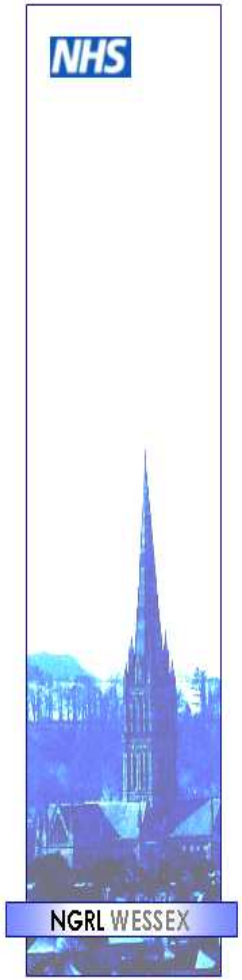
Salisbury NHS
NHS Foundation Trust



Non-invasive prenatal detection of Down syndrome:

an update and overview of the use of new genetic technologies

Helen White, PhD

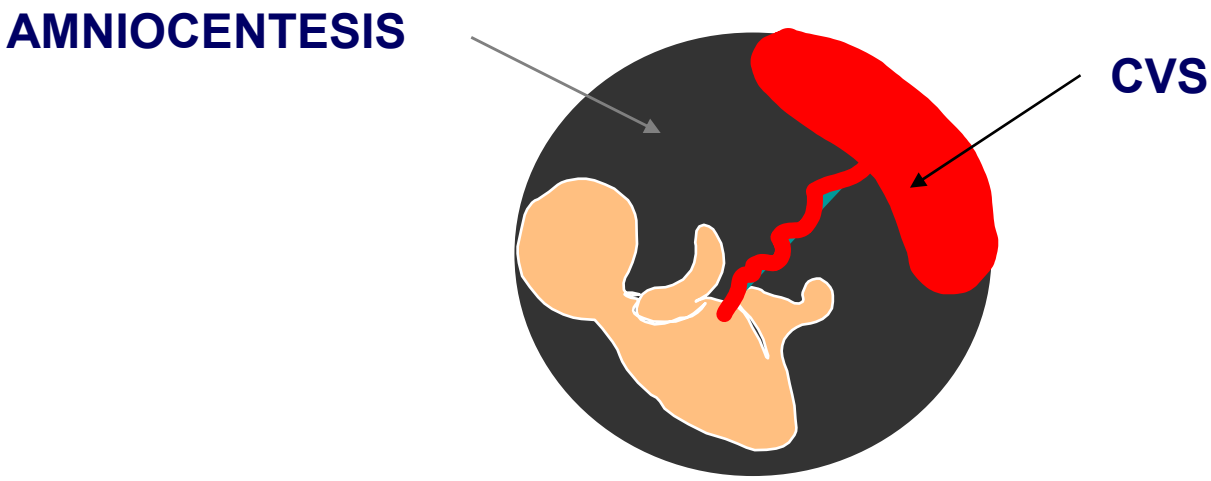
National Genetics Reference Lab (Wessex)

DH Department of Health

Outline of talk

- Current prenatal screening for aneuploidy
- How can cell free fetal nucleic acids be used for DS testing?
- New non-invasive techniques for detection of DS
  - Quantitative SNP analysis from cffRNA
  - Digital PCR
  - Shot gun sequencing of cfDNA
- RAPID: Plans to develop NIPD for aneuploidy

Current prenatal screening for aneuploidy



- Prenatal screening for is offered to all pregnant women
- Undertaken in two phases:

  screening and risk assessment

  invasive prenatal diagnosis of high risk cases
- Gold standard for diagnosis of chromosomal abnormalities is karyotyping

Current prenatal screening for aneuploidy

Due to a small but significant risk to the pregnancy, many women are reluctant to opt for these procedures

In 2006-7:

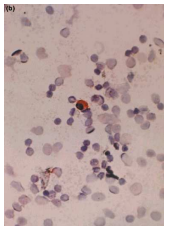
~700,000 pregnant women a year underwent antenatal screening

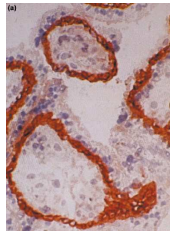
20,000 amniocentesis and 5,200 CVS were performed

Estimated associated procedural related pregnancy loss of ~250

Other sources of fetal tissue for non-invasive prenatal diagnosis

Fetal cells in maternal circulation





erythroblasts
trophoblastic cells
leucocytes

Difficult to isolate and persist for years after pregnancy

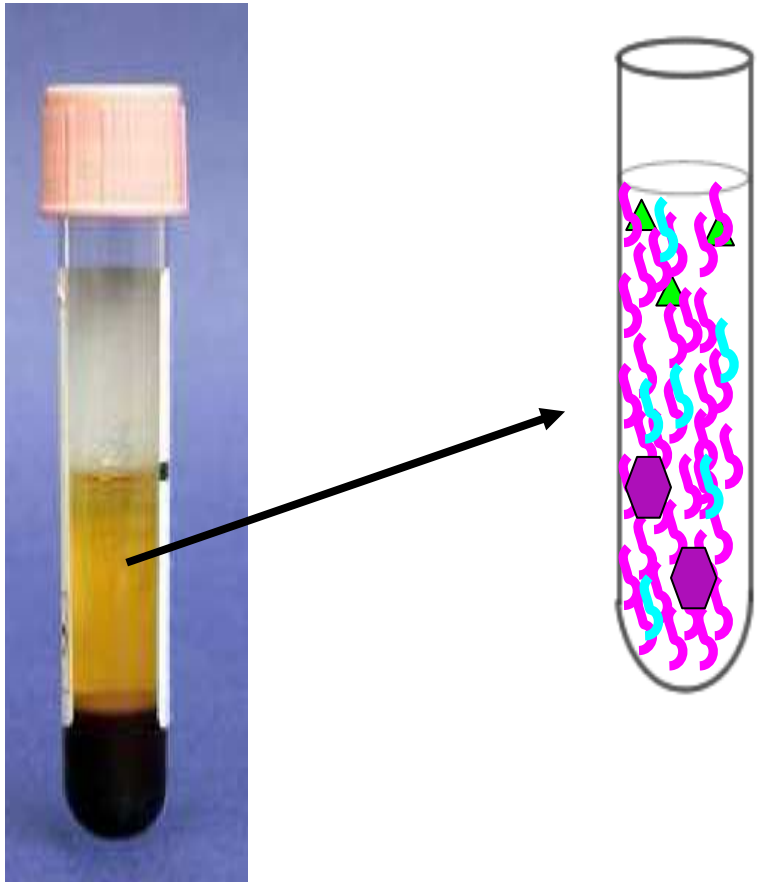
Cell free fetal nucleic acid in the maternal circulation

Originates from trophoblast and 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

Extraction of cell free fetal nucleic acids from maternal plasma



Cell free maternal DNA (96.6%)

Cell free fetal DNA (3.4%)

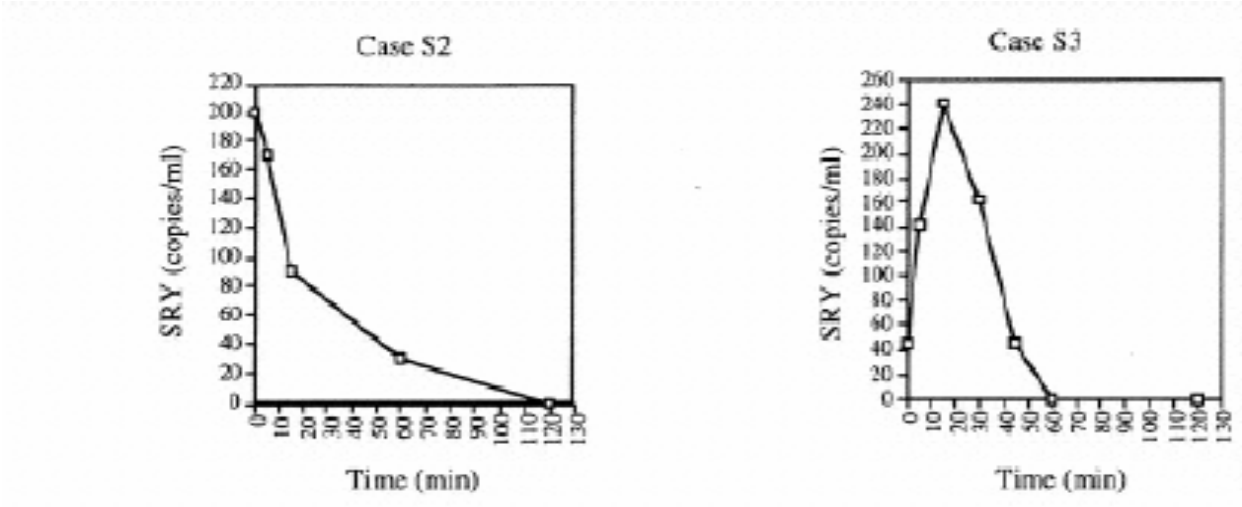
Amount of cf fetal DNA extracted is equivalent to 25 genomes / ml plasma

Cell free maternal RNA

Cell free fetal RNA

Clearance of cell free fetal nucleic acids after delivery

ffDNA



ffRNA

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’

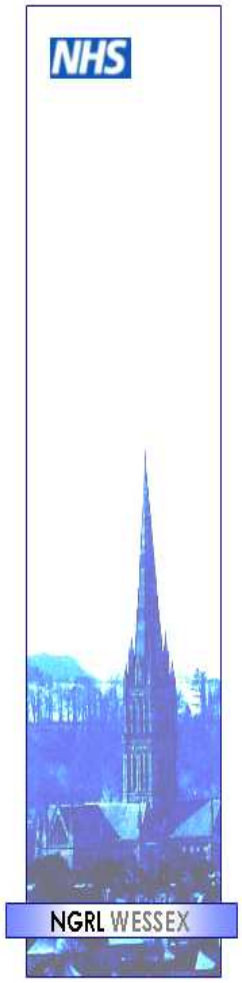
- **Recent major breakthroughs**

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

  Digital PCR of cfRNA and cfDNA

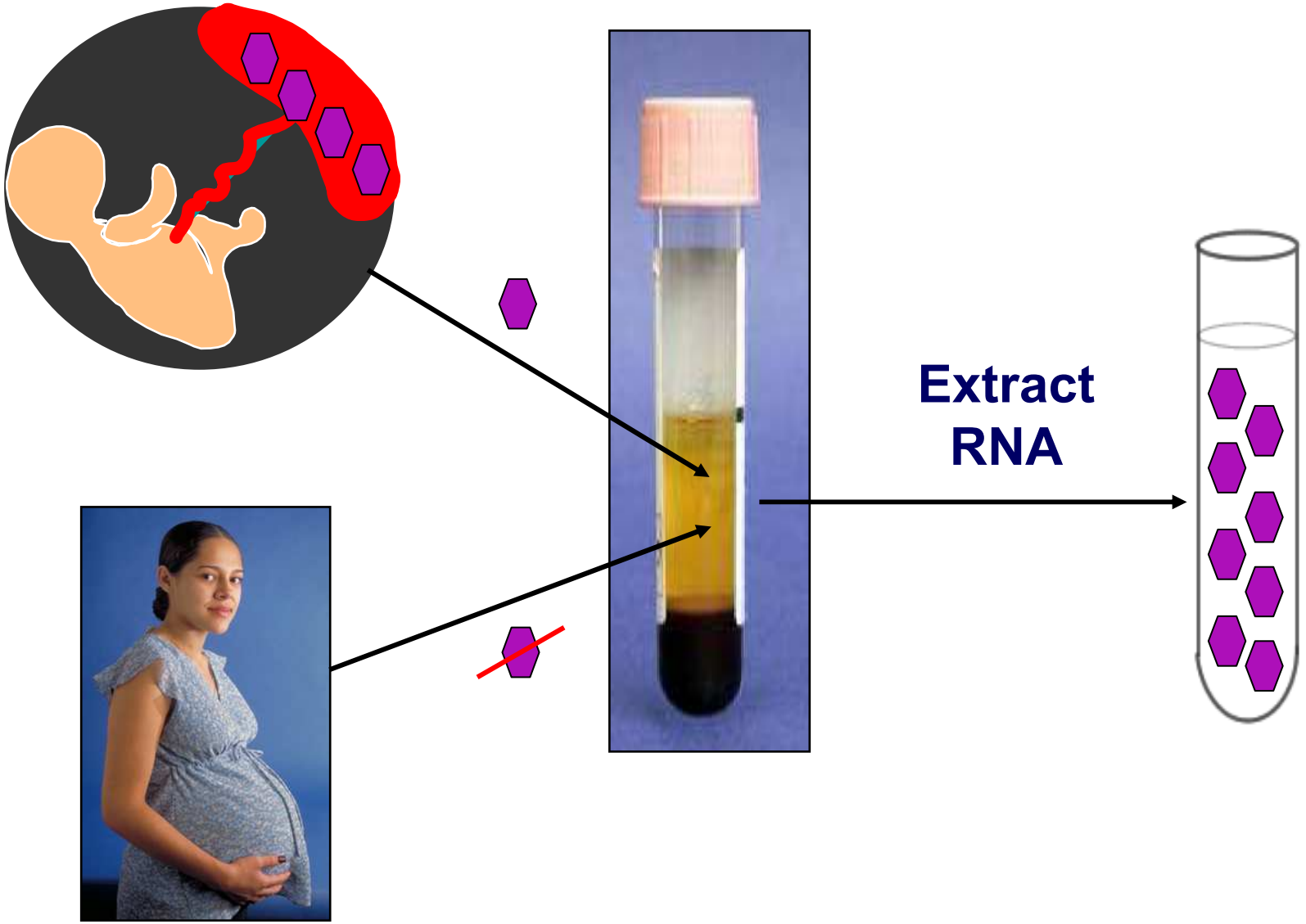
  Massively parallel sequencing of cfDNA

  Epigenetic markers



Quantitative analysis of Single Nucleotide Polymorphisms in fetal specific mRNAs

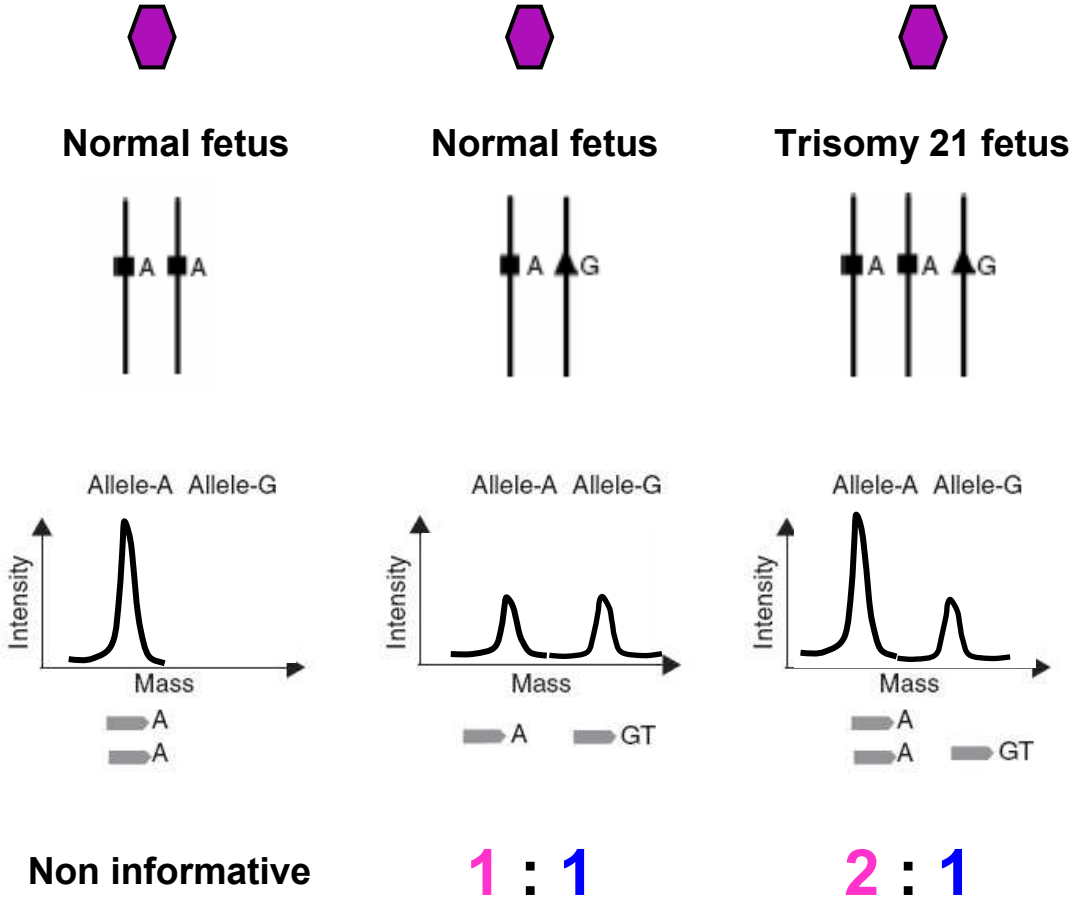
Quantitative analysis of SNPs in fetal specific mRNA



Quantitative analysis of SNPs in fetal specific mRNA

Analysis by MALDI-TOF (mass spectrometry)

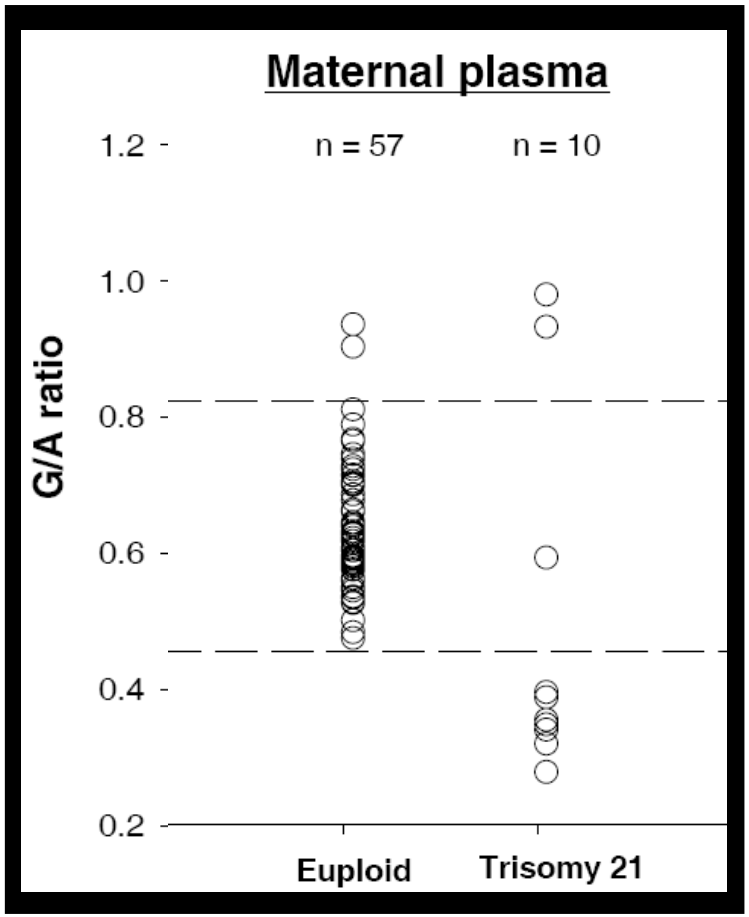
- PLAC4 mRNA ( ) is derived exclusively from fetal chromosome 21
- PLAC4 mRNA expressed in the placenta and is found in the plasma of pregnant women



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

  • Compatible with high throughput screening

- **DISADVANTAGES**

  • Fetus has to be informative for SNP analysed

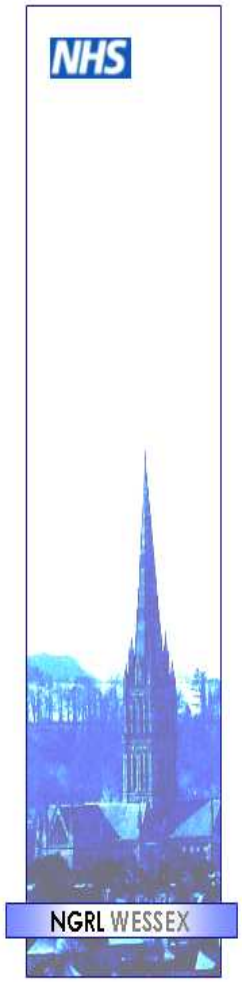
  • RNA can be unstable – implications for sample collection

- **FUTURE REQUIREMENTS**

  • Identification of more polymorphic loci to increase informative cases

  • Multi centre large scale validation required

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



Digital PCR

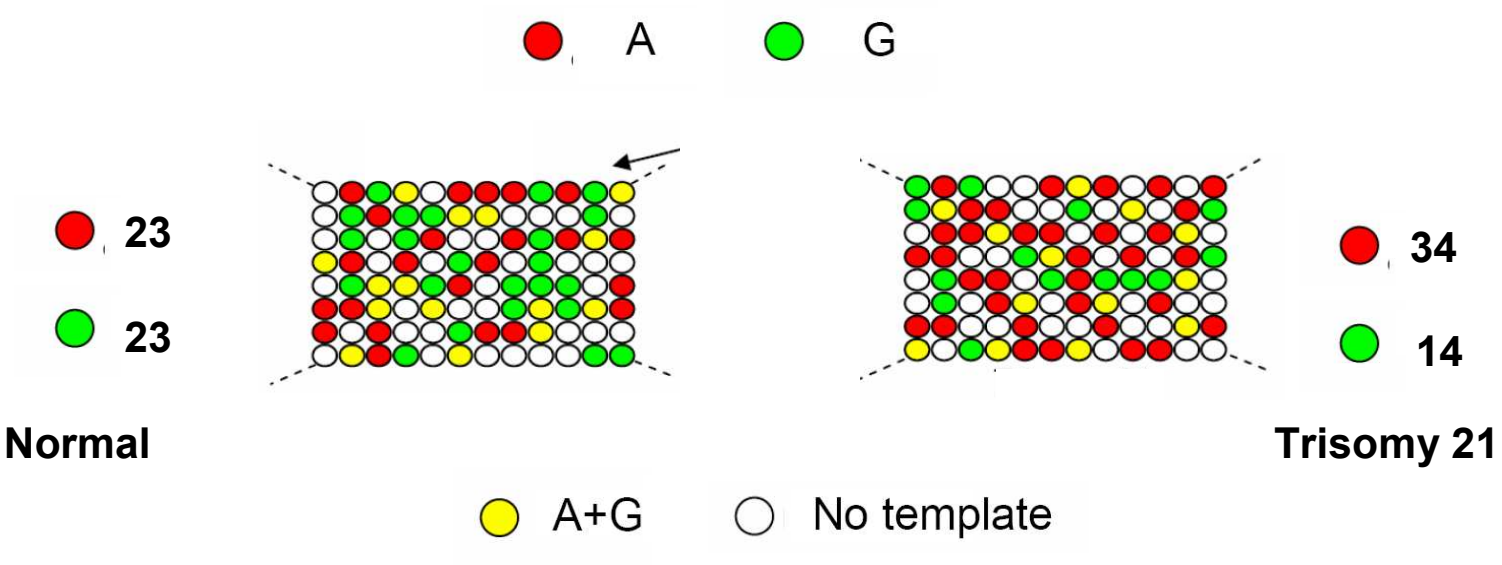
Digital RNA SNP strategy

Relative chromosome dosage

Quantitative analysis of SNPs in fetal specific mRNA

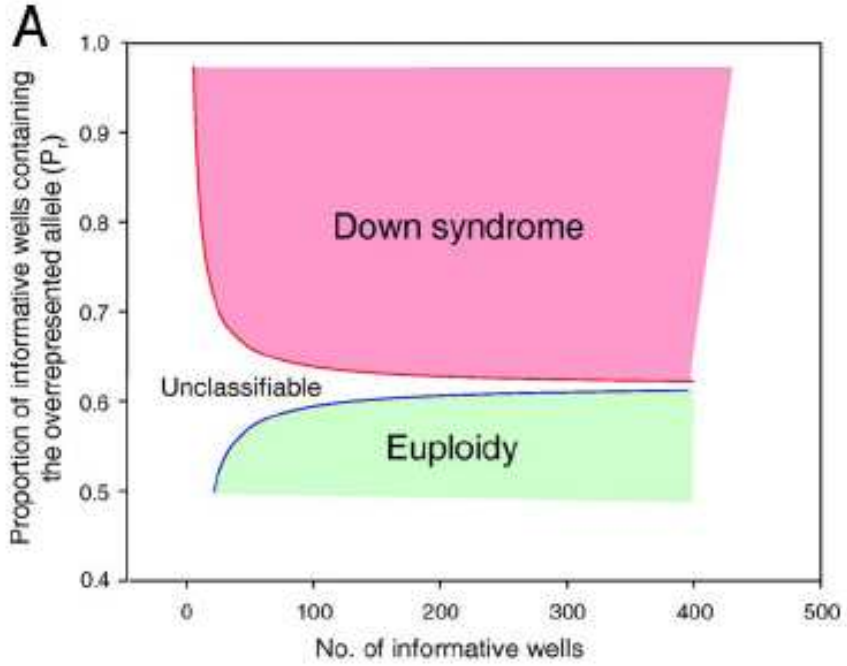
Digital PCR

- 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

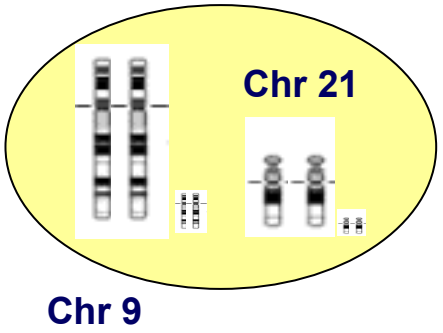


Genotype	No. of wells positive for individual alleles				m_r	SPRT result		
	A only	G only	AG	All negative		P_r	Unclassifiable region	Classification
AG	90	100	97	97	0.67	0.526	0.62–0.64	Euploid
AG	97	105	65	117	0.55	0.520	0.61–0.63	Euploid
AG	66	92	34	192	0.30	0.582	0.59–0.62	Euploid
AG	29	28	3	324	0.08	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

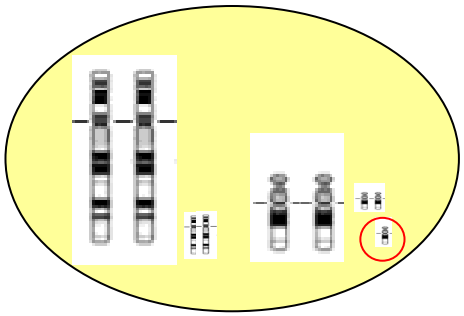
Lo et al., PNAS, 2007

DNA testing preferable: is universal i.e. polymorphism independent

- 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%



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



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

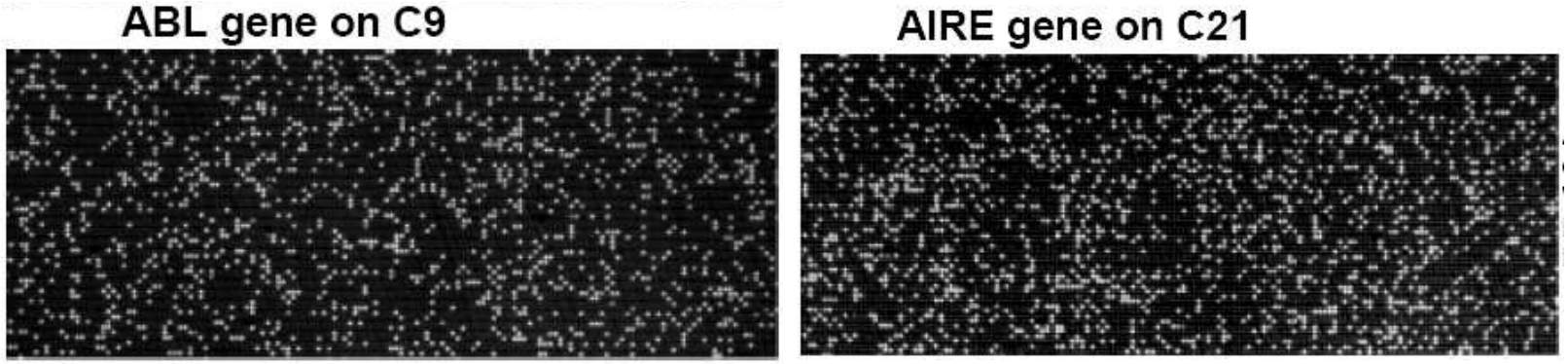
SOLUTIONS:

- 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

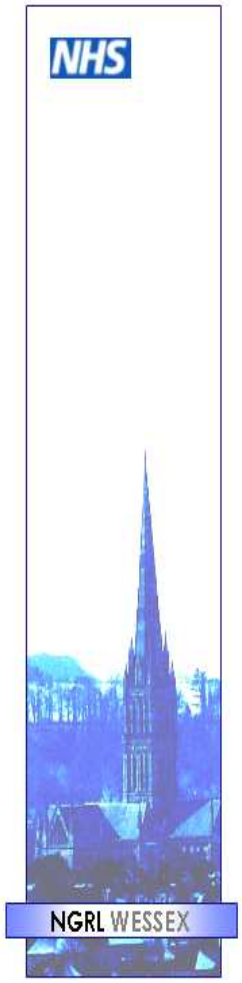
  • At present using relative chromosome dosage can only detect trisomy 21 if fetal DNA component is 25%

- **FUTURE REQUIREMENTS**

  • For relative chromosome dosage and high throughput RNA SNP analysis higher density digital PCR equipment needs to be developed

  • Enrichment of fetal DNA

  • Multi centre large scale validation would be required



Massively parallel / shotgun sequencing

An alternative method for digital quantification of DNA

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

H. Christina Fan*, Yair J. Blumenfeld†, Usha Chitkara†, Louanne Hudgins‡, and Stephen R. Quake*§

*Department of Bioengineering, Stanford University and Howard Hughes Medical Institute, 318 Campus Drive, Clark Center, Room E300, Stanford, CA 94305; †Division of Maternal and Fetal Medicine, Department of Obstetrics and Gynecology, Stanford University, 300 Pasteur Drive, Room HH333, Stanford, CA 94305; and ‡Division of Medical Genetics, Department of Pediatrics, Stanford University, 300 Pasteur Drive, Stanford, CA 94305

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 women:

  9 trisomy 21

  2 trisomy 18

  1 trisomy 13

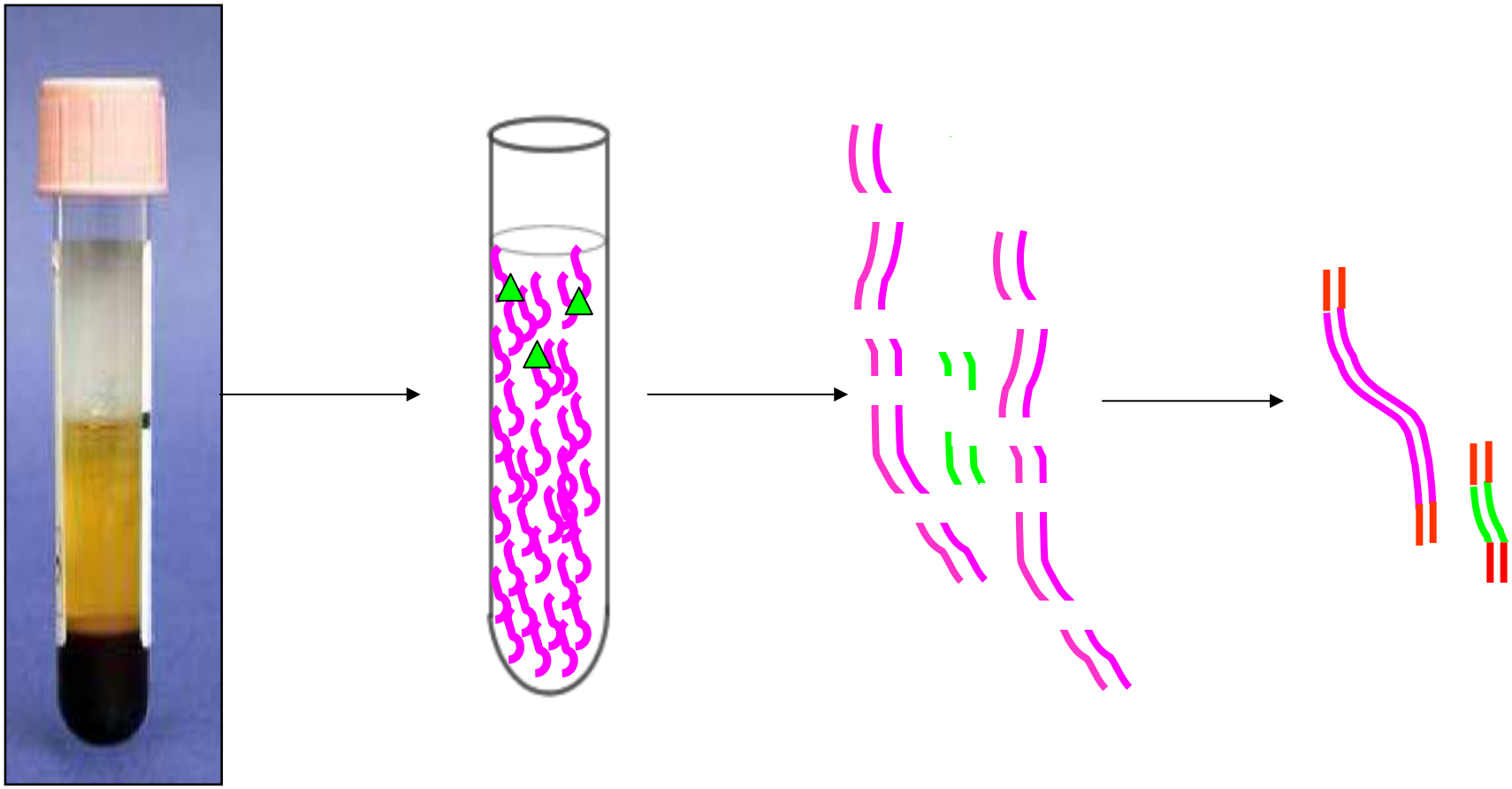
  6 normal

  and 1 genomic DNA sample from a male control

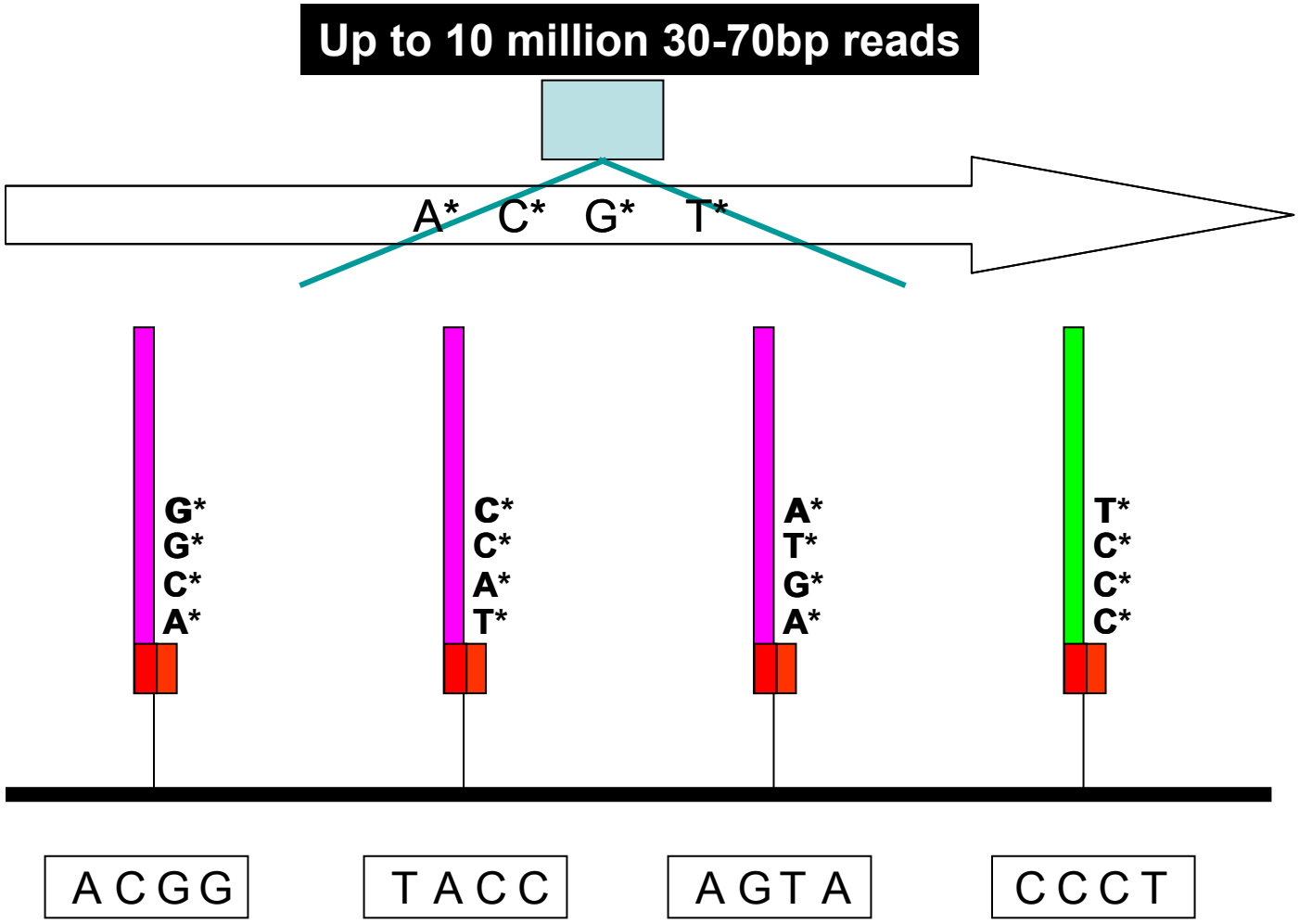
- Gestational age 10 – 35 weeks (earliest trisomy case 14 weeks)
- 5 million sequencing reads for each patient
- Compared density of reads on each chromosome to those obtained from a normal genomic DNA sample
- Also compared density of Chr 21 reads from disomy and trisomy 21 samples
- Coverage of Chr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

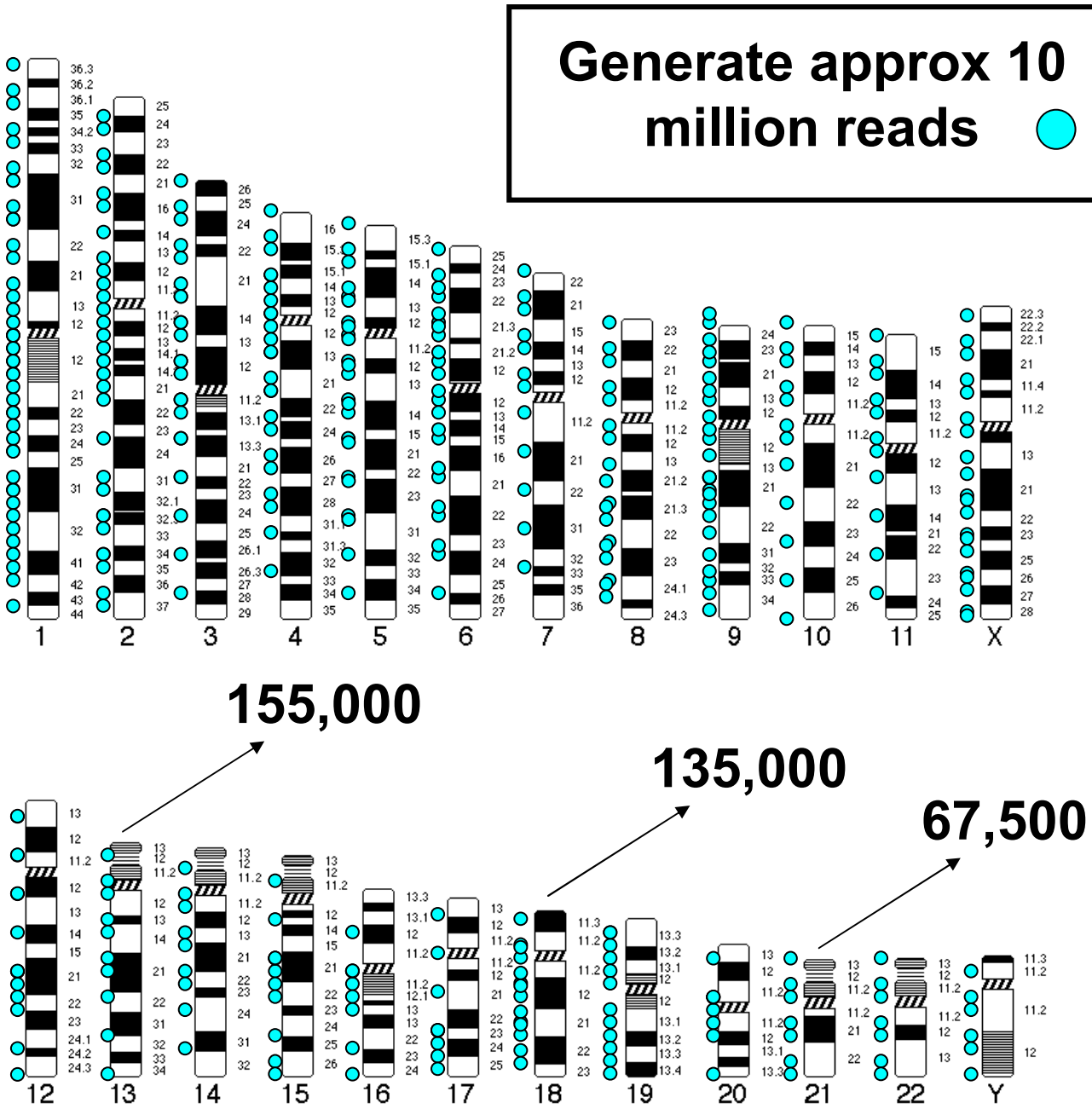
Fan et al., PNAS Oct 2008

Shot gun sequencing

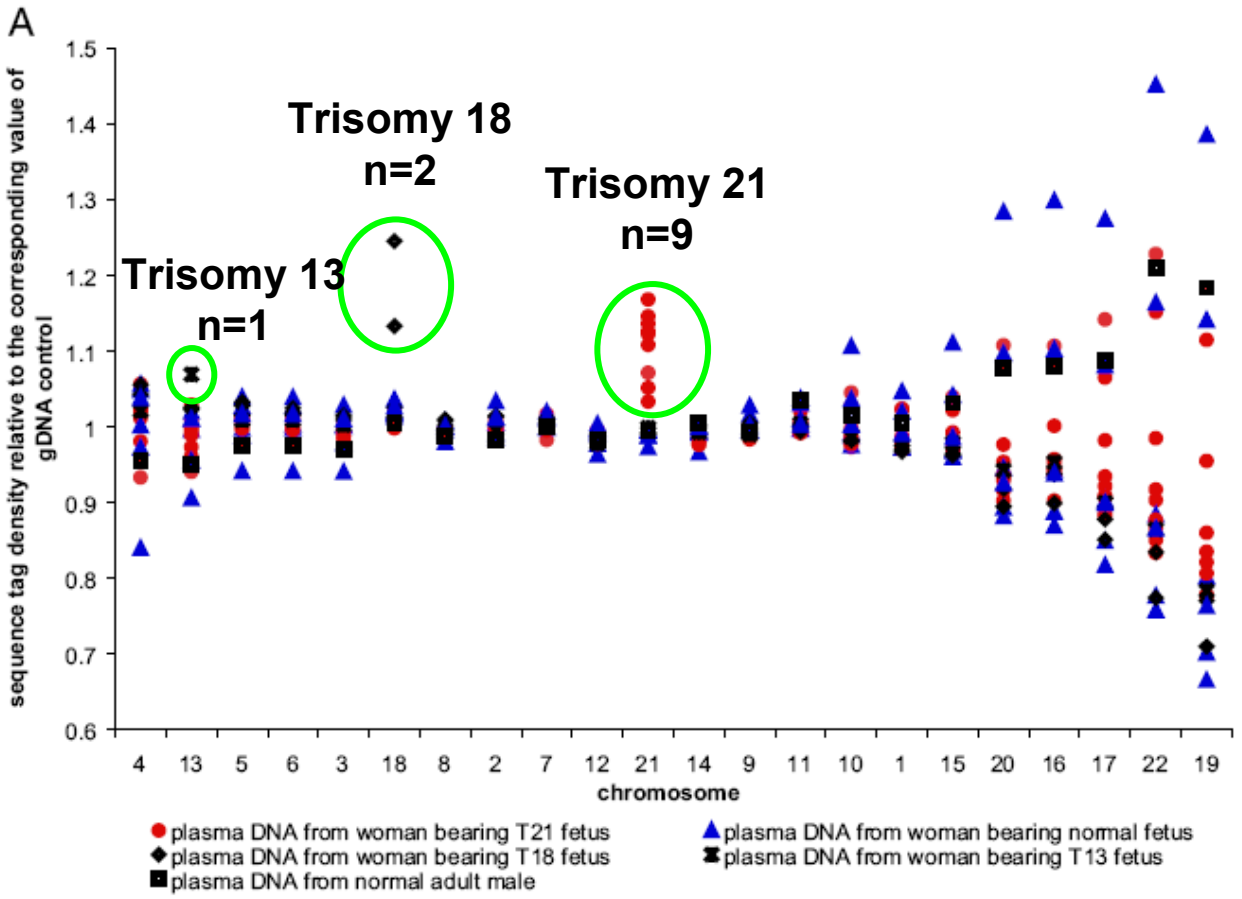


Sequencing by Synthesis



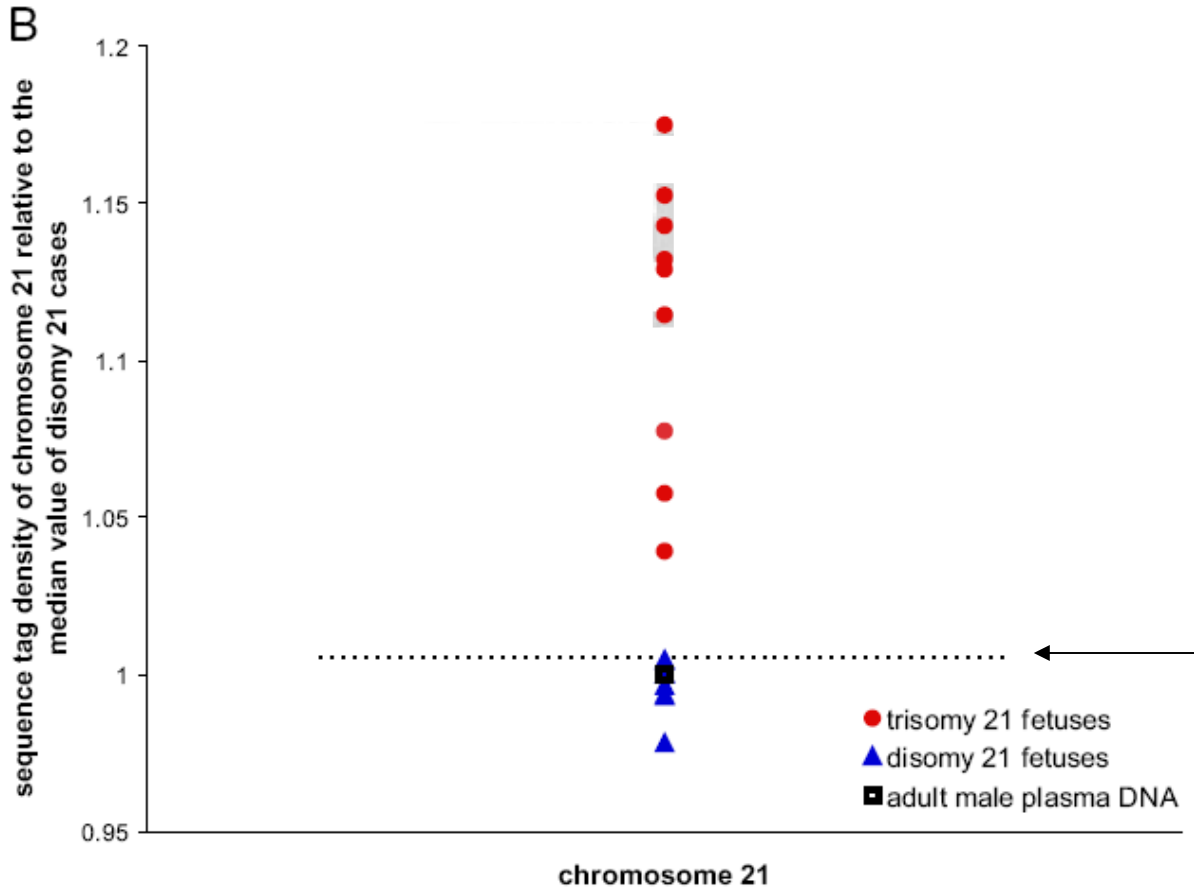

Generate approx 10 million reads
155,000
135,000
67,500
1
2
3
4
5
6
7
8
9
10
11
X
12
13
14
15
16
17
18
19
20
21
22
Y

Results of shotgun sequencing of maternal plasma DNA



Fan et al., PNAS Oct 2008

Results of shotgun sequencing of maternal plasma DNA



Upper 99% confidence limit for normal samples

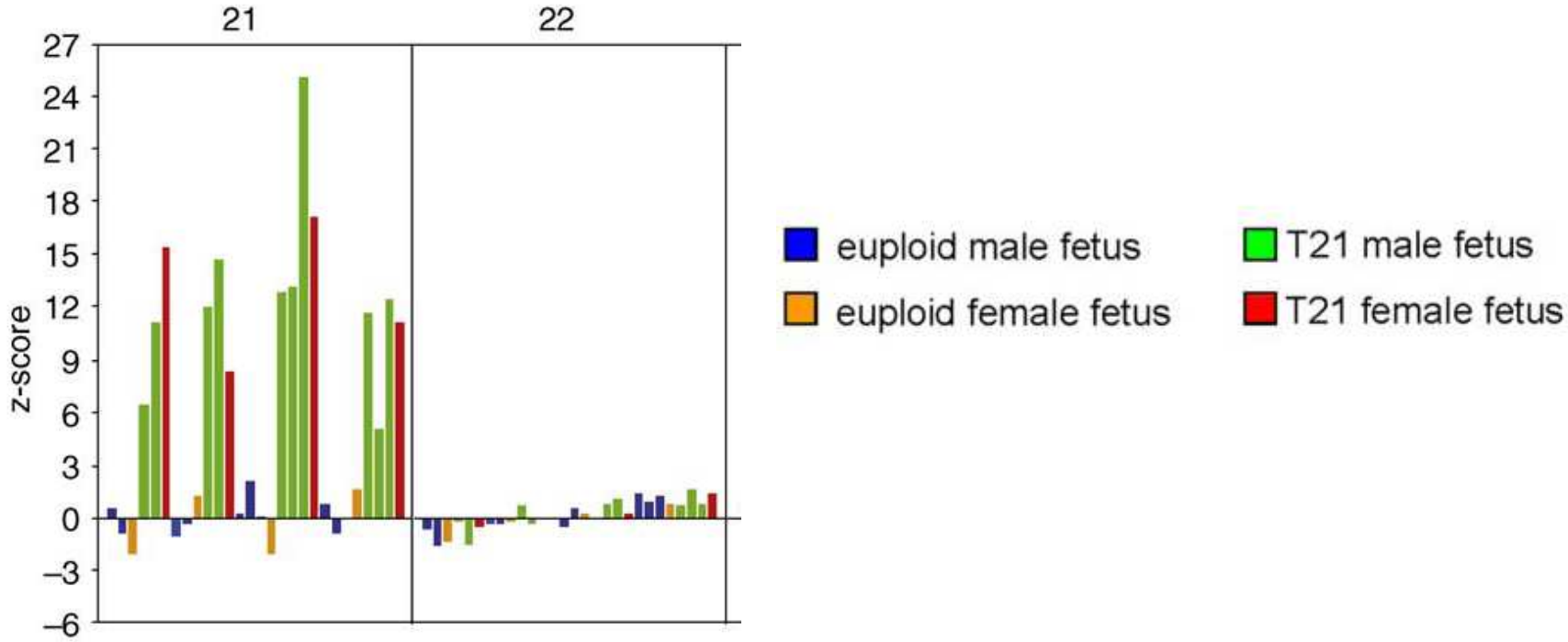
Fan et al., PNAS Oct 2008

Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma

Rossa W. K. Chiu[a,b], K. C. Allen Chan[a,b], Yuan Gao[c,d], Virginia Y. M. Lau[a,b], Wenli Zheng[a,b], Tak Y. Leung[e], Chris H. F. Foo[f], Bin Xie[c], Nancy B. Y. Tsui[a,b], Fiona M. F. Lun[a,b], Benny C. Y. Zee[f], Tze K. Lau[e], Charles R. Cantor[g,1], and Y. M. Dennis Lo[a,b,1]

[a]Centre for Research into Circulating Fetal Nucleic Acids, Li Ka Shing Institute of Health Sciences, Departments of [b]Chemical Pathology and [e]Obstetrics and Gynaecology, and [f]Centre for Clinical Trials, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong SAR, China; [c]Center for the Study of Biological Complexity and [d]Department of Computer Science, Virginia Commonwealth University, Richmond, VA 23284; and [g]Sequenom, Inc., San Diego, CA 92121

Sequenced maternal plasma: 14 trisomy 21 and 14 normal cases correctly identified



Chiu, PNAS, Dec 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

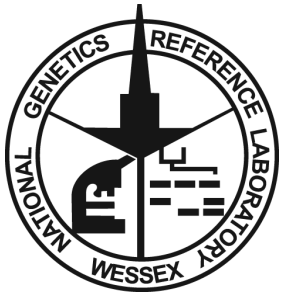
- Important to ensure that women and healthcare professionals understand the changes and women fully understand the implications of these tests

- 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

RAPID: Plans to develop NIPD for aneuploidy

- Matched maternal blood samples and cultured fetal cells are currently being collected from women undergoing invasive prenatal testing in London and the Wessex region
- Assessment of analytical and clinical validity of tests for the non-invasive detection of DS (and trisomy 13 & 18):
  - SNP allele ratios using MALDI-TOF mass spectrometry
  - digital PCR
  - development of targetted new generation sequencing assays
- cffDNA and cffRNA will be used to evaluate NIPD tests and cultured fetal cells will be used to confirm the NIPD result
- Sensitivity and specificity of each assay will be determined

More information



hew@soton.ac.uk

www.ngrl.org.uk/Wessex

www.rapid.nhs.uk

rapid@ich.ucl.ac.uk

l.chitty@ich.ucl.ac.uk