Module 6:
NGS and Fetal Fraction

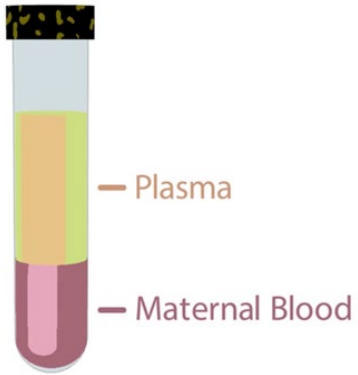
St George's University Hospitals
NHS Foundation Trust

Module 6 will cover:

- Next Generation Sequencing (NGS)
    - An overview of the technology
    - Analysis software
- Fetal fraction (FF)
    - What is fetal fraction?
    - Why fetal fraction is used
    - Dynamic fetal fraction

the SAFE test
St George's Antenatal Fetal Evaluation

Next generation sequencing (NGS)

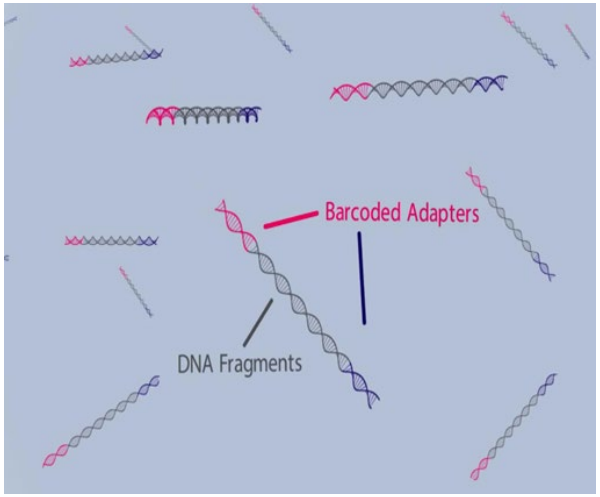


- In an unaffected pregnancy, chromosome 21 represents 1.36% of the total cell-free DNA (cfDNA) in the maternal circulation.
- If the pregnancy is affected by trisomy 21, this percentage rises to about 1.42% - only 0.06% difference.
- To distinguish such small differences in the amount of cfDNA found, incredibly accurate DNA counting and sorting methods are required.
- NGS sequencing enables millions of DNA strands to be sequenced in parallel, this is cheaper, quicker and generates more data.

NGS workflow for cfDNA screening

1. DNA extraction: DNA fragments are extracted from blood plasma. In each 1 ml of blood there are millions of cfDNA fragments from all 23 chromosome pairs from both the fetus and the mother.

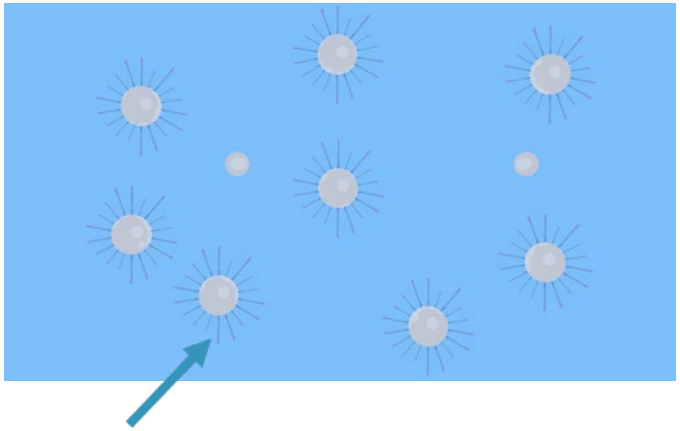
2. Library preparation: DNA fragments from the sample are modified, labelled with barcode and amplified using PCR – referred to as a library. The barcodes are specific to each individual patient sample and are used to identify each patient's DNA after sequencing.



NGS workflow for cfDNA screening

3. Pooling: 12 patient samples are pooled into a single tube. Prior to this samples are measured (quantified) to ensure that there is an equal representation of each of the 12 samples in the pool.

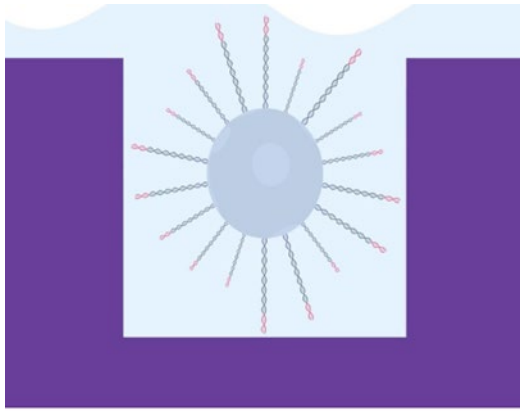
4. Template preparation: Each DNA sequence in the pool is bound to a bead (ion sphere) and amplified. This allows for the sequencing of each fragment separately and strengthens the signal.



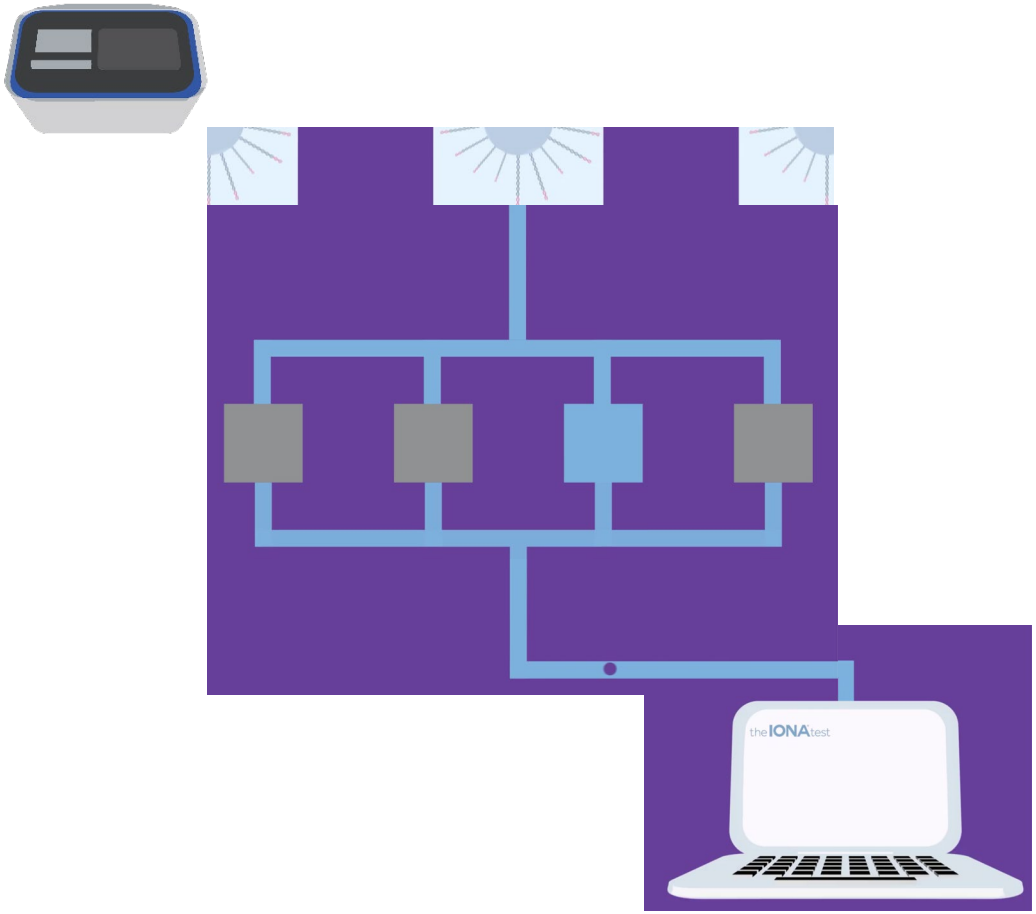
Ion Sphere with clonal amplification

NGS workflow for cfDNA screening

5. Sequencing: Ion spheres (one per well) are loaded onto the Sequencing chip. Each chip holds pooled DNA from 12 patients. The chip is sequentially washed with nucleotides. DNA fragments are incorporated (bound) if they are complementary.



Sequencing: Base calls



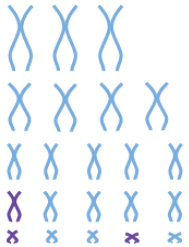
- As nucleotides (A, T, C and G) are incorporated (bound) a hydrogen ion (H^+) is released.
- This changes the pH in the well - measured by the sequencer.
- Multiple repetitive nucleotides (eg TAAG) will create a stronger signal for that particular base.
- The software assembles the signals and converts them to base calls.

Analysis of sequencing data

The sequenced cfDNA fragments are aligned to a reference genome.

The fragments are then counted and an overall amount of DNA is assigned to each chromosome.

The amount of chromosome 13, 18 and 21 is then compared against the total amount.

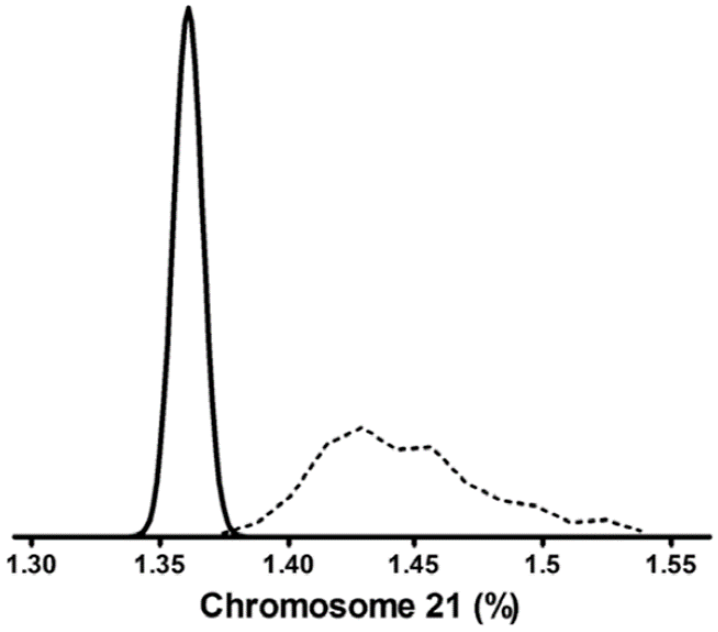


$$\frac{\text{Chr. 21 or 18 or 13}}{\text{Total amount of DNA}} = ?$$

Analysis & mapping distribution

The likelihood that a particular sample is from an affected pregnancy is assessed by looking at the sequence quality, count density, controls and fetal fraction.
This is all performed and analysed using advanced computer technologies to report on:

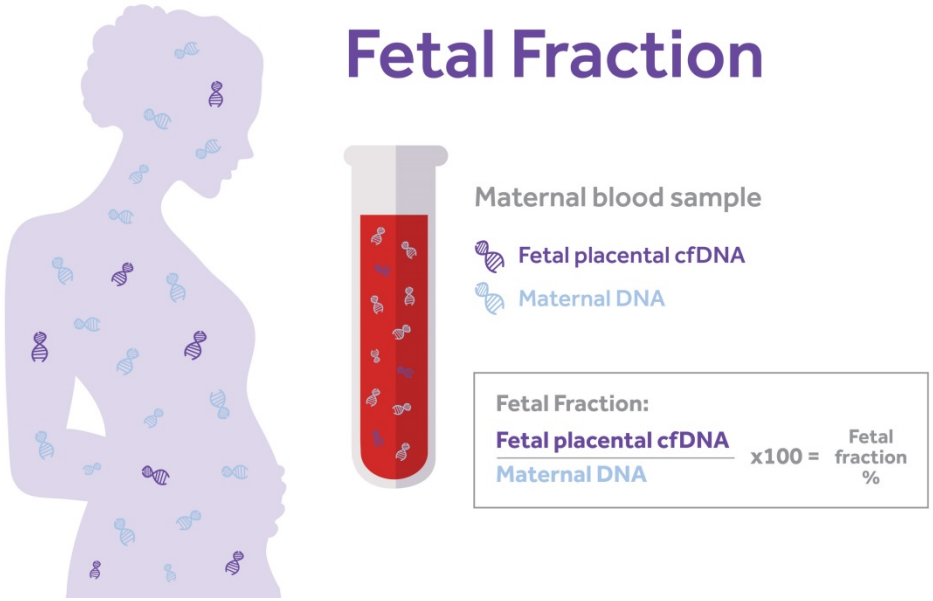
- How well the chip was loaded
- If there is enough DNA in the wells
- How many wells have good quality data



Fetal Fraction

- cfDNA in maternal plasma is a mixture of maternal and placental cfDNA. The proportion of cfDNA from the placenta is known as ‘fetal fraction’.

- All NIPT tests are affected by the fetal fraction of cfDNA. Most, but not all, cfDNA screening tests measures fetal fraction in analysis.

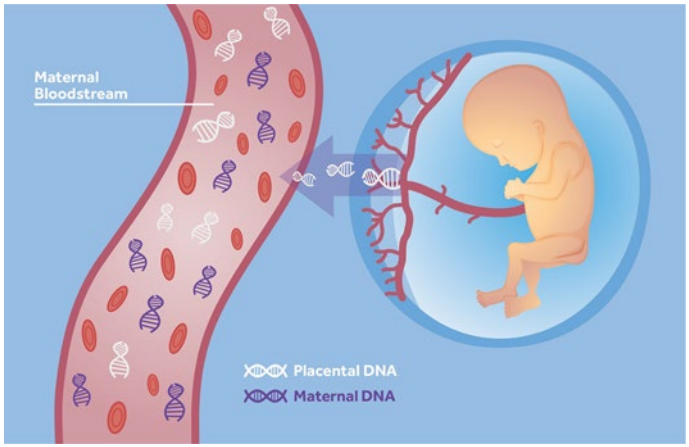


cfDNA screening tests and measuring fetal fraction

- One study involved analysis of non-pregnant blood samples. Laboratories that did not measure fetal fraction reported a ‘female infant – no aneuploidy’ because the test analysed only maternal cfDNA and presumed fetal cfDNA present.

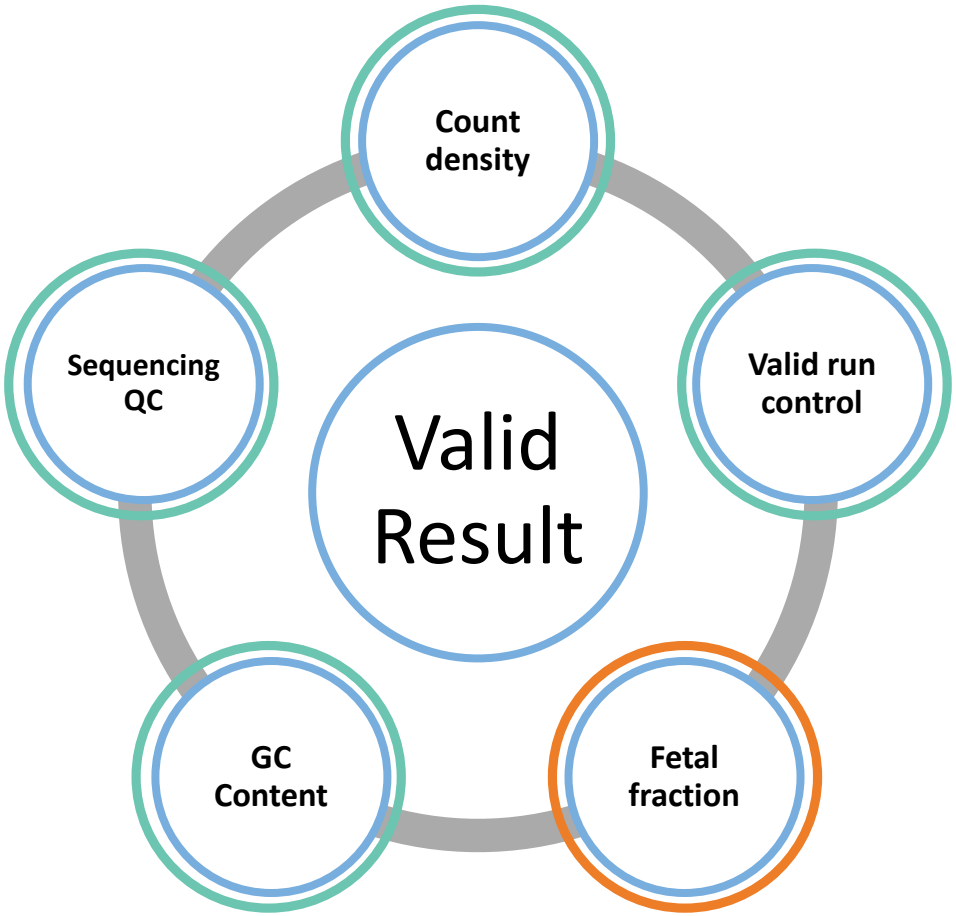
- If a mother has a “low fetal fraction” it can impact the ability to generate a result on the sample.

- Studies have shown that pregnant women with a large BMI, earlier in pregnancy and some other factors too, can have a low fetal fraction.



the SAFE test
St George's Antenatal Fetal Evaluation

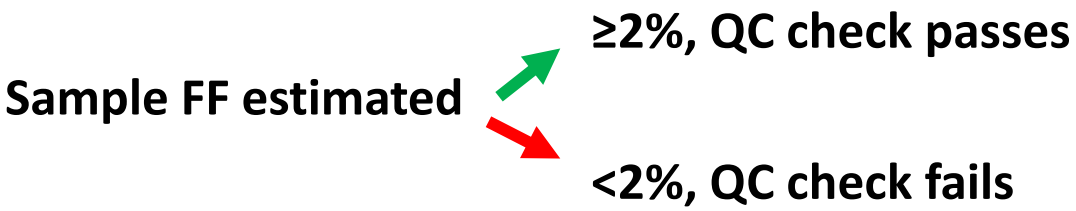
Dynamic fetal fraction
Sample/run validity



The fetal fraction estimation and QC check is the final step in the analysis process

Fetal Fraction in the SAFE test

- The FF estimate needs to be applied with consideration for (i) other available data and (ii) the technology used
- The SAFE test firstly, uses a hard cut-off of 2%



- Accounts for <0.5% of the sample population

the SAFE test
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Fetal Fraction: Sample/run validity

- A valid SAFE test result is a balance of fetal fraction (FF), count density and chromosome ratio
- First, determination of whether the dynamic check is required (FF of 2-4%)
- If it is required, the number of aligned sequencing reads is assessed (count density) which allows for the required level of FF to be determined.
- A higher count density means the FF% required can be lower. Conversely, a lower count density will require a higher fetal fraction.

	Fetal Fraction	Count Density	Result
Scenario 1	2-8%		Valid
Scenario 2	<2%		Invalid
Scenario 3	>8%		Invalid