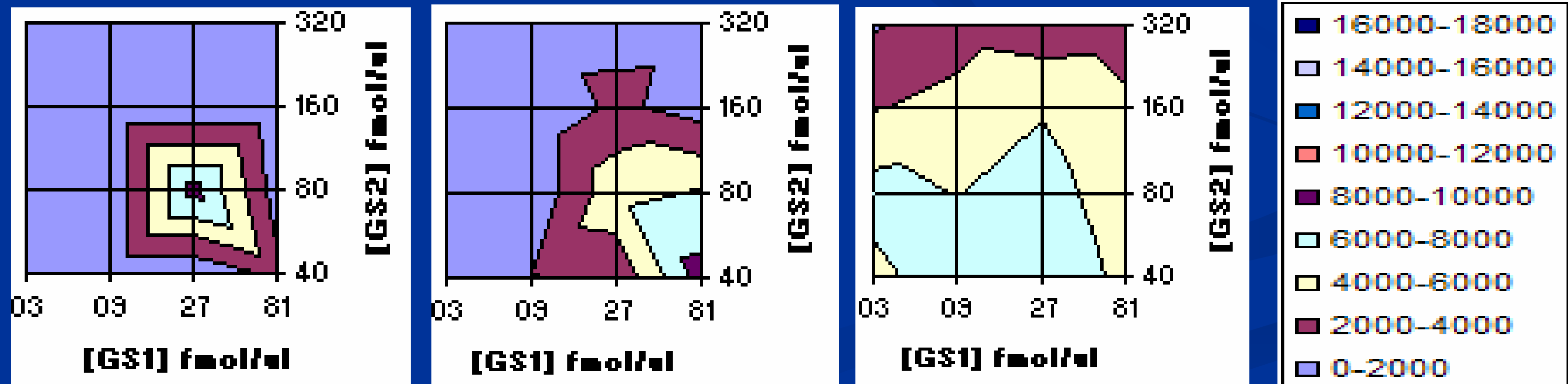
Aim: <u>clean trace with single peak within analysis window (1000 to 25000 RFU for 3730)</u>

- Primary optimisation [US1F]:[GS1]:[GS2]
  - Determine fixed [US1F] (15 fmol/µl reaction)
  - Determine titration ranges for individual optimisations

    [GS1] 3, 9, 27, 81 fmol/µl reaction

    [GS2] 40, 80, 160, 320 fmol/µl reaction

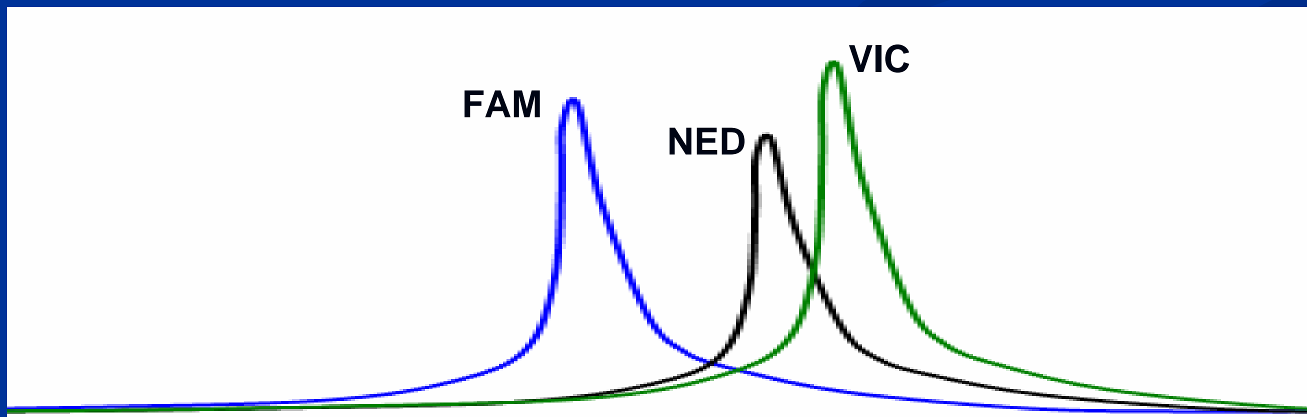
- Individual fragment optimisation [GS1]:[GS2]



Dyes

- ABI fluorescent dyes
  - FAM
  - VIC
  - NED
  - PET gives a split signal.

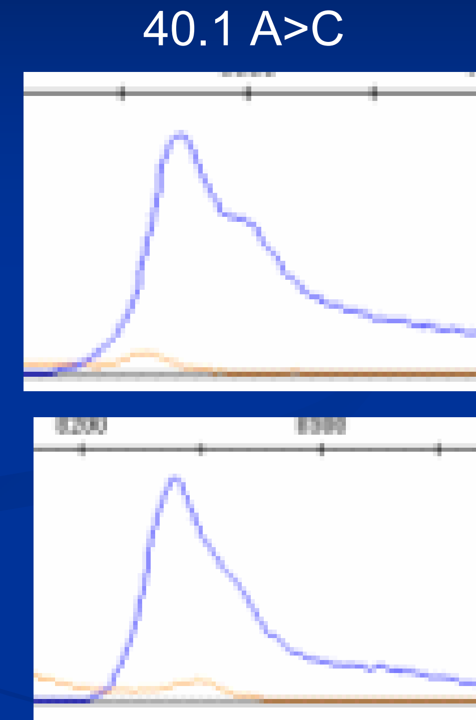
Comparable signals but altered mobility (FAM, VIC, NED)



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)

40.1 A>C



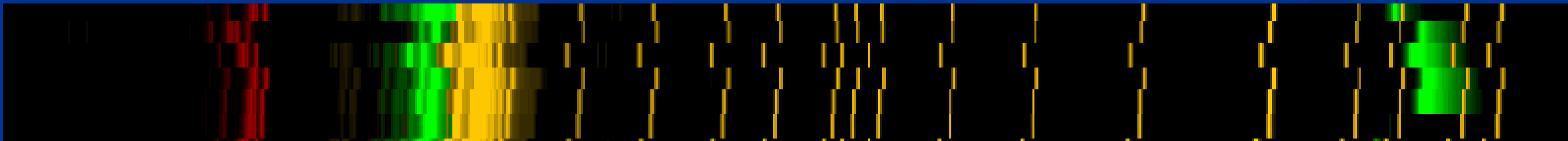
- Heteroduplexing
  - 95°C x10 mins
  - Reduce to 55 °C over 40 mins

Post PCR processing

- Dilution 1:100 to 1:2000 in H_2O(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 H_2O > 1:40
  - 2 µl + 8 µl of H_2O + 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

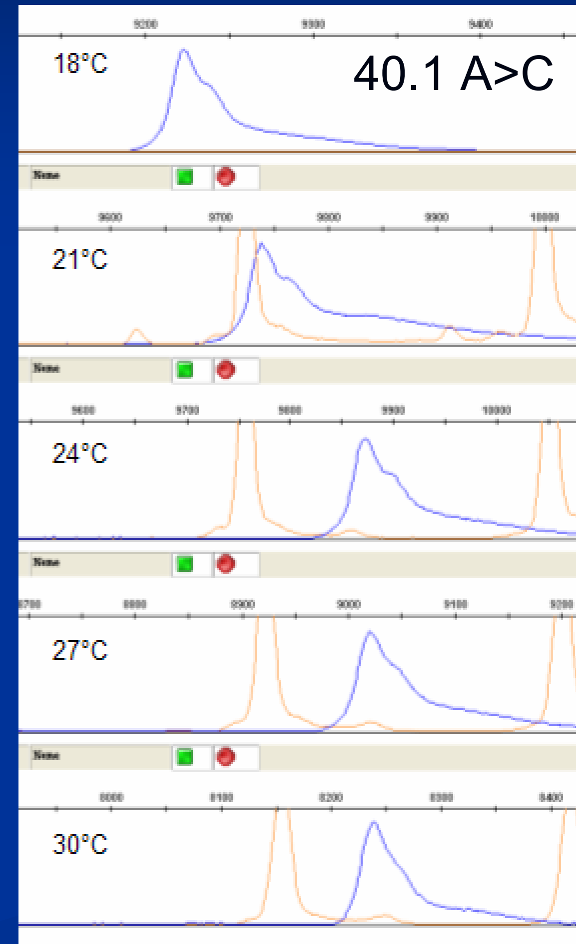
Template: GeneMapper50_POP7_Template

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

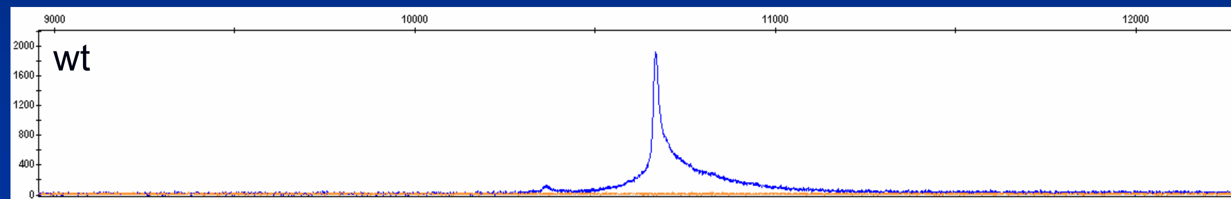


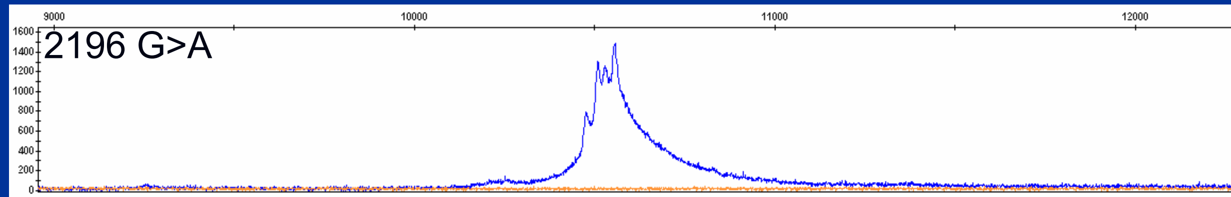
Analysis

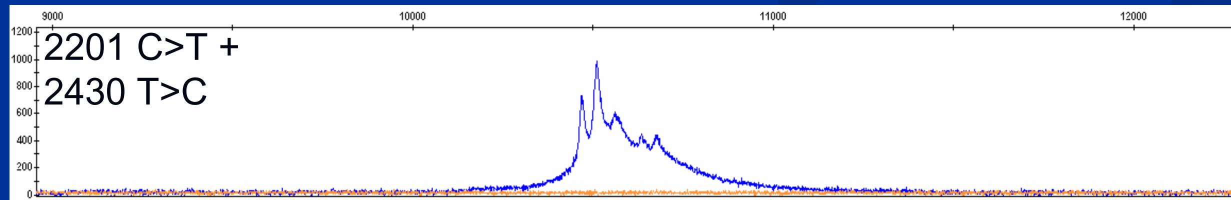
- Analysis window
  - 3730: 1000-25000 RFU
  - 3130: 500-5000 RFU
- Manual ~30mins per 96 well plate
- Automated (Bionumerics – Applied Maths)
  - Direct analysis from instrument output
  - Trace identity recognition
  - 6 difference parameters
  - Objective analysis
  - LIMS compatible output

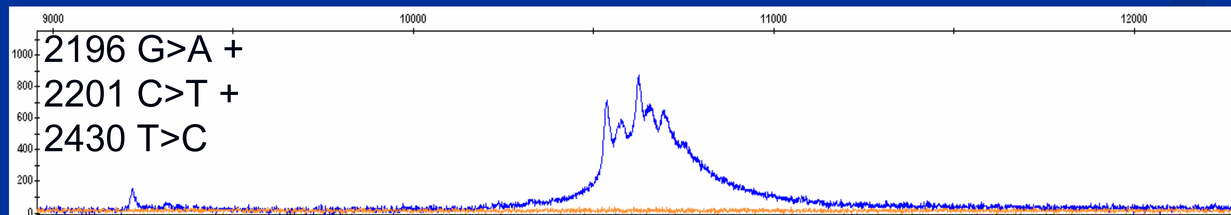
Special cases

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)