Advancing NIPT Workflows:

Using Size Selection to Enrich Fetal Fraction, Enabling Extended Blood Storage in EDTA tubes

Prenatal screening for Down's Syndrome and other chromosomal imbalances by the analysis of a blood sample has been available since the 1980s. The original methods utilised maternal pregnancy markers, such as alpha-fetoprotein (AFP) and human chorionic gonadotropin (hCG), which lacked specificity (too many false positives) and sensitivity (too many false negatives).[1] In 2011, the field was revolutionised by the introduction of non-invasive prenatal testing (NIPT), which directly measures the presence of placental-derived DNA, representing the fetus, circulating in the mother's blood.[2] This method is used today to screen pregnancies, minimising the number of people who needlessly undergo invasive testing, which has a higher risk of spontaneous miscarriage and has greatly improved sensitivity and specificity over the maternal markers.[3,4]

Cell-free DNA and Non-invasive Prenatal Testing

The technology we know today as NIPT was made possible by the first discovery of circulating cell-free fetal DNA (cffDNA) in the mother's blood by Dennis Lo in 1997.[5,6] The proportion of cffDNA in the mother's blood that is derived from the fetus is referred to as the fetal fraction. It was immediately apparent that this had the potential to open the door to improved prenatal screening; however, at that time, no one could identify a method to analyse the fetal DNA amongst the maternal DNA circulating in the blood because of the relatively low fetal fraction, typically around 10% of cell-free (cfDNA) is cffDNA at 12 weeks gestation.

The insight that solved this problem was made, again, by Dennis Lo in 2008, who realised that it wasn't necessary to separate the fetal and maternal DNA – instead he developed a method based on counting chromosomal markers.[7] Down's Syndrome is caused by an extra copy of chromosome 21; therefore, a woman pregnant with a fetus affected by Down's Syndrome will show a small increase in the amount of chromosome-21 DNA in circulation. Chromosome 21 represents about 2.1% of the human genome but in a pregnancy affected by Down's Syndrome, this is increased to around 2.2% (depending on the fetal fraction). If it was feasible to measure this small but significant increase, then it would allow prenatal screening of trisomy 21. Technical advances in DNA sequencing that allowed the analysis and chromosomal mapping of millions of cffDNA fragments made it possible to accurately count and calculate the proportion of chromosome-21-derived DNA. A proportion of 2.1% was good evidence of an unaffected pregnancy; a higher proportion indicated the presence of trisomy 21. This insight led to the establishment of NIPT as we know it today, meaning fetal trisomy can be screened for from a maternal blood sample without the need for an invasive procedure which carries a risk of spontaneous miscarriage of healthy pregnancies.

Challenges with Fetal Fraction

Over the years this theory has been translated into a plethora of NIPT methodologies, greatly improving the performance of prenatal screening; however, some challenges remain. Fetal fraction is highly variable but is broadly accepted as lower in lower gestational age and mothers with a higher BMI. As fetal fraction decreases, the amount of chromosome-21 DNA derived from the fetus also decreases, meaning the difference between affected and unaffected results becomes more difficult to measure reliably. Many NIPT providers have a minimum fetal fraction requirement and below this level the test is deemed to have failed.

Increasing the depth of DNA analysis can partially help to overcome low fetal fraction by generating more data points to analyse, but this substantially increases the cost of testing and doesn't overcome the issue that the relationship between count density (the number of individual measurements of chromosomes) and accuracy is not linear: at very low fetal fractions even a significant increase in sequencing levels will not provide a reliable result. At fetal fractions below 5%, there is a significant increase in the required depth of sequencing. A better alternative to increasing sequencing depth would be to increase fetal fraction, which would have the combined benefits of improved accuracy at lower cost.

Enriching Fetal Fraction with Size-selection Methodologies

Cell-free fetal DNA fragments are generally shorter than those of cell-free maternal DNA and, therefore, offer a means to select and enrich for fetal DNA using size-selection methodologies. However, this approach has been limited by the unavailability of a size-selection method which has both the accuracy and precision to separate maternal and fetal DNA, and with a high enough DNA recovery to ensure that the sample is preserved for downstream

testing. Recent advances in DNA size-selection technology have seen the emergence of instruments that can more accurately and precisely separate DNA fragments, giving reproducible, high yielding size-selected DNA with enriched fetal fraction.[8] An example is Ranger® Technology from Yourgene Health which is able to accurately and reliably prepare a specific size fraction of the cfDNA.

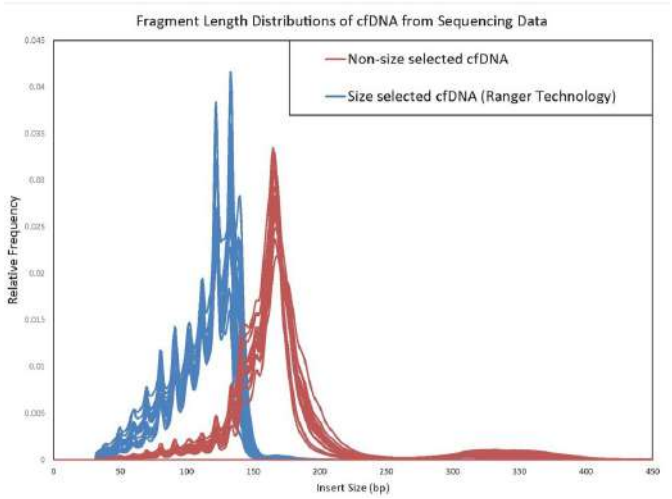

Figure 1: Histogram profiles for size-selected (blue) and non-size-selected (red) DNA libraries used in NIPT applications. Size-selection methodologies enrich for fetal DNA fragments as the fragments are shorter than those of maternal origin.

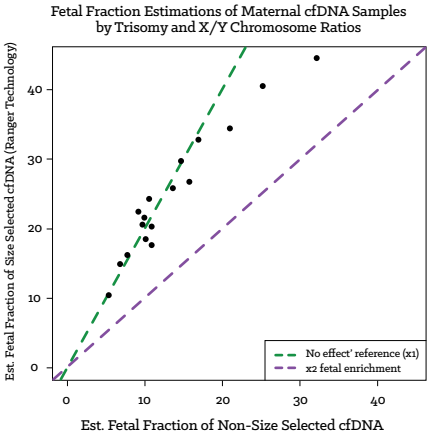

Figure 2: Comparison of fetal fraction between samples size selected as opposed to those that are processed without size selection.

Size-selection technologies help to capture the shorter fetal DNA fragments, reducing the proportion of maternal DNA content (Figure 1). Applying size-selection technologies to NIPT samples can achieve up to two-fold fetal fraction increases compared with non-size-selected samples (Figure 2).

Fetal Fraction Recovery Following Storage in EDTA Blood Collection Tubes

An additional benefit of size selection on cfDNA is that it is possible to consider the use of standard EDTA tubes rather than expensive specialised preservation blood collection tubes (BCTs) for NIPT. Size selection can reduce the maternally derived cfDNA, maintaining, and even enriching, the fetal fraction of these samples stored in EDTA gel tubes, as demonstrated by the Centre de recherche du CHU de Quebéc-Université Laval using Ranger Technology.[8]

When using EDTA tubes, cell lysis due to sample instability can result in the release of maternally derived genomic DNA, the presence of which can cause dilution of the cffDNA, leading to reduced fetal fraction. Use of clinical-grade technologies offering scalable and precise DNA size selection with >97% reproducibility and 80% recovery, helps to ensure consistent enrichment of the fetal fraction in NIPT. These can provide call results from as little as 2% fetal fraction, compared to the industry standard of 4%. Having the ability to increase the fetal component of the cfDNA by reducing the maternal DNA content within a sample will help to lower failure rates and deliver more reliable NIPT results, meaning more pregnant women will have the opportunity to benefit from NIPT early in their pregnancies.

A recent collaborative study with Tommy's charity and St Mary's NHS Hospital (Manchester, UK) assessed the ability of Ranger Technology to recover the fetal fraction from whole blood stored in EDTA BCTs over a prolonged period using the IONA® Nx NIPT Workflow (Yourgene Health). The ability to detect trisomy samples was unchanged across the extended time periods, up to 7 days, and the study concluded that utilising size selection in NIPT workflows allows the use of EDTA BCTs for prolonged periods over 8 hours.[9]

Cost Savings and Avoidance of IP Litigation

Streck BCTs are expensive, with a single tube costing $8–10 USD. In contrast, EDTA BCTs cost around $0.55. We conducted a cost-benefit analysis which demonstrated that for mid- to high-throughput laboratories, the actual value attached to that cost-saving could be significant from as early as year one. An NIPT laboratory running 8,000 samples per year could expect to see savings of over $55,000 by year three from switching to an EDTA/Ranger Technology workflow. A lower-throughput laboratory processing 2,000 samples per year could expect to see nearly $29,000 saved per year by choosing EDTA over Streck BCTs. For ultra-high-throughput laboratories looking to run >100,000 samples per year, the overall saving in year one alone is estimated to be just over $700,000 (Table 1). Further cost savings would also be expected due to a reduction in failure rates, although this cannot be quantified at this time until further planned studies are completed.

Scenario	Year 1 Savings ($)	Year 2 Savings ($)	Year 3 Savings ($)
High-throughput library (100,000/year) (EDTA BCT savings only)	706,630	757,500	757,500

Table 1: Predicted cost savings years 1–3 for a high-throughput (100,000 samples/year) laboratory utilising Ranger Technology and EDTA BCTs. The calculation is based on internal data and considers licensing from Yourgene Health, technology implementation costs, as well as BCTs used per patient per sample run. The cost of EDTA BCTs was set at $0.55 per tube, and Streck at $9.64. These values represent an average from publicly available list prices and have been verified using external customer data.

There are also annual cost-benefits derived from a reduction in test failures due to insufficient fetal fraction. Enrichment by size selection can salvage up to 50% of samples which would otherwise be failed due to low fetal fraction. The average laboratory might expect around 3–4% of all samples tested to have a fetal fraction below the analytical sensitivity. By using size selection to salvage these low fetal fraction samples, laboratories stand to make significant cost savings in addition to those achieved by switching to EDTA BCTs.

Another consideration is the fragility of glass Streck BCTs compared to plastic EDTA BCTs; using plastic tubes for sample transport will reduce the number of lost samples due to breakages and is more compatible with high-throughput automation.

In the US, an additional financial concern surrounds future IP licensing payments for use of the cell stabilisation BCT, adding significant costs to these tests. This is a huge financial burden for all

molecular diagnostic tests performed by US labs if they continue to use cell stabilisation blood tubes.

The Next Generation of NIPT Workflows

The results of the studies referenced in this article show that utilising size selection in NIPT workflows allows the use of EDTA BCTs for prolonged periods over 8 hours. Data indicate that not only is fetal fraction maintained, but that the results of NIPT are concordant across the 7 days of the study.

Many NIPT clinics and hospitals take a maternal blood sample with an expensive BCT, such as a Streck tube, and the sample is stable for up to 14 days. However, the data show the capability to use EDTA tubes, which are commonly used and a significantly lower cost, with proven stability up to 7 days. The use of EDTA blood tubes in NIPT could offer a considerable saving in the cost of the stabilising BCTs, and in the US where there is IP in the use of stabilising BCTs, avoid costly licensing fees.

By enabling EDTA BCTs to be used for collection, transport and analysis of samples, these technologies provide clinicians and laboratories with reduced costs and improved flexibility, making NIPT more accessible whilst ensuring patients receive highly accurate prenatal screening results.

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