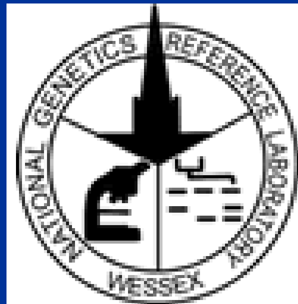
Salisbury Health Care NHS

NHS Trust

Health Technology Assessment of the Nanogen® Molecular Biology Workstation and Pyrosequencing™ PSQMA System



Helen White

National Genetics Reference Laboratory (Wessex)

Salisbury District Hospital

Nanogen® Molecular Biology Workstation

October 2002 – August 2003

Automated multi-purpose instrument which uses the NanoChip® Electronic Microarray for:

- SNP detection
- STR analysis
- gene expression
- unknown mutation screening

3 major subsystems

NanoChip® Loader:

96 patient samples loaded onto 1 - 4 NanoChip® Cartridges

NanoChip® Reader:

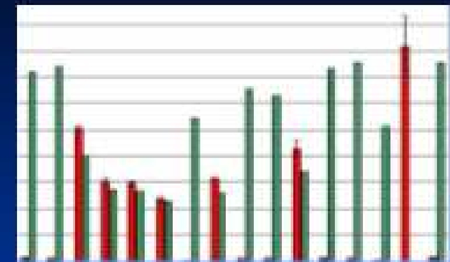
laser-based fluorescence scanner for detection of assay results

Computer hardware and software:
automates import, analysis and export of data

Instrumentation Process

Blank Cartridge

Microtiterplate with Samples



Detection and Analysis

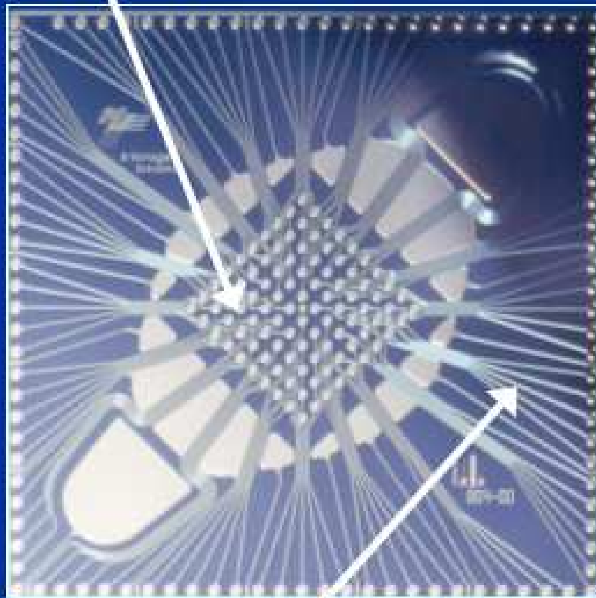
Addressing
Hybridization

Loaded Cartridge

NanoChip® Electronic Microarray Structure: Microelectrode and Permeation Layer

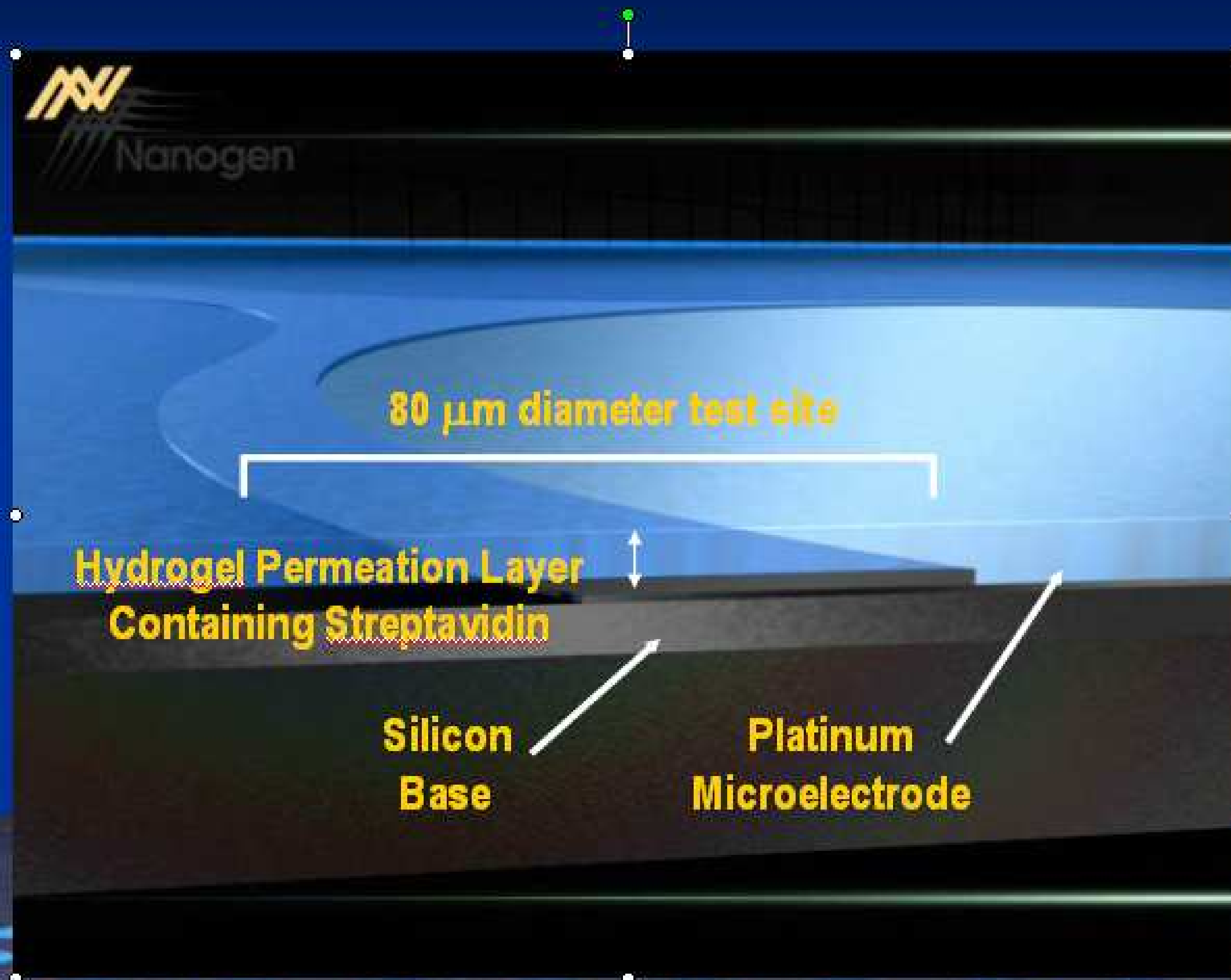
Charged molecules placed at specific test sites on a NanoChip® microarray:

Test site (Electrodes)

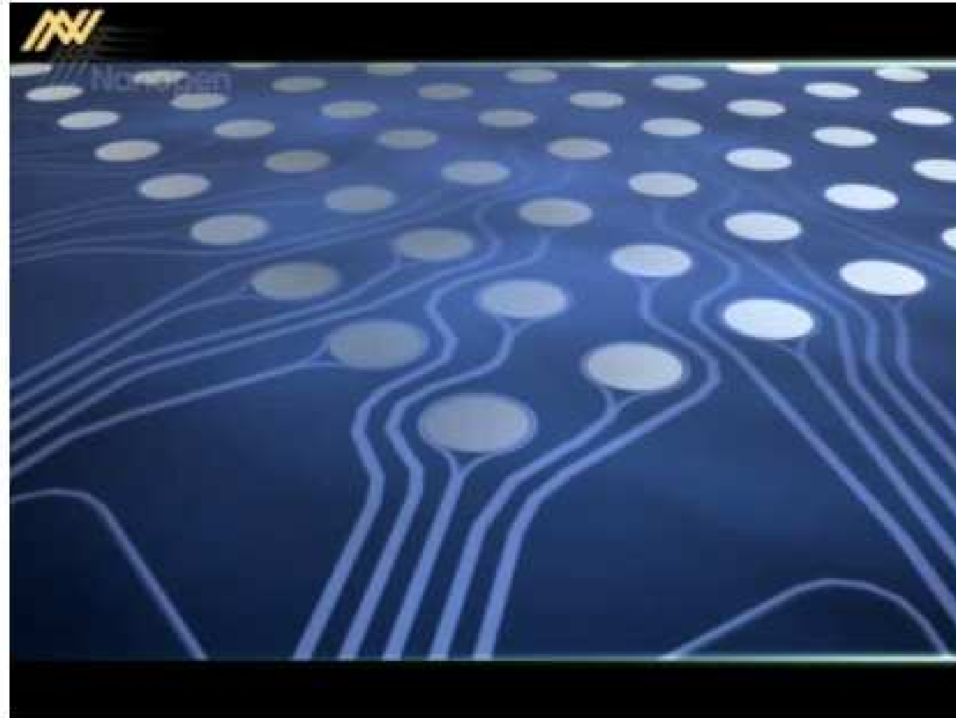


Connections (platinum wires)

NanoChip® Electronic Microarray Structure:



Electronic Addressing



- One or more sites is electronically activated
- The sample is electronically guided to the test site(s)
- The biotinylated sample then binds to the streptavidin
- The pH at each test site is controlled electrochemically allowing for binding at only specified sites.

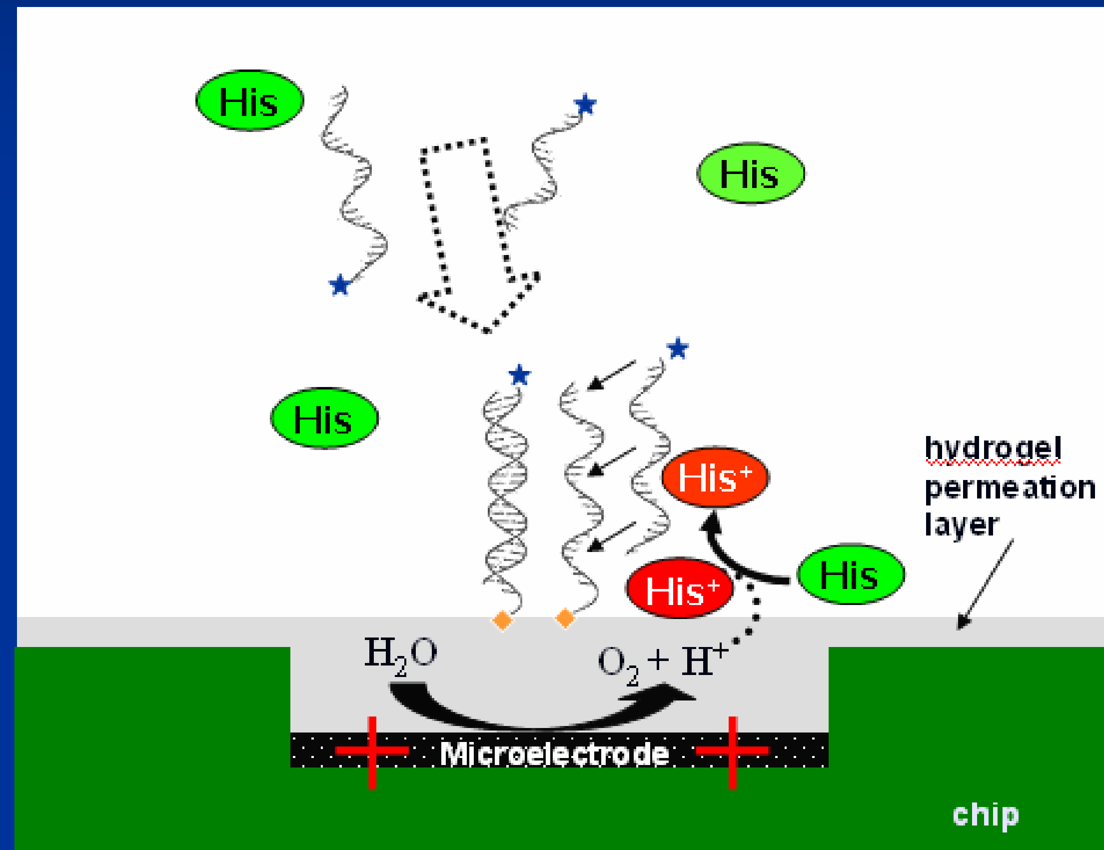
Effect of Histidine Buffer On Hybridization

Positive charge is applied:

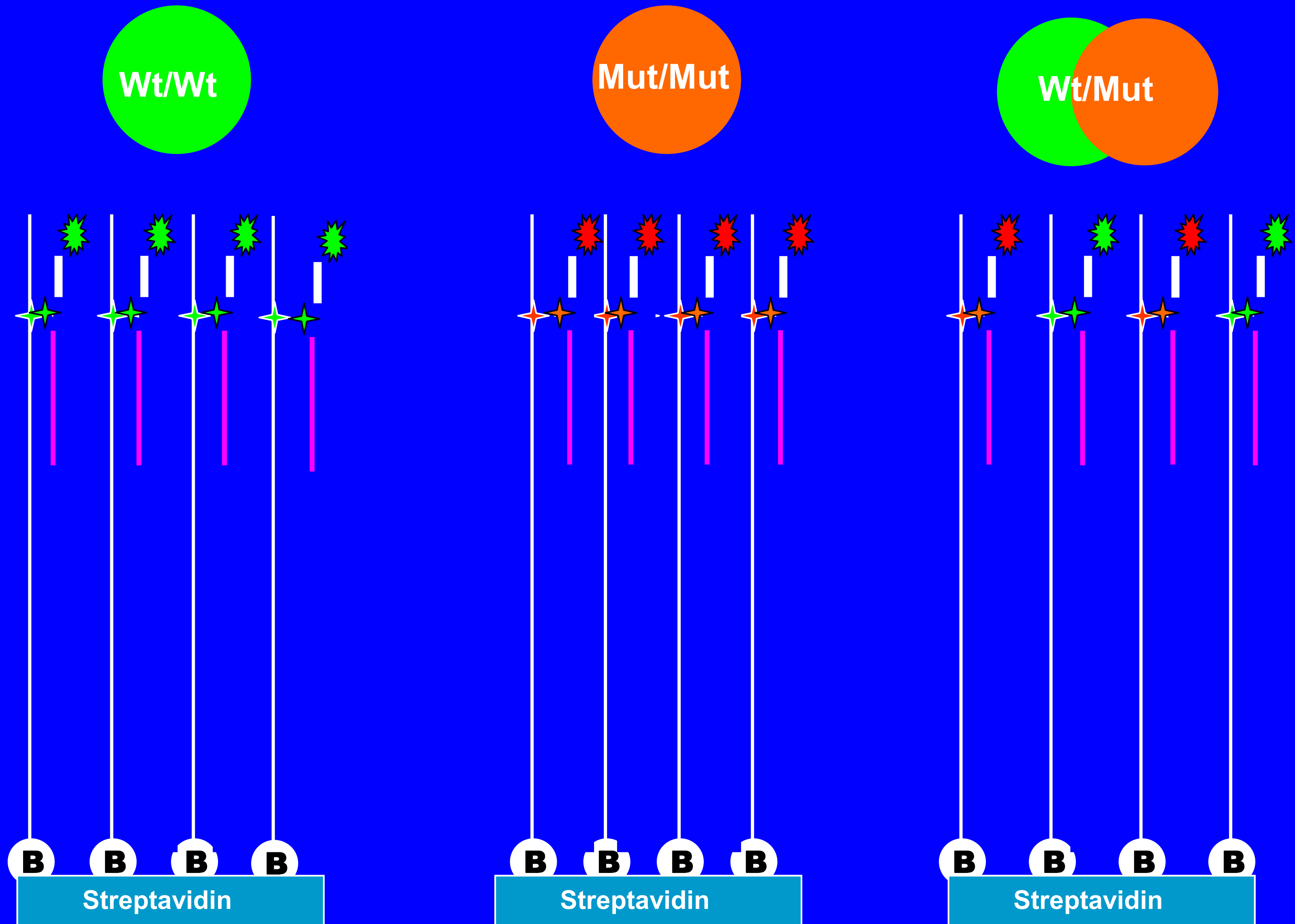
Water is oxidized into oxygen gas and H+ ions at the microelectrode surface

Neutral histidine becomes Histidine+

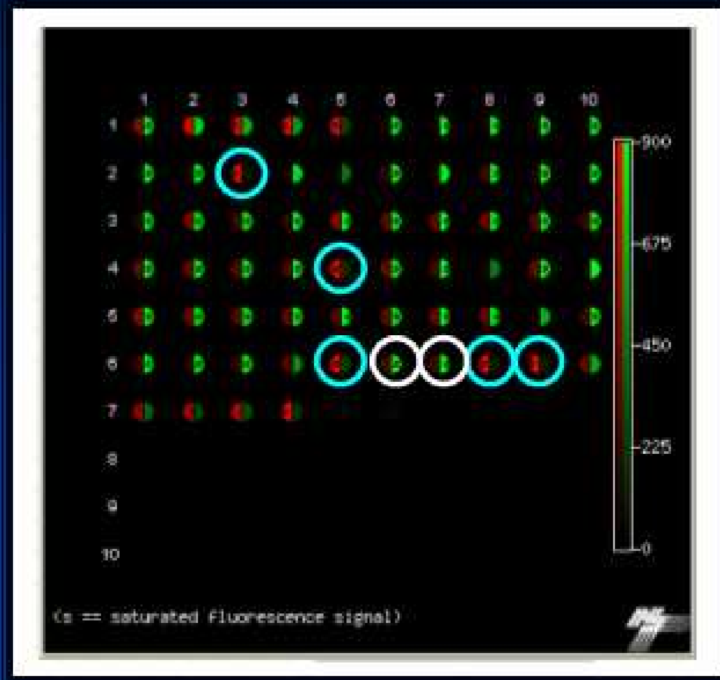
The Histidine+ neutralizes the negatively charged phosphate backbone of the DNA, allowing hybridization to occur.



How does SNP Detection Work?



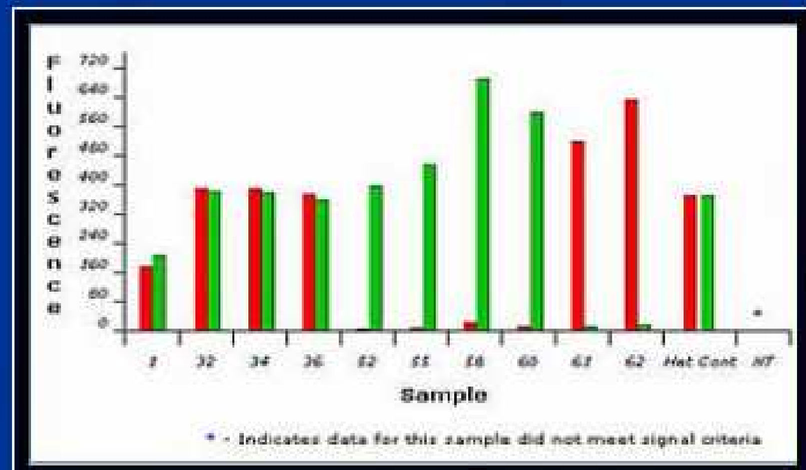
Examples of formats for data output



Fluorescence Scan Results:

	1	2	3	4	5	6	7	8	9	10
1	161/319	273/260	349/214	382/178	497/68	25/494	20/490	21/481	17/478	15/389
2	19/355	22/342	400/32	27/285	18/141	47/552	22/307	59/594	33/496	22/444
3	37/461	120/533	75/583	49/546	146/611	94/587	95/569	104/492	83/527	65/549
4	102/520	104/507	95/536	83/461	481/59	121/542	71/510	21/167	107/558	18/327
5	149/520	111/525	103/419	109/506	94/484	111/552	111/559	106/588	17/210	57/565
6	62/485	35/338	66/402	96/197	498/71	91/488	77/545	411/37	457/38	121/207
7	181/123	208/84	244/102	372/64	21/17	16/15	—	—	—	—
8	—	—	—	—	—	—	—	—	—	—
9	—	—	—	—	—	—	—	—	—	—
10	—	—	—	—	—	—	—	—	—	—

Results for factorV (27 scan, 1024, low)					
Sample	Red	Green	Pads	Ratio (R::G)	Probe Designation
1	187.56	218	1	1 :: 1.16	snp/wt
32	407.86	402	1	1.01 :: 1	snp/wt
34	407.86	398	1	1.02 :: 1	snp/wt
36	392.98	377	1	1.04 :: 1	snp/wt
52	5.95	416	1	1 :: 69.87	wt/wt
55	11.91	479	1	1 :: 40.22	wt/wt
58	26.79	727	1	1 :: 27.13	wt/wt
60	14.89	628	1	1 :: 42.19	wt/wt
61	547.79	15	1	36.52 :: 1	snp/snp
62	666.87	18	1	37.05 :: 1	snp/snp
Het Cont	390	390	1	1 :: 1	snp/wt
NT	N/A	N/A	0	N/A	N/A



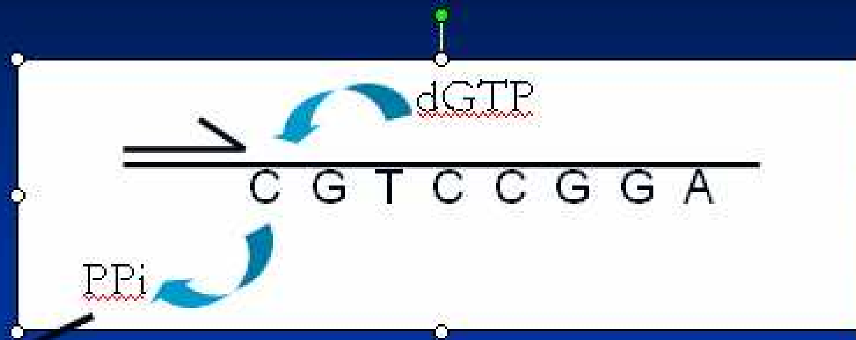
Pyrosequencing™ PSQMA System

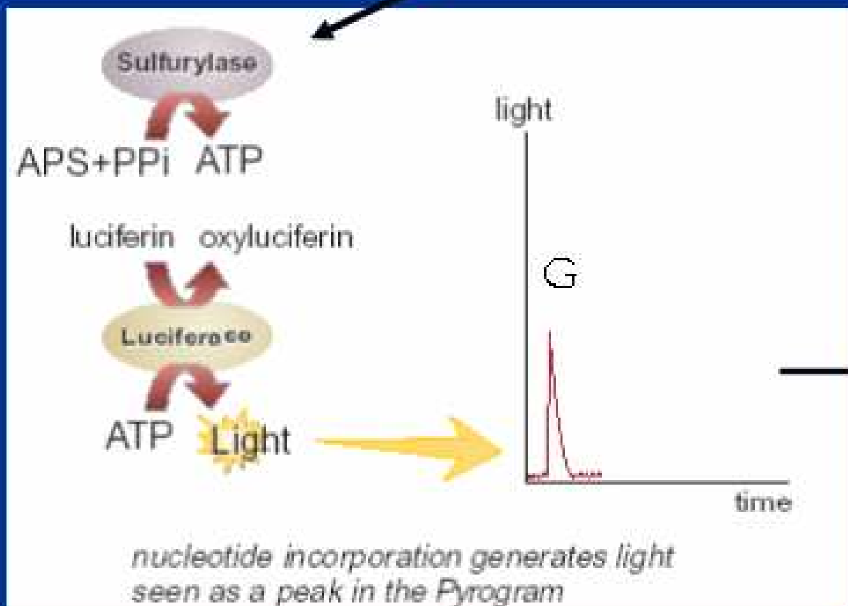
May 2003 – ongoing

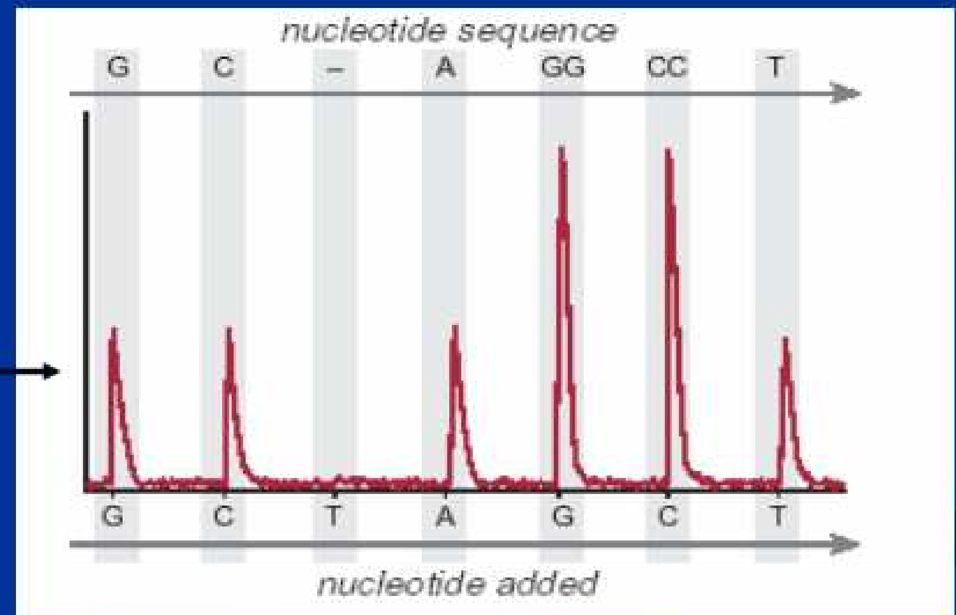
Real time sequencing method for the analysis of short to medium length DNA sequences. Can be used for many applications including:

- SNP analysis
- Allele frequency quantification
- STR analysis
- CpG methylation analysis
- Gene dosage
- Microbiological typing

Principle of Pyrosequencing







Evaluation of SNP detection

- **90 DNA samples were genotyped using triplex PCR assays for Factor V Leiden G1691, Prothrombin G20210A and MTHFR C677T.**

Samples had been previously genotyped using PCR RFLP analysis by Molecular Pathology Unit at Southampton General Hospital

- **50 DNA samples were genotyped for six mitochondrial mutations and the degree of heteroplasmy was also determined.**

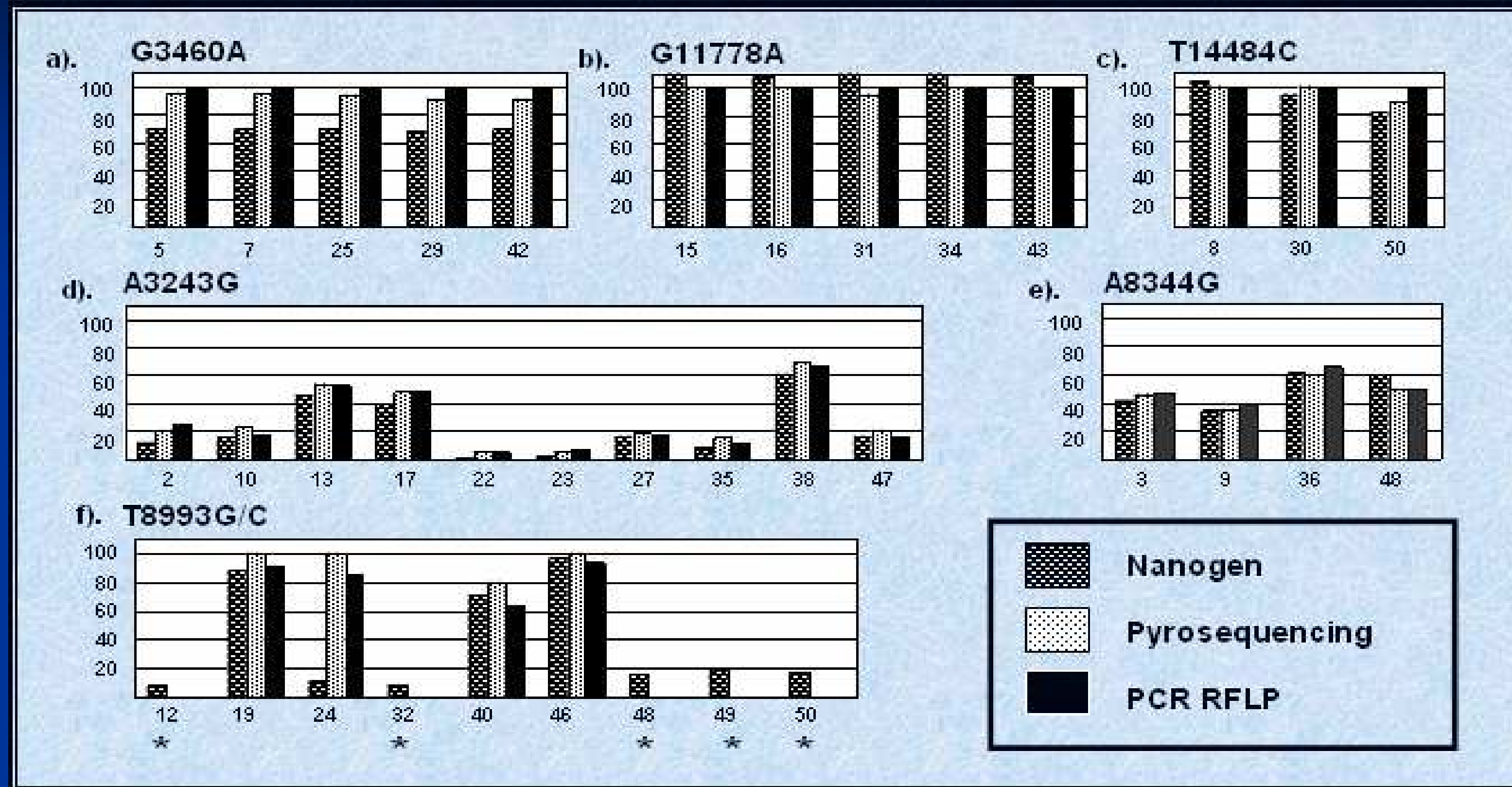
Samples had been previously genotyped using PCR RFLP analysis by Oxford Medical Genetics Laboratory

SNP Detection for FV / FII / MTHFR

90 DNA samples were genotyped using triplex PCR assays

	Nanogen	Pyrosequencing
Accuracy	99 %	100 %
Samples repeated to obtain full genotype	14.4 %	5.5%
Failure rate	3.3%	0 %
Time for analysis of 96 samples	10 hrs 45 min	3 hrs 30 min
Cost per genotype	£1.63 / sample	£0.65 / sample

Detection and estimation of heteroplasmy



Pyrosequencing is less expensive and more efficient than the Nanogen System:

- system costs - 36% less expensive
- analysis costs - 58% less expensive
- total run time - 67% faster

Conclusions for SNP detection

SNP Detection

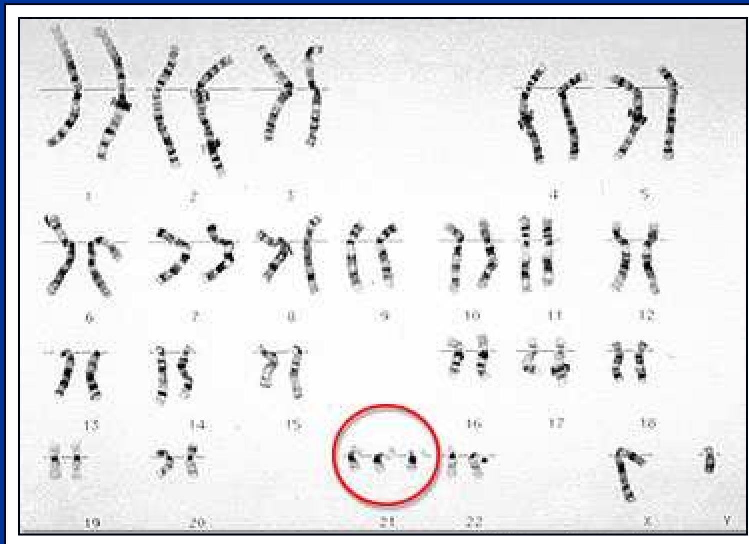
- Both technologies accurate for SNP genotyping
- Pyrosequencer faster and cheaper
- Pyrosequencer had lower failure rate

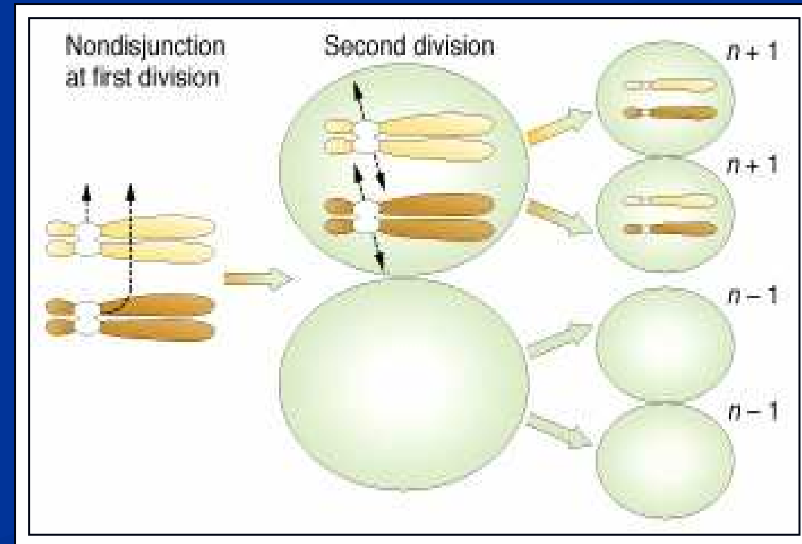
Detection and estimation of Heteroplasmy

- Both technologies provided accurate genotyping
- Pyrosequencing more accurate at estimating heteroplasmy
- Pyrosequencer more efficient and economical

Detection of trisomy 13, 18 & 21 and sex chromosome aneuploidy for prenatal diagnosis using Pyrosequencing™ technology

In collaboration with Prof Antonarakis and Sam Deutsch (University of Geneva)





Existing fast screening techniques for detection of aneuploidy

Interphase fluorescent in situ hybridisation (FISH)

- labour intensive
- 50 – 100 interphases need to be scored

Quantitative fluorescent PCR (QF-PCR)

- relies on amplification of polymorphic microsatellite repeats
- less expensive than FISH
- many samples can be treated in parallel
- Multiple markers need to be analysed to ensure that at least 2 informative markers can be analysed for each individual
- Requires optimisation of multiplex PCRs

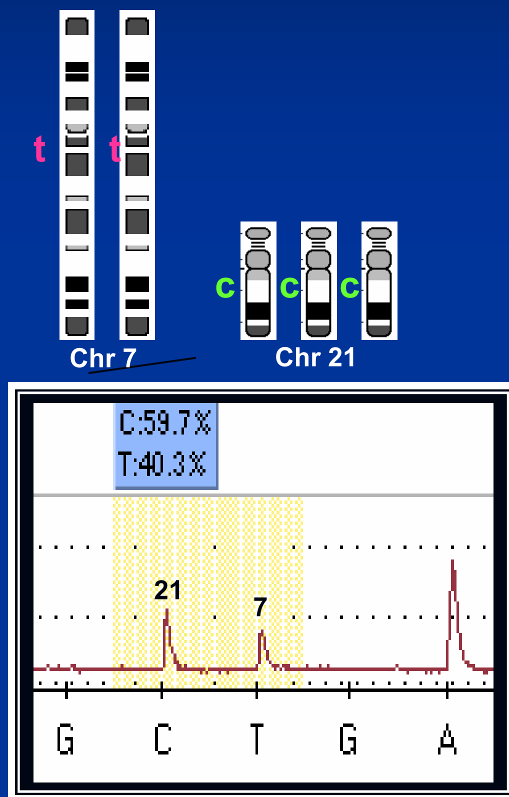
Multiplex Ligation probe amplification (MLPA)

- simultaneous analysis of up to 40 loci
- 8 probes per chromosome needed for reliable results
- Still under evaluation

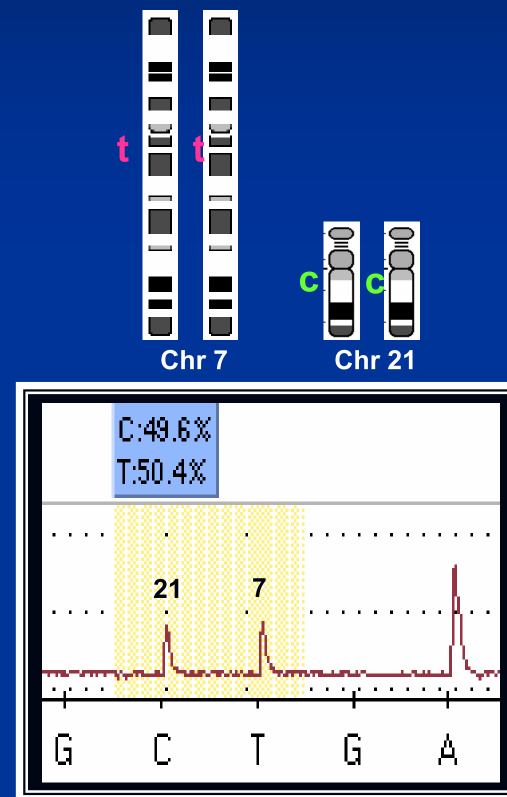
Detection of aneuploidy using paralogous gene quantification by Pyrosequencing™

- Paralogous sequences are located on different chromosomes
- High sequence identity but will accumulate sequence differences over time
- PCR primers are designed to co-amplify paralogous sequences located on different chromosomes
- PCR products (of identical size) will contain a number of sequence differences
- Known as paralogous sequence mismatches (PSM)
- Quantification of PSMs can be used to determine the dosage of chromosomes in which the paralogous sequences are located

Paralogous gene quantification



Trisomy 21
(59.7%)

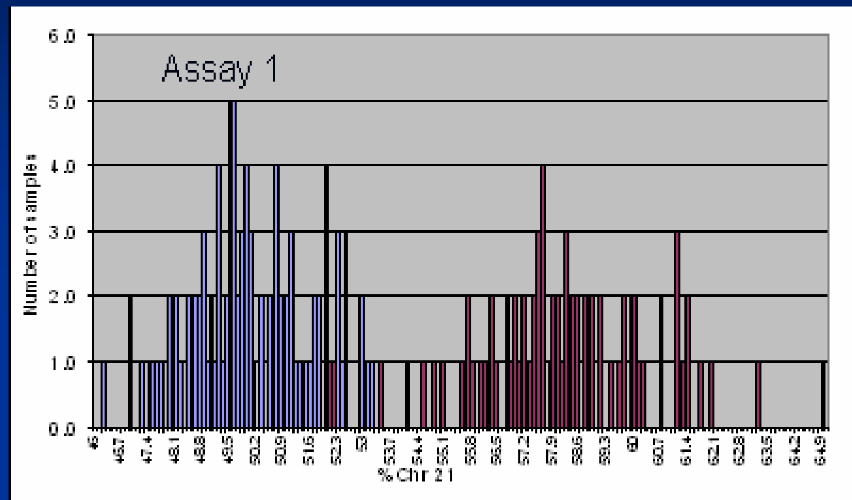


Normal Control
(49.6%)

The ratio of the SNPs reflects the relative frequency of the chromosomes tested.

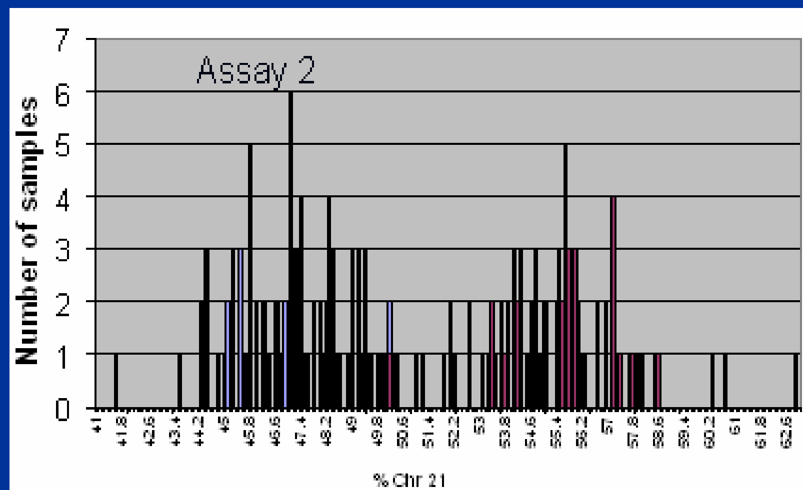
Initial Analysis of trisomy 21 assays

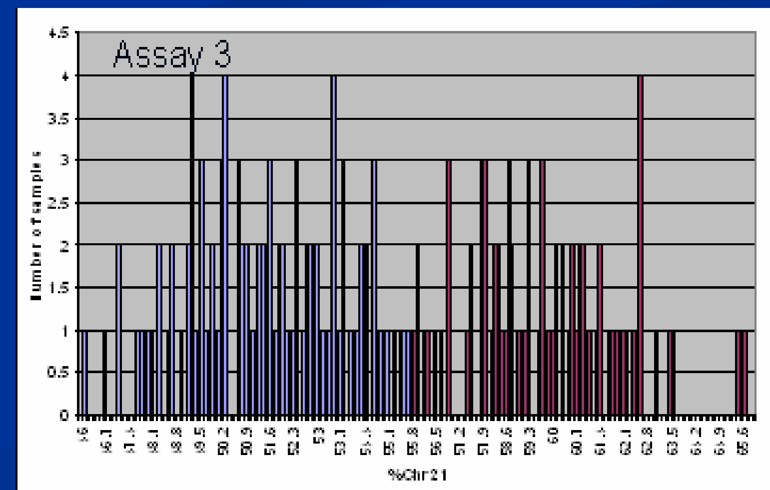
Data from 103 normal controls and 73 Trisomy 21 samples



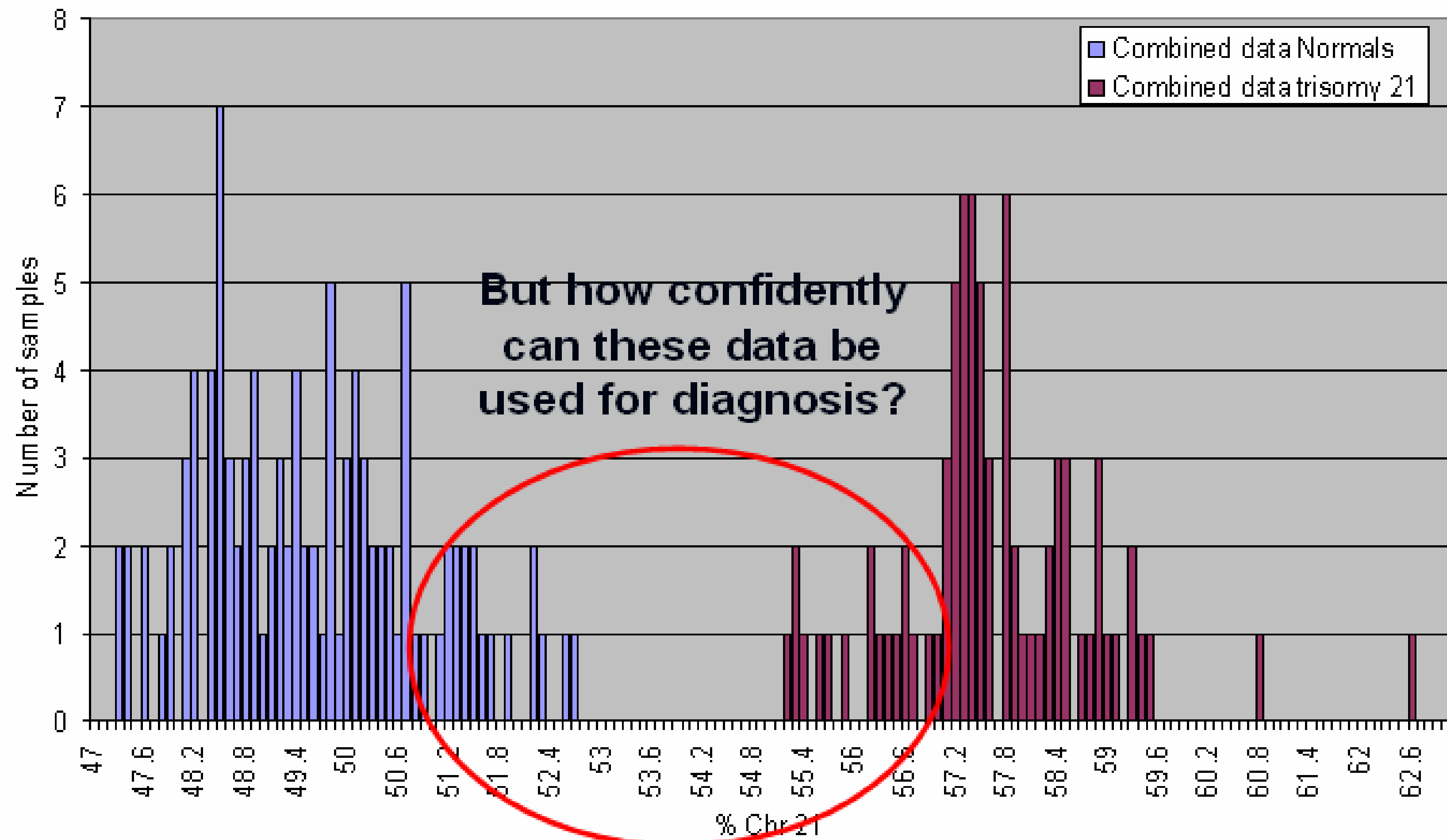
Normal

Trisomy 21





Combined data from three trisomy 21 assays



Mathematical model to assign % risk based on AQ frequency

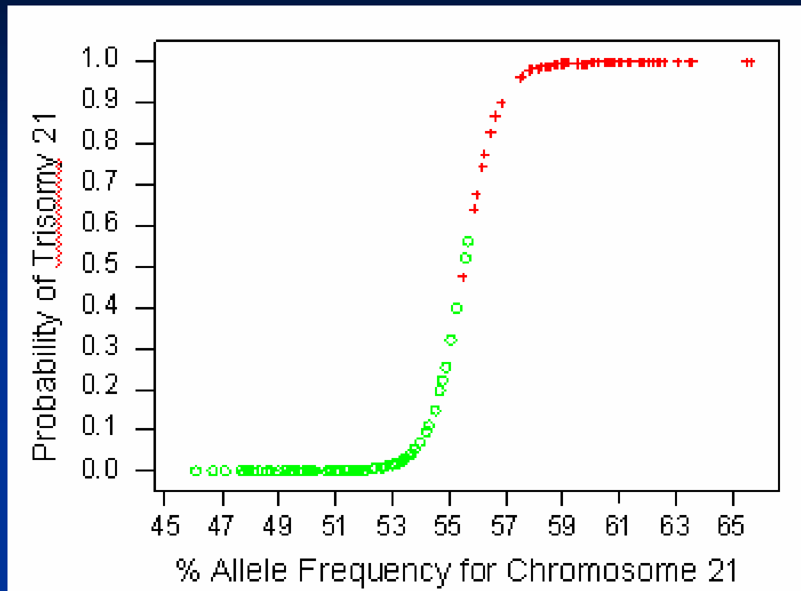
- To minimise the impact of inter assay variation a mathematical model has been devised which assigns a probability of the patient sample being trisomic based on the % AQ frequency

- At least 10 normal and 10 known trisomy controls are run per batch of tests

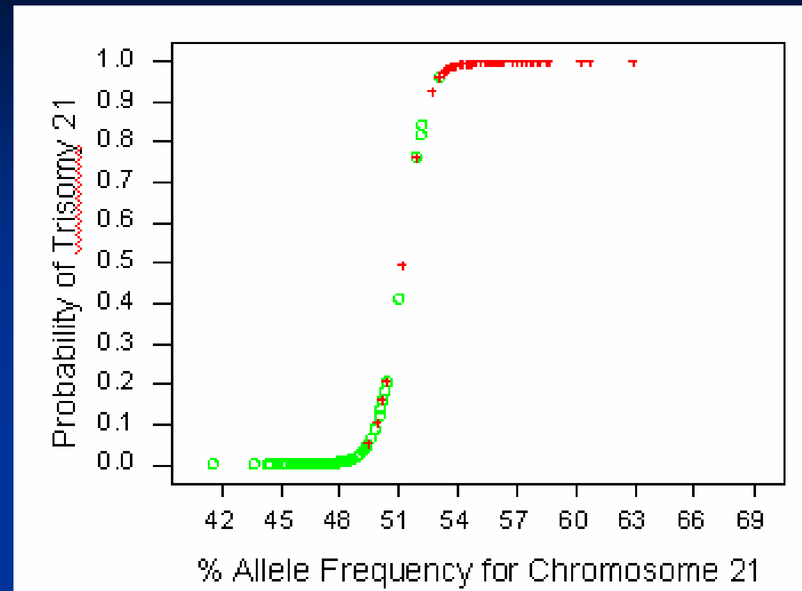
- The mean and standard deviation of the two populations are normalised and used to determine a probability of trisomy given a pre-screen risk of 0.5

- The AQ percentage value for each assay is entered and the % risk is calculated.

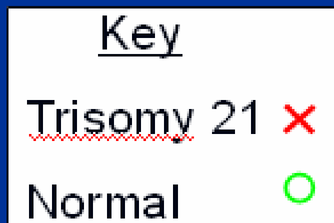
Trisomy 21 Results

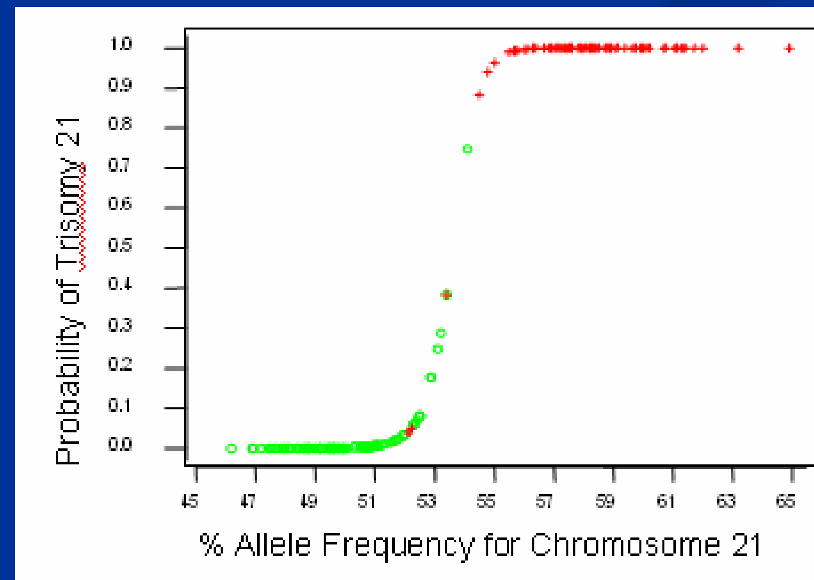


Assay 1



Assay 2

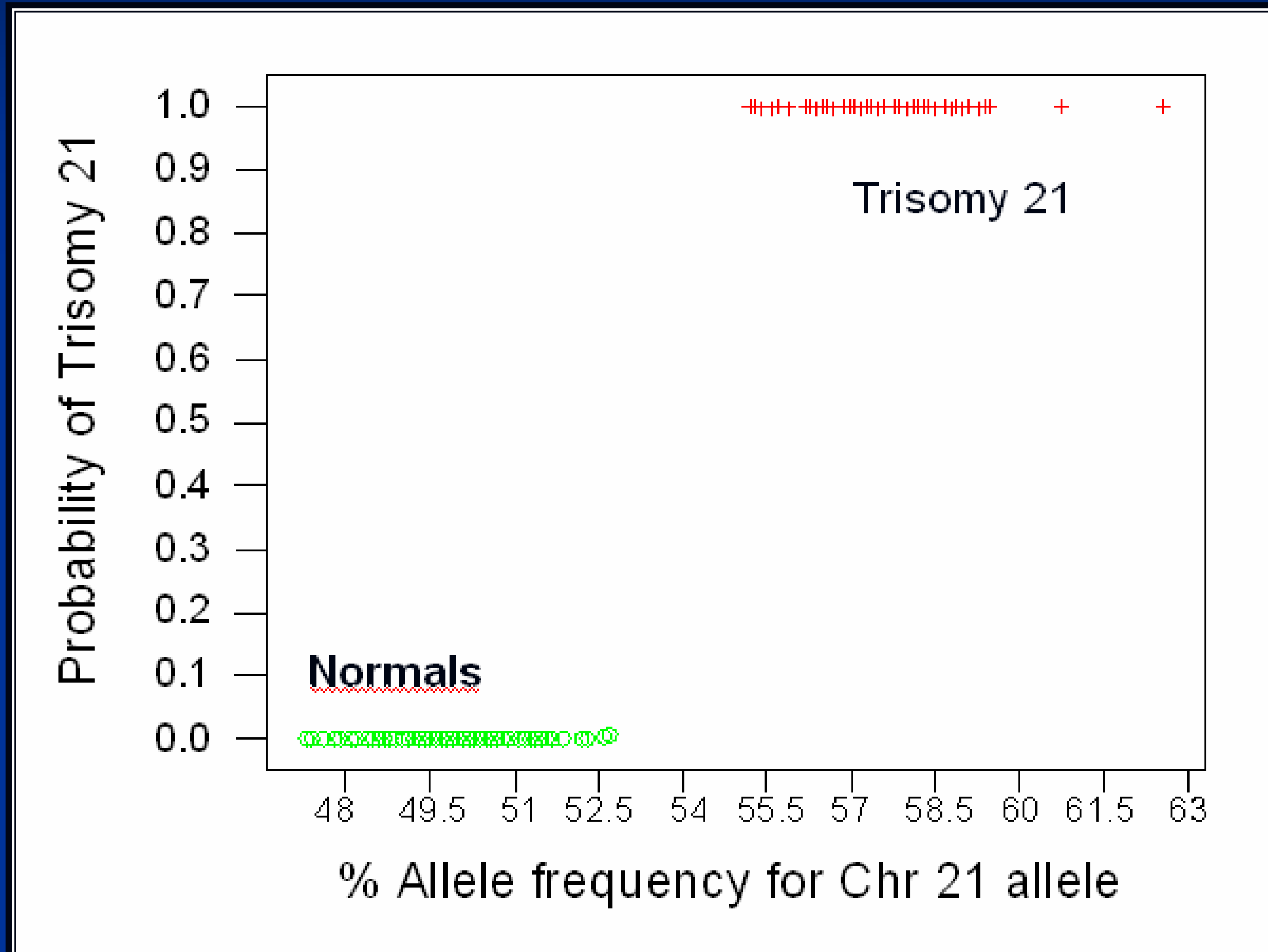




Assay 3

Trisomy 21 Evaluation

Data from 103 normal controls and 73 Trisomy 21 samples



Paralogous gene quantification for prenatal diagnosis of aneuploidy - Conclusions

- Results from analysis of trisomy 21 assays are promising
- Assays are robust, easy to set up and interpret
- Use of the mathematical model alleviates problems of inter assay variation and standardises interpretation of data
- Triploidy, rare chromosomal abnormalities and other structural abnormalities will not be detected
- Continuing analysis for detection of trisomy 13, 18 and sex chromosome aneuploidy using 440 known aneuploid samples (tissue / peripheral blood/ amniotic cultures) and 300+ amniotic fluid samples collected prospectively from diagnostic prenatal lab
- Mathematical model will be extended to included all data and tested for robustness
- Should represent an competetive alternative to other techniques for use in routine diagnostic laboratories

Acknowledgements

Vicky Durston, NGRL (Wessex)

Gemma Potts, NGRL (Wessex)

Wessex Regional Genetics DNA Laboratory (WRGL)

Dr John Harvey, WRGL / NGRL

Prof Nick Cross, WRGL / NGRL

Monica Petterson, Biotage AB

Bjorn Hihn, Nanogen Europe BV

FV/FII/MTHFR and Mitochondrial Heteroplasmy

Louise Lavender, Molecular Pathology Unit, Southampton

Anneke Seller & Carl Fratter, Oxford Medical Genetics

Pre Natal Aneuploidy Diagnosis

Prof Stylianos E Antonarakis & Samuel Deutsch, University of Geneva

Paul Strike, Research and Development Support Unit, SDH