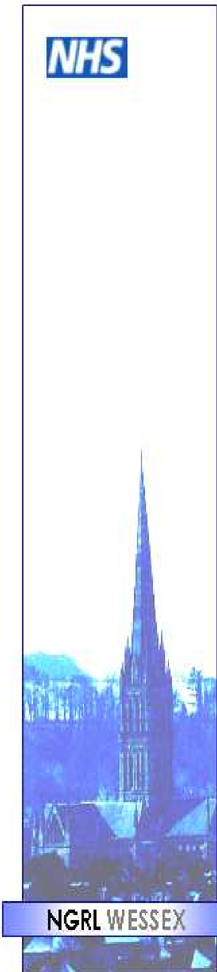
Salisbury NHS
NHS Foundation Trust



New genetic technologies for non-invasive detection of Down syndrome

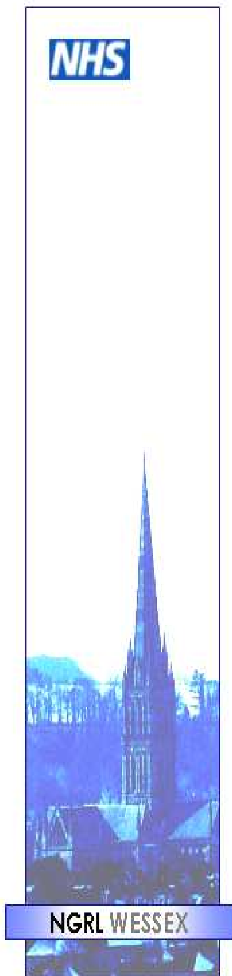
Helen White, PhD

Senior Scientist

National Genetics Reference Lab (Wessex)

DH Department of Health

Outline of talk



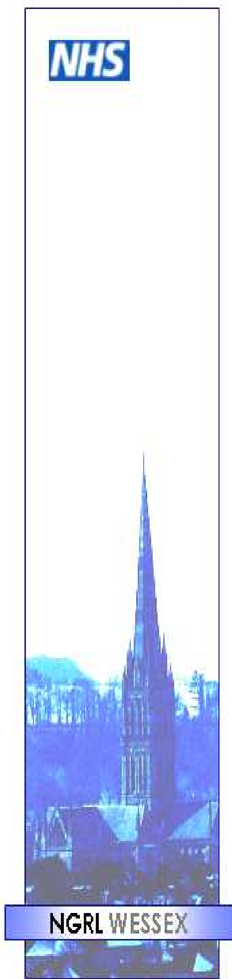
- Cell free fetal nucleic acids in blood of pregnant women

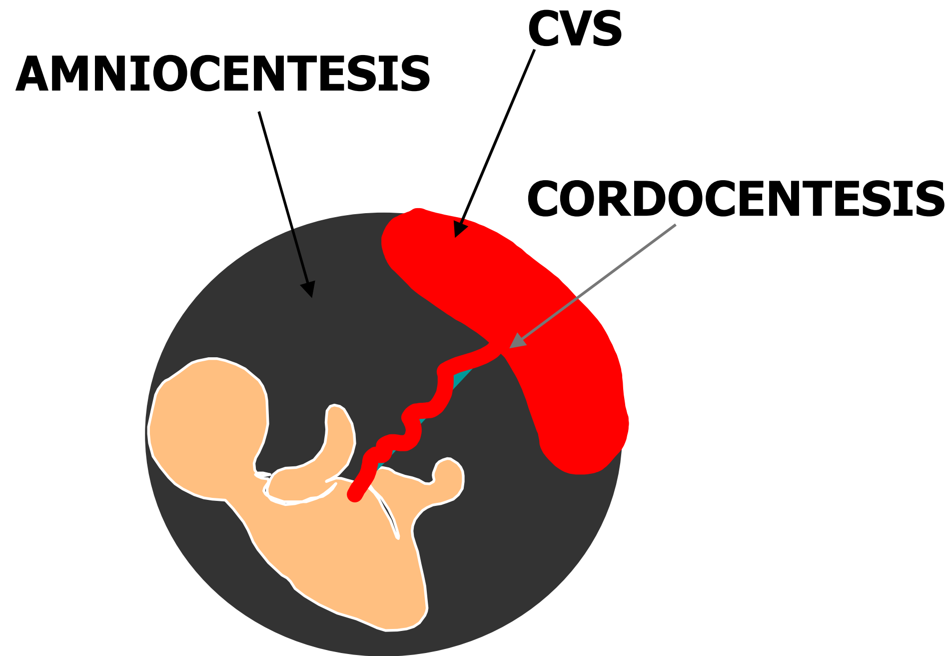
  - how can they be used for DS testing?

- New non-invasive techniques for detection of DS

  - Quantitative SNP analysis from cffRNA
  - Digital PCR of cfDNA and cffRNA
  - Shot gun sequencing of cfDNA

Current prenatal diagnosis requires invasive procedures





1% risk of miscarriage

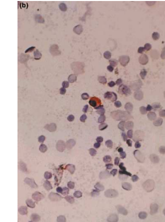
Not possible before 11 weeks' gestation

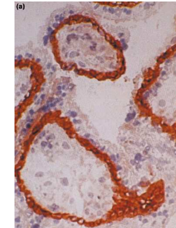
Other sources of fetal tissue for non-invasive prenatal diagnosis

NHS

NGRL WESSEX

Fetal cells in maternal circulation





erythroblasts
trophoblastic cells
leucocytes

Difficult to isolate and persist for years after pregnancy

Cell free fetal nucleic acid in the maternal circulation

Detectable from 5 weeks' gestation

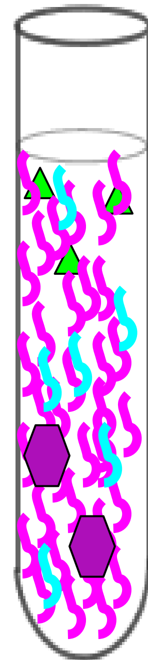
Cell free fetal DNA: 3 - 6% of total circulating cell free DNA
94 - 97% of cell free DNA is maternal

Cell free fetal RNA: fetal specific transcripts identified

Both DNA and RNA cleared from circulation within 30 minutes of delivery

Originates from trophoblast

Extraction of cell free fetal nucleic acids from maternal plasma



Amount of cf fetal DNA extracted is equivalent to 25 genomes / ml plasma

How can cell free fetal nucleic acids be used for non-invasive Down syndrome testing?

- **Major technical challenge**

Background of cell free maternal **DNA** means direct quantification of fetal chromosome copy number is problematic and technically demanding

Ideally need:

targets that are free from maternal background interference

and / or

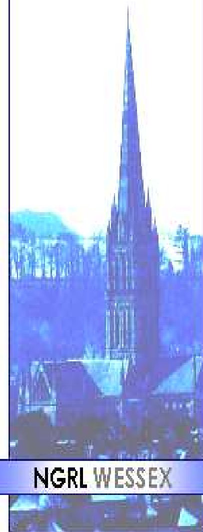
technologies that enable extremely accurate copy number 'counting'

- **Recent major breakthroughs**

Quantitative analysis of Single Nucleotide Polymorphisms in **fetal specific** mRNAs

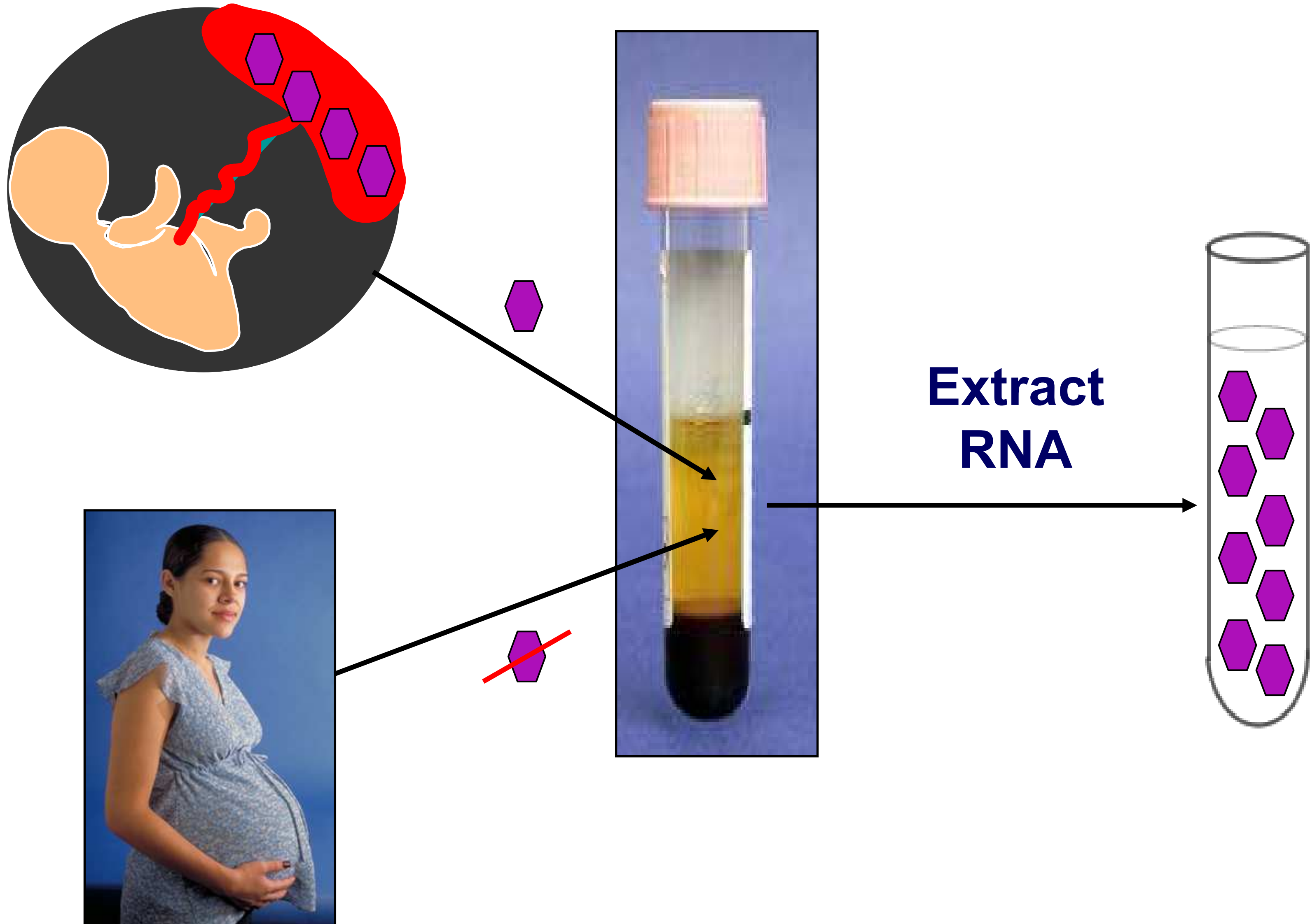
Digital PCR of cfRNA and cfDNA

Shotgun sequencing of cfDNA

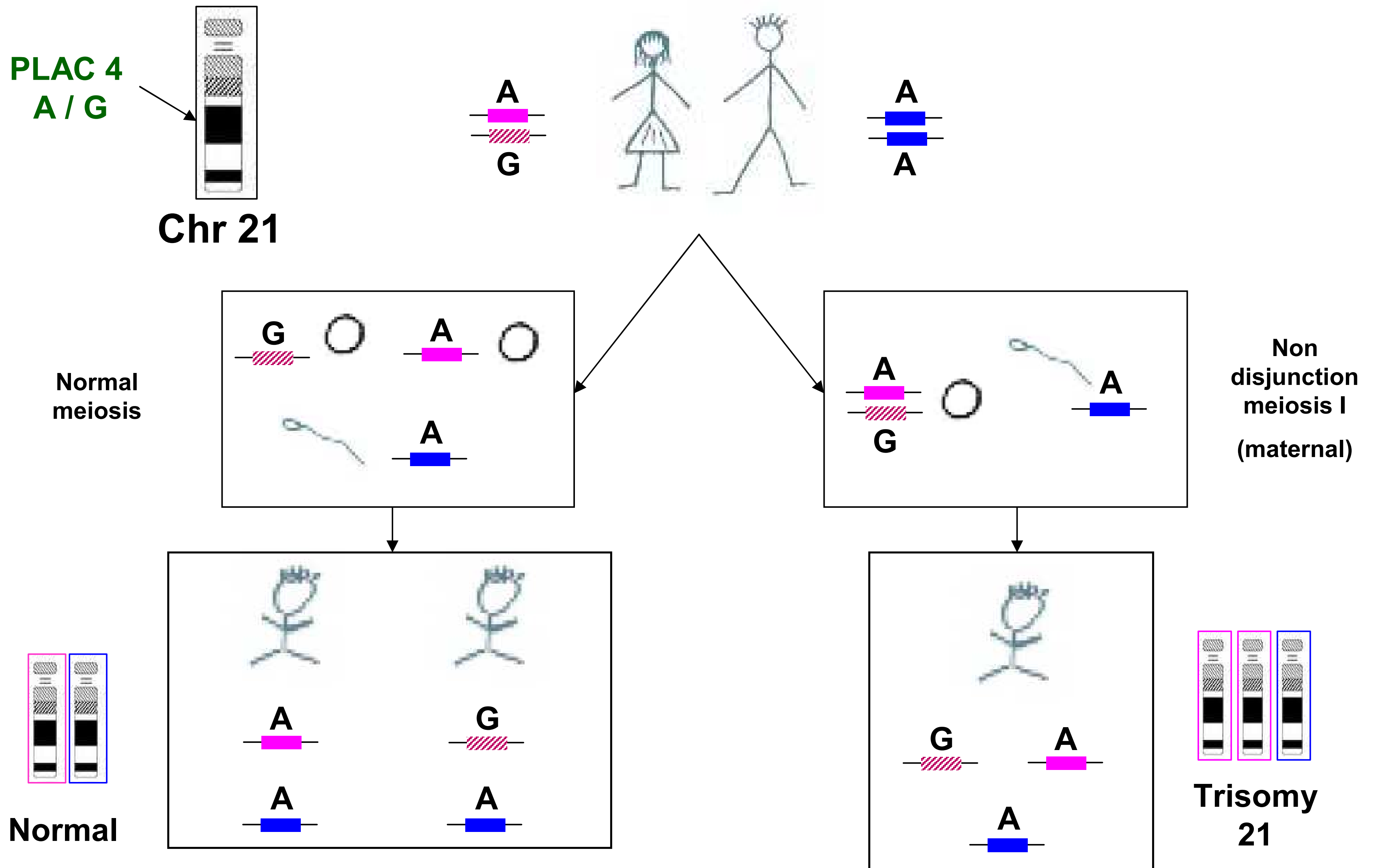


Quantitative analysis of Single Nucleotide Polymorphisms in fetal specific mRNAs

Quantitative analysis of SNPs in fetal specific mRNA

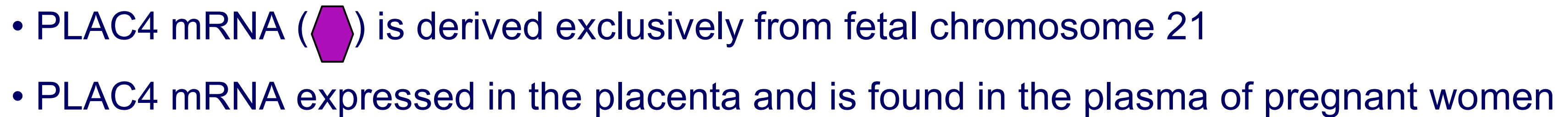


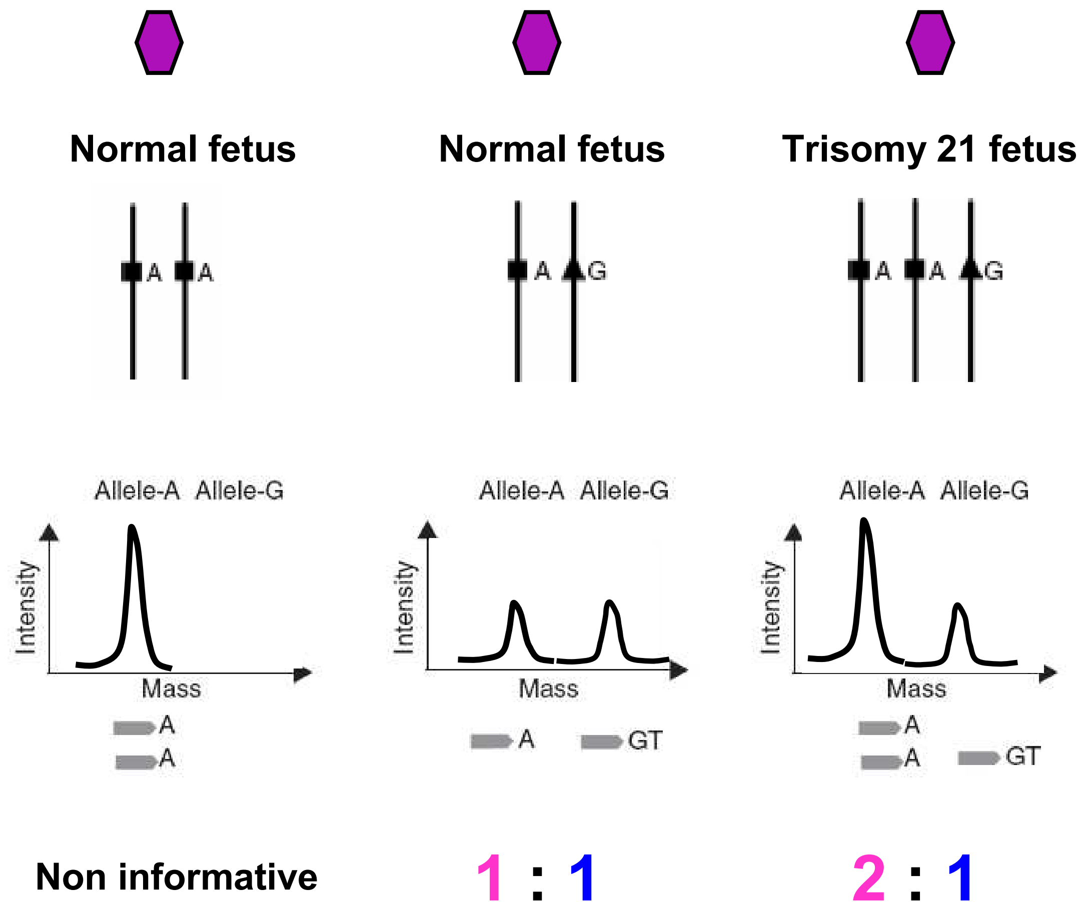
Quantitative analysis of SNPs in fetal specific mRNA



Quantitative analysis of SNPs in fetal specific mRNA

Analysis by MALDI-TOF (mass spectrometry)

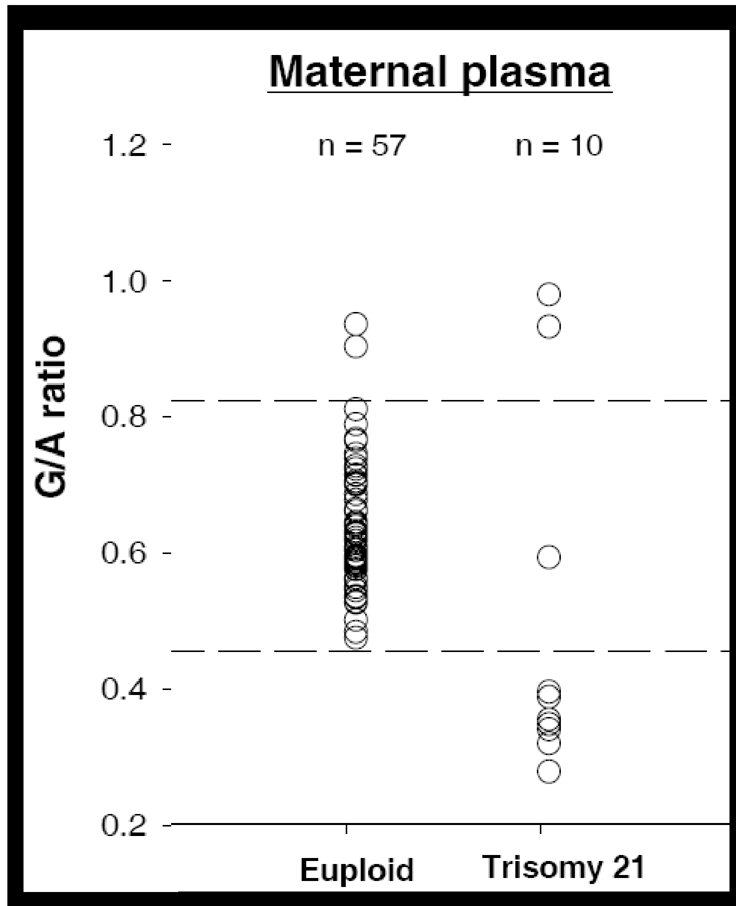




Lo et al., Nature Med & PNAS, 2007

Quantitative analysis of SNPs in fetal specific mRNA

Analysis by MALDI-TOF (mass spectrometry)



- Correctly diagnosed fetal trisomy 21 in **90%** of +21 cases (n=10)
- Excluded diagnosis of trisomy 21 in **96.5%** of normal controls (n=57)

- Sensitivity: 90%
- Specificity: 96.5%

Lo et al., Nature Med & PNAS, 2007

Quantitative analysis of SNPs in fetal specific mRNA

- **ADVANTAGES**

  • Diagnostic sensitivity and specificity using one marker are comparable to multiple marker screening tests for DS currently in practice (if informative)

  • Test is insensitive to gestational age and can be offered early in pregnancy

  • Target free of maternal background

- **DISADVANTAGES**

  • Fetus has to be informative for SNP analysed

    - testing of the PLAC4 SNP will only give a result for 40% of pregnant women

- **FUTURE REQUIREMENTS**

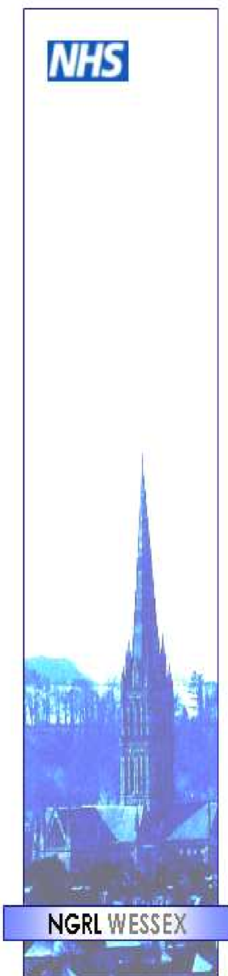
  • Identification of more polymorphic loci to increase informative cases – population dependent

  • Multi centre large scale validation required

  • Expand testing to include fetal specific transcripts from chromosomes 18 & 13

Quantitative analysis of SNPs in fetal specific mRNA

Sequenom Inc, SEQureDx(TM) Technology



- Developed quantitative RNA SNP test analysing multiple (>10) SNPs on chr 21:

  - increases test coverage to **greater than 95%** in the US population

- Complete concordance with clinical results in 619 samples tested to date
- Initiating multi-site 5000-sample laboratory developed test (LDT) validation study
- Acquired Center for Molecular Medicine, a CLIA-certified molecular diagnostics lab and anticipate commercial launch of **primary screening test** in **June 2009**
- Sponsoring RNA Noninvasive Aneuploidies ("RNA") study:

  - multi-center, prospective study involving 10,000 samples from first and second trimester pregnancies using the SEQureDx technology, managed and analysed by an independent third-party

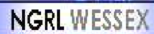
- Identified novel markers for Trisomy 18 that have passed initial selection criteria

Press release: Sequenom, Inc Sept. 23, 2008

Digital PCR

Digital RNA SNP strategy

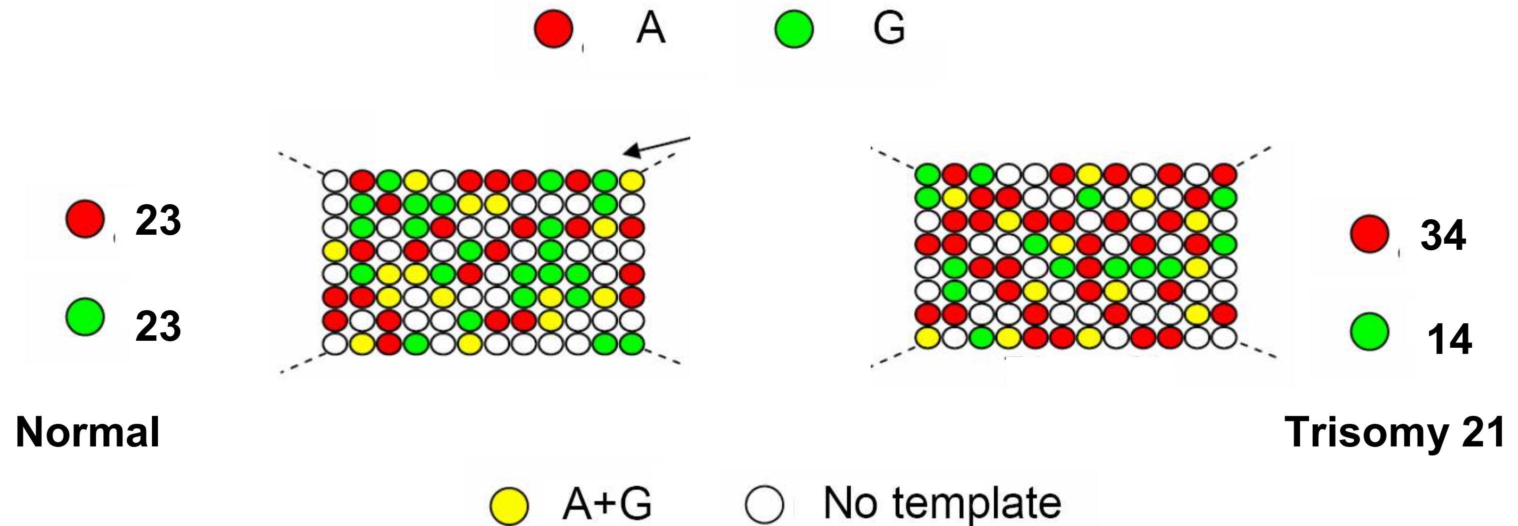
Relative chromosome dosage



Quantitative analysis of SNPs in fetal specific mRNA

Digital PCR

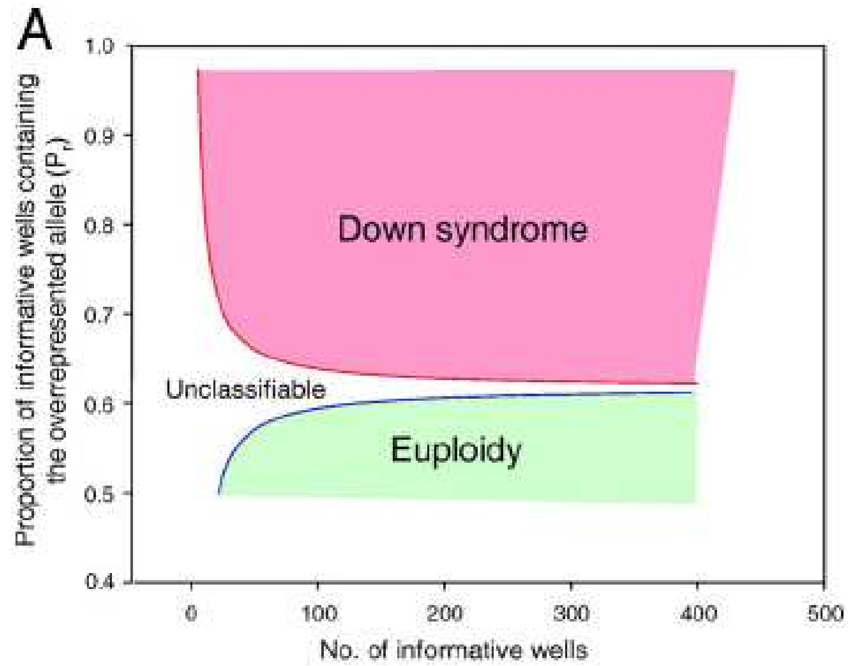
- Dilute cffRNA sample to < 1 copy per reaction well using 384 well plates
- Perform real time PCR for PLAC4 SNP using two coloured probes



- Count red and green wells
- Determine statistically whether an allele is over represented

Lo et al., PNAS, 2007

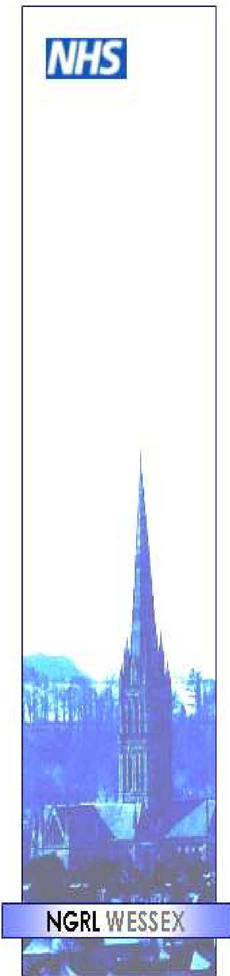
NGRL WESSEX



Genotype	No. of wells positive for individual alleles				m_r	SPRT result		
	A only	G only	AG	All negative		P_r	Unclassifiable region	Classification
AG	90	100	97	97	0.67	0.526	0.62–0.64	Euploid
AG	97	105	65	117	0.55	0.520	0.61–0.63	Euploid
AG	66	92	34	192	0.30	0.582	0.59–0.62	Euploid
AG	29	28	3	324	0.08	0.509	0.54–0.64	Euploid
AG	112	85	44	143	0.41	0.569	0.60–0.62	Euploid
AG	90	101	72	121	0.55	0.529	0.61–0.63	Euploid
AG	73	91	57	163	0.41	0.555	0.60–0.63	Euploid
AG	66	90	52	176	0.37	0.577	0.59–0.62	Euploid
AG	71	56	17	240	0.21	0.559	0.58–0.62	Euploid
AAG	110	53	21	200	0.21	0.675	0.58–0.61	T21
AAG	246	127	112	283	0.37	0.660	0.60–0.61	T21
AGG	66	114	66	138	0.42	0.633	0.60–0.62	T21
AGG	58	130	54	142	0.34	0.691	0.59–0.62	T21

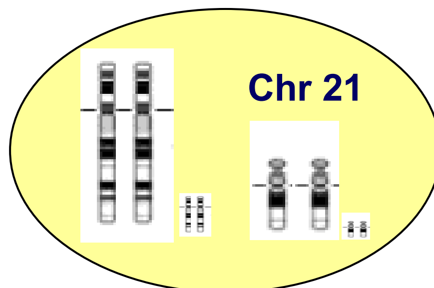
Lo et al., PNAS, 2007

Can digital PCR be used in a polymorphism independent way?

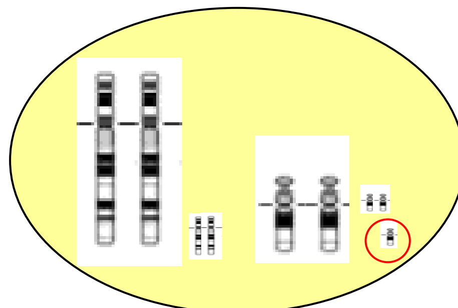


PROBLEMS:

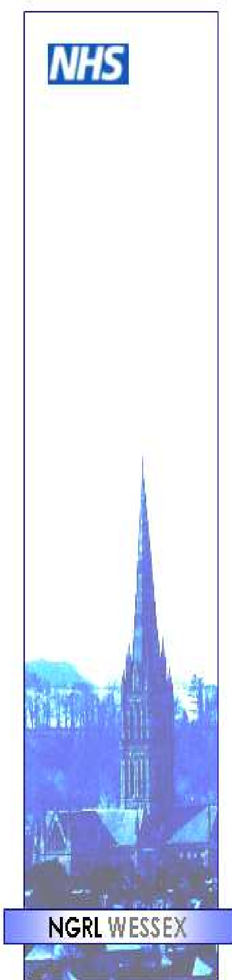
- Only 3-6% of the cell- free DNA fraction is fetal
- Expected enrichment of chromosome 21 lies within the range of 1.5% to 3%
- Relative chromosome dosage: can we compare amount of chr 21 present with amount of another autosome?



$$\frac{\text{Total amount of Chr 21 (0.94 + 0.06)}}{\text{Total amount of Chr 9 (0.94 + 0.06)}} = 1$$

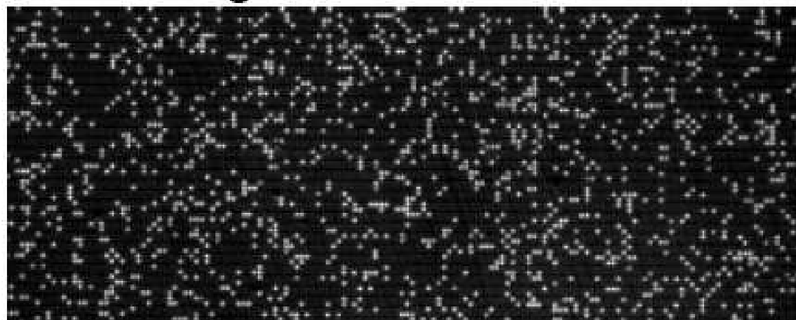


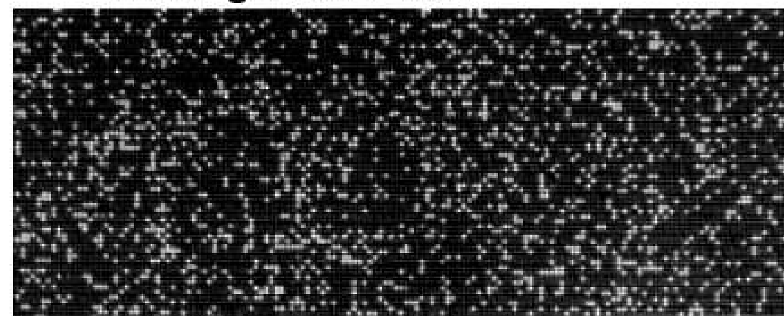
$$\frac{\text{Total amount of Chr 21 (0.94 + 0.09)}}{\text{Total amount of Chr 9 (0.94 + 0.06)}} = 1.03$$



SOLUTIONS:

- Digital PCR provides an alternative method for quantifying the relative abundance of two alleles
- Using existing commercially available microfluidic systems for digital PCR it would be possible to detect T21 if fetal DNA component was 25% (7680 rxns)
- Theroretically it is possible to detect a 1% difference by ‘counting’ a large number of digital PCR reactions
- Using a prototype “MegaPixel” digital PCR device that allows for 1,000,000 simultaneous single molecule reactions a 3% increase in chromosome 21 has been detected

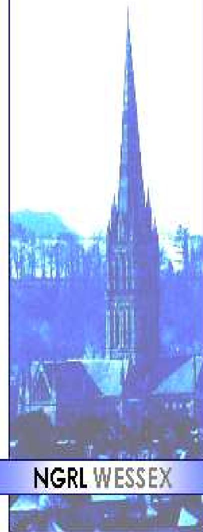




Tropini and Hansen ISPD poster 2008

Digital PCR

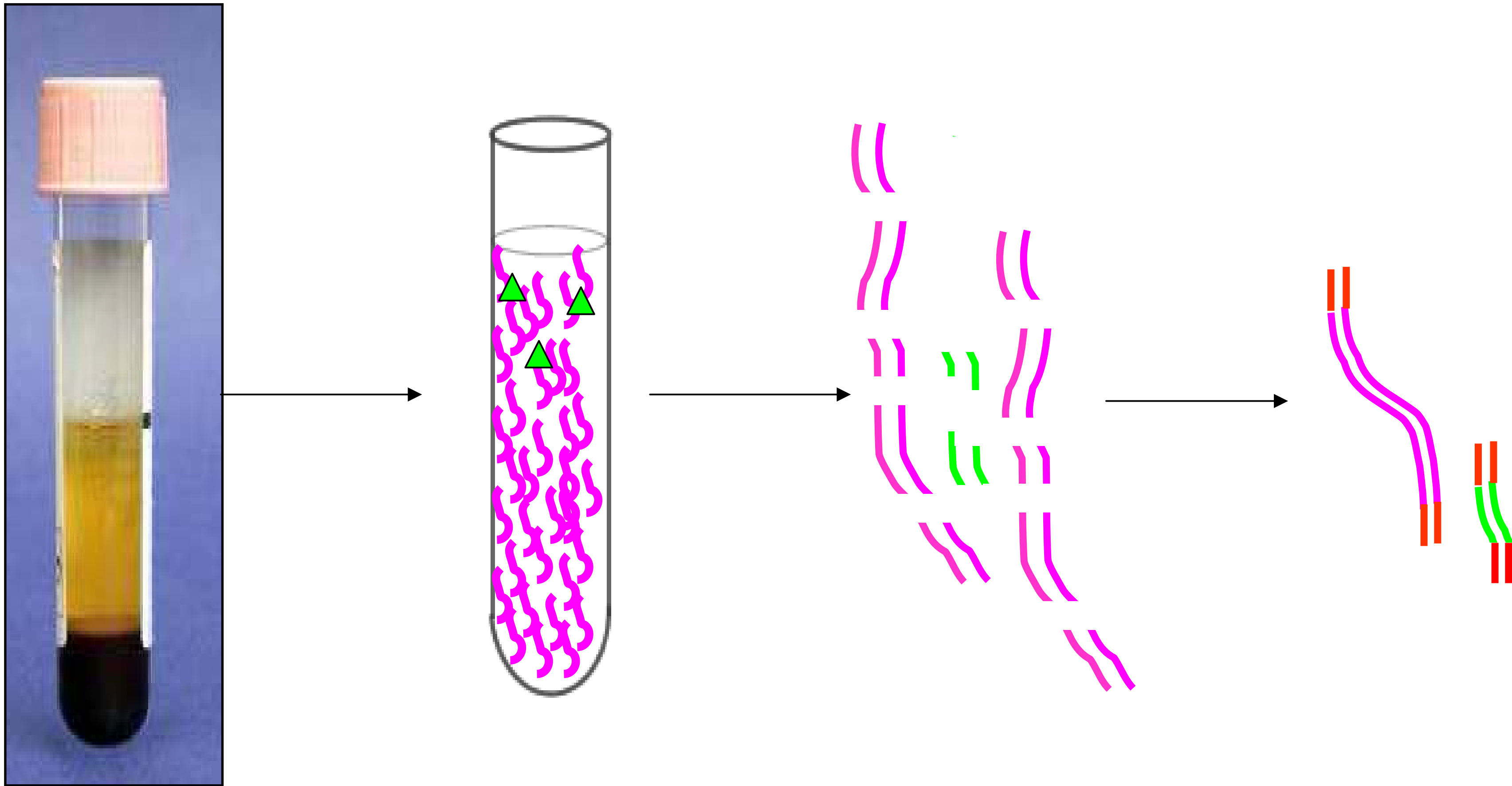
- **ADVANTAGES**
  - Successful proof of principal studies shown have shown utility for quantitative RNA SNP analysis and relative chromosome dosage
  - Relative chromosome dosage is polymorphism independent and could be used in all pregnancies
- **DISADVANTAGES**
  - For RNA SNP analysis the fetus has to be informative for SNP analysed
  - At present using relative chromosome dosage can only detect trisomy 21 if fetal DNA component is 25%
- **FUTURE REQUIREMENTS**
  - For relative chromosome dosage and high throughput RNA SNP analysis higher density digital PCR equipment needs to be developed
  - Multi centre large scale validation would be required



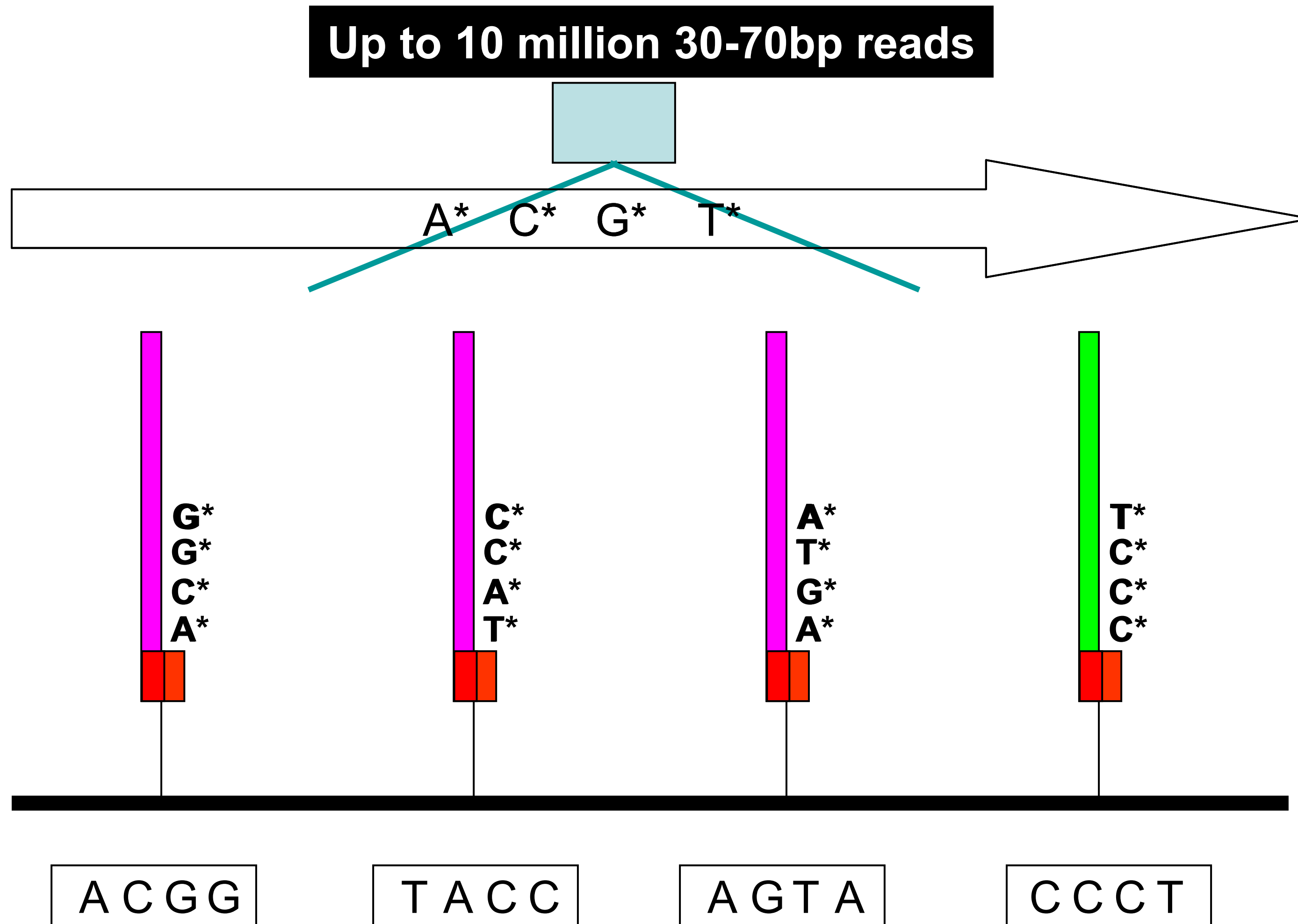
Shot gun sequencing

An alternative method for digital quantification of DNA

Shot gun sequencing



Sequencing by Synthesis



D Homo sapiens chromosome 10 genomic contig, alternate assembly

```
Query  1          TCGATCGTCAGCTAC
                  |||||||||||||||
Sbjct  1779498    TCGATCGTCAGCTAC   1779484
```

AGCGAACTAGCGGGCATGTCAGTCG
AGCGAACTAGCGGGCATGTCAGTCG
AGCGAACTAGCGGGCATGTCAGTCG
ACTAGCATGTCGTTCTTCTTTTAGATCG
AGCGAACTAGCGGGCATGTCAGTCG
ATCGATCAGTTAGCCCTCTGATCGA
ACTAGCATGTCGTTCTTCTTTTAGATCG
AGCGAACTAGCGGGCATGTCAGTCG
ATCGATCAGTTAGCCCTCTGATCGA
ACTAGCATGTCGTTCTTCTTTTAGATCG
AGCGAACTAGCGGGCATGTCAGTCG
ACTAGCATCGATCAGCTACGACT
ATCGATCAGTTAGCCCTCTGATCGA
ACTAGCATGTCGTTCTTCTTTTAGATCG
AGCGAACTAGCGGGCATGTCAGTCG
ACTAGCATCGATCAGCTACGACT
ATCGATCAGTTAGCCCTCTGATCGA
ACTAGCATGTCGTTCTTCTTTTAGATCG
AGCGAACTAGCGGGCATGTCAGTCG
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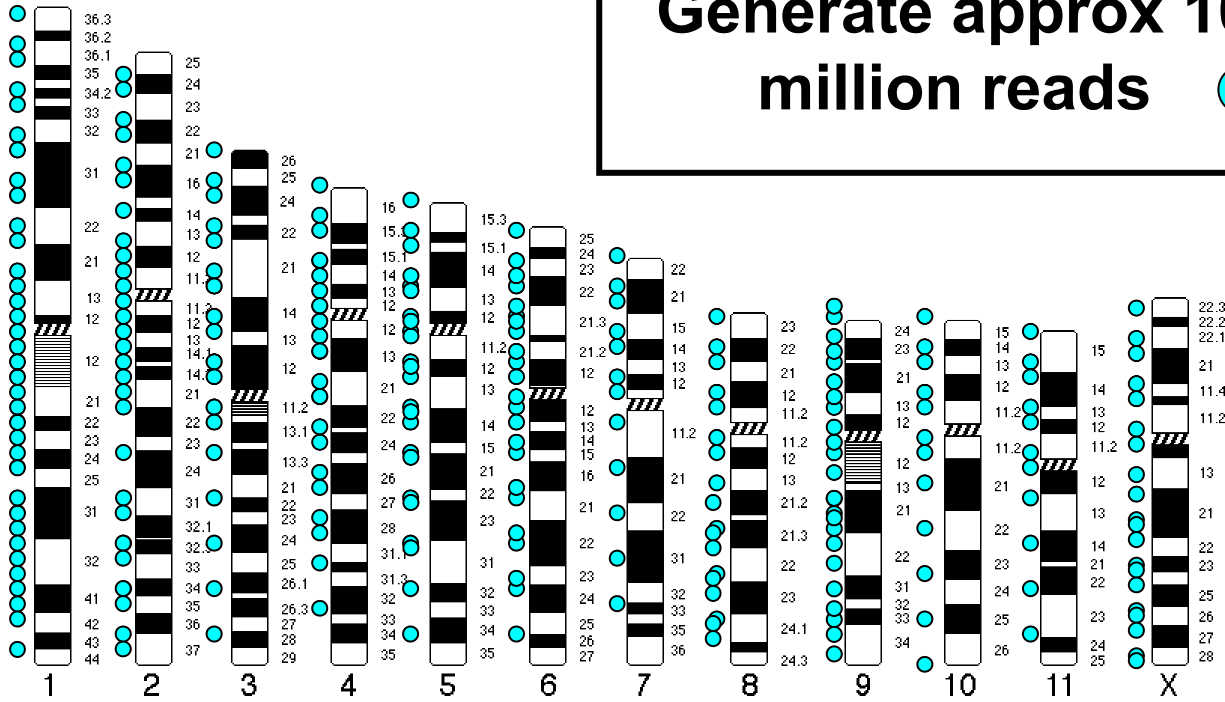
ATCGATCAGTTAGCCCTCTGATCGA
ACTAGCATGTCGTTCTTCTTTTAGATCG
ACTAGCATCGATCAGCTACGACT
ATCGATCAGTTAGCCCTCTGATCGA
ACTAGCATCGATCAGCTACGACT

ACGATCAGCTACGATC
AGCTAGATCGATCAGC
TACGATCAGCTACGAT
CAGCGATCGACTAGCA
TCGAGCATCGACTAGC
TACGATCGATAGCATC
GATCAGCTACGA

CHAT
CXXC6
STOX1
ABLIM2
STK32C
C10orf92

10p15.3 10p15.2 10p15.1 10p14 10p13 10p12.33 10p12.32 10p12.31 10p12.2 10p12.1 10p11.23 10p11.22 10p11.21 10p11.1 10q11.1 10q11.21 10q11.22 10q11.23 10q21.1 10q21.2 10q21.3 10q22.1 10q22.2 10q22.3 10q23.1 10q23.2 10q23.31 10q23.32 10q23.33 10q24.1 10q24.2 10q24.31 10q24.32 10q24.33 10q25.1 10q25.2 10q25.3 10q26.11 10q26.12 10q26.13 10q26.2 10q26.3

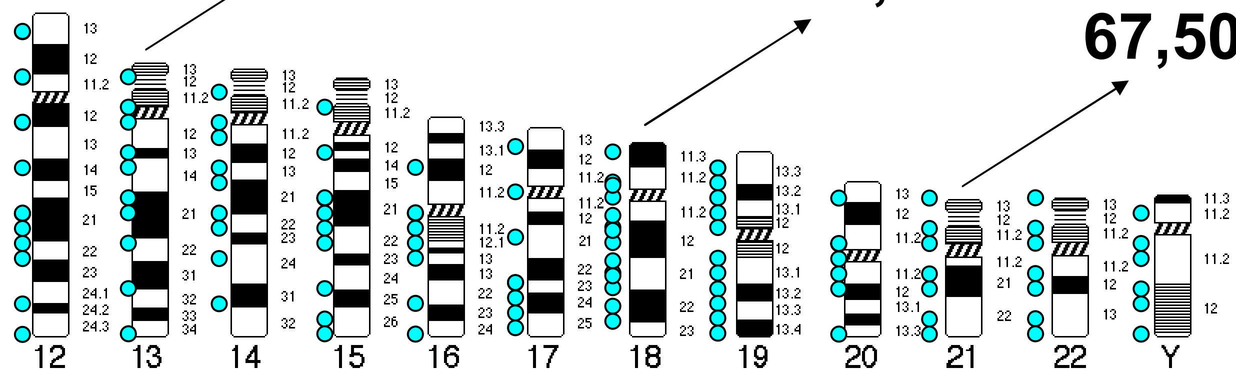
Generate approx 10 million reads

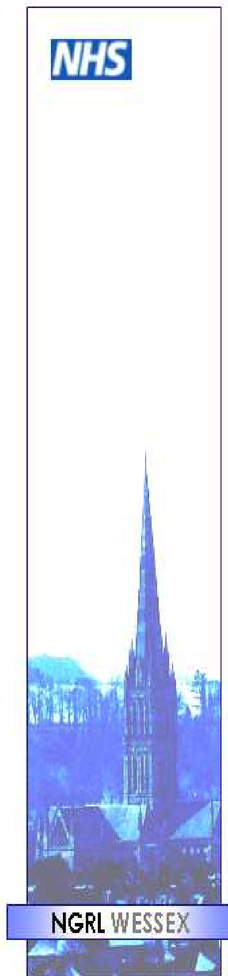


155,000

135,000

67,500





Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood

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Communicated by Leonard A. Herzenberg, Stanford University School of Medicine, Stanford, CA, August 22, 2008 (received for review July 13, 2008)

- Shotgun sequenced plasma DNA samples from 18 pregnant women:

  9 trisomy 21

  2 trisomy 18

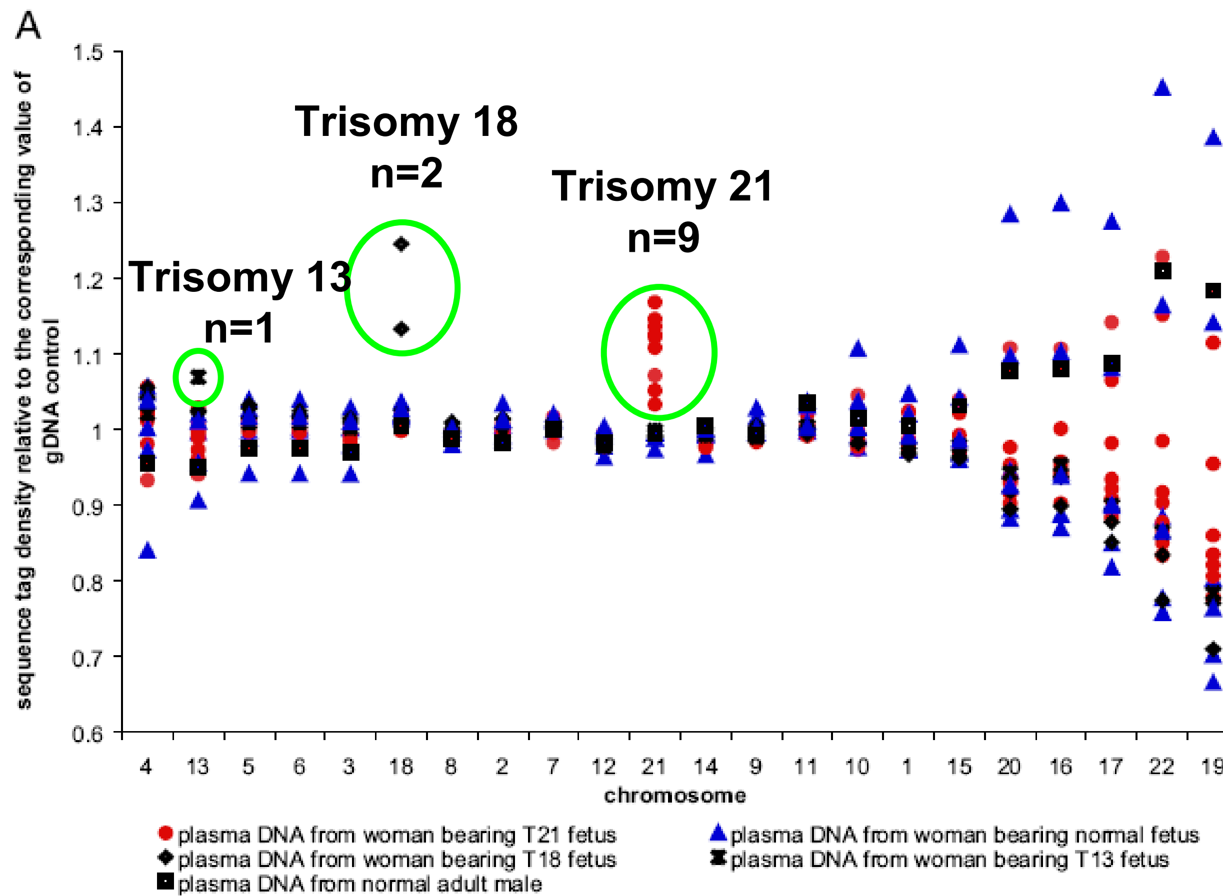
  1 trisomy 13

  6 normal

  and 1 genomic DNA sample from a male control
- Gestational age 10 – 35 weeks (earliest trisomy case 14 weeks)
- 5 million sequencing reads for each patient
- For each patient compared density of reads on each chromosome to those obtained from a normal genomic DNA sample
- Also compared density of Chr 21 reads from disomy and trisomy 21 samples
- Coverage of Chr 21 sequences in trisomy 21 was 4 – 18% higher than disomic cases

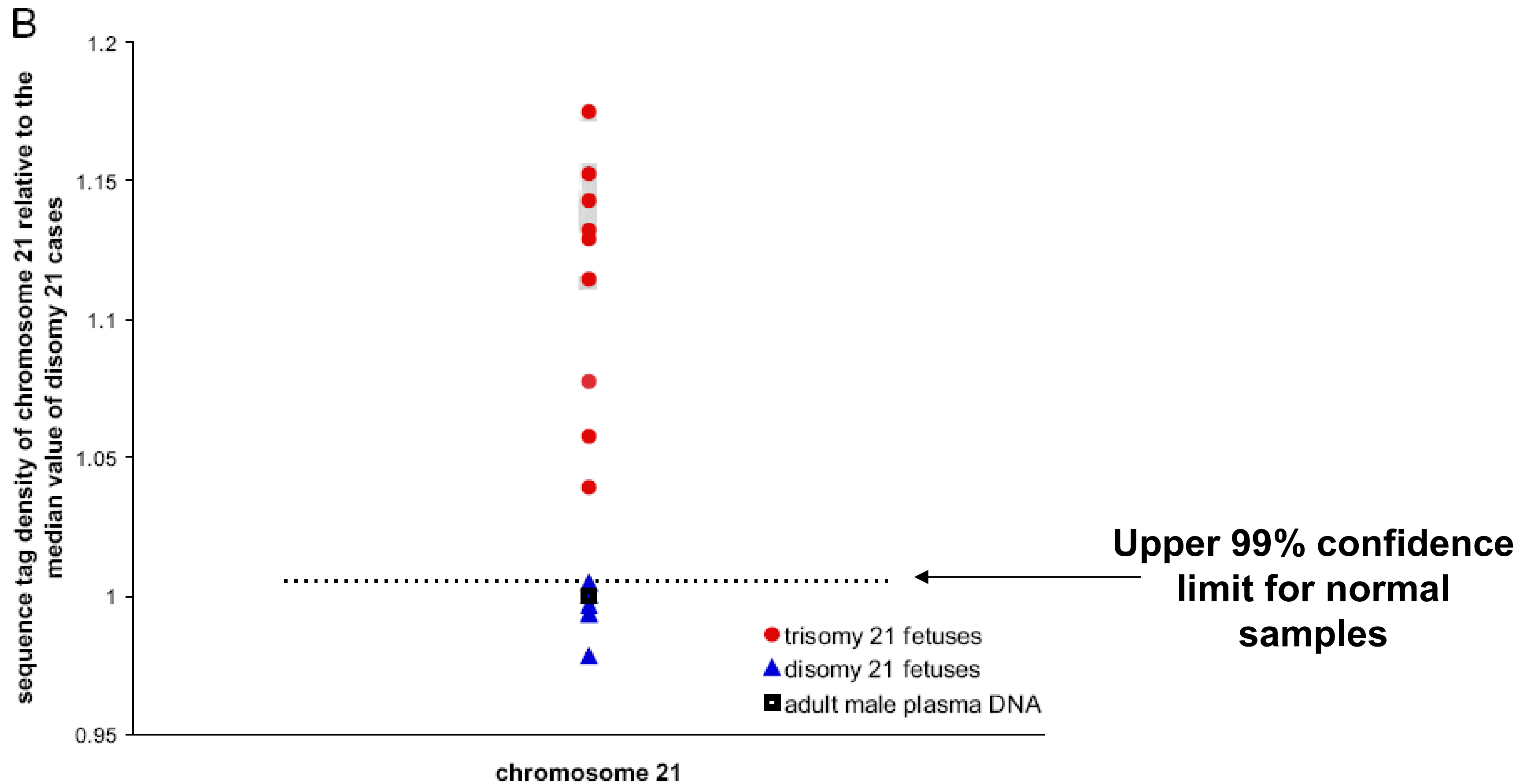
Fan et al., PNAS Oct 2008

Results of shotgun sequencing of maternal plasma DNA



Fan et al., PNAS Oct 2008

Results of shotgun sequencing of maternal plasma DNA



Fan et al., PNAS Oct 2008

Shot gun sequencing

- **ADVANTAGES**
  - Successful proof of principal study for detection of major trisomies; 13, 18 and 21
  - Polymorphism independent and could be used in all pregnancies
  - Has potential to detect unbalanced chromosome rearrangements
- **DISADVANTAGES**
  - Expensive and large amount of data processing – interpretation.
  - In current form would not be feasible to adapt to high throughput screening
- **FUTURE REQUIREMENTS**
  - Technological development required to produce machines and workflow protocols that could cope with a high throughput of samples

Summary

- New technologies need to be validated in large UK patient cohorts to determine accuracy
- The limits of gestation for testing using all techniques need to be determined
- Laboratory standards need to be developed

- Have the potential to replace current Down syndrome screening tests with a test that would be diagnostic

- Tests unlikely to replace invasive testing / current screening for some time although Sequenom may offer primary screening / diagnostic test in the US from June 2009

- Important to ensure that women and healthcare professionals understand the changes and women fully understand the implications of these tests

- NIHR funding secured to evaluate NIPD in more detail and determine the infrastructure and resources that will be required for timely implementation into NHS practice