# Health Policy Advisory Committee on Technology

## Technology Brief

## Non-invasive prenatal testing (NIPT)

**May 2013**

HealthPACT

*emerging health technology*

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This brief was commissioned by Queensland Health, in its role as the Secretariat of the Health Policy Advisory Committee on Technology (HealthPACT). The production of this brief was overseen by HealthPACT. HealthPACT comprises representatives from health departments in all States and Territories, the Australian and New Zealand governments and MSAC. It is a sub-committee of the Australian Health Ministers' Advisory Council (AHMAC), reporting to AHMAC's Hospital Principal Committee (HPC). AHMAC supports HealthPACT through funding.

This brief was prepared by Linda Mundy from the HealthPACT Secretariat.

## Technology, Company and Licensing

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|---|---|
| **Register ID** | **WP142** |
| **Technology name** | **Non-invasive prenatal testing (NIPT) of chromosomal abnormalities such as trisomy-21 (Down syndrome)** |
| **Patient indication** | **Women considered to be at high-risk of carrying a fetus with a chromosomal abnormality such as trisomy-21** |

### Description of the technology

Down syndrome, the most common aneuploidy, and results from the presence of a third copy, either full or partial, of chromosome 21. Similarly, trisomy 13 and 18 result from the presence of an extra copy of chromosomes 13 and 18. Methods for the non-invasive prenatal testing (NIPT) of trisomy 21 and 18 were assessed by HealthPACT in 2008 and then updated in 2009. These assessments, complete with more detailed descriptions of the methods employed, can be found at the end of this current assessment.

In the past, attempts have been made to isolate intact fetal cells from the maternal circulation, though results have been discouraging due to the rarity of these cells (1 cell/mL of maternal blood). In addition, fetal cells such as lymphocytes and leukocytes can persist for years in the maternal circulation from previous pregnancies. Fetal DNA, however, can be identified in the maternal plasma from five weeks post-conception, with the level increasing with gestational age, and is then cleared from the circulation following birth. Several NIPT methods have been investigated using fetal nucleic acids, either by isolating cell-free fetal DNA or RNA derived from the placenta, transcribed from genes located on the chromosome of interest (13, 18 and 21). Isolated fetal nucleic acids are then identified using techniques including PCR[1] and shotgun sequencing.[2]

Several methods were discussed previously including:

- detection of paternally inherited SNP[2]s, as discussed by Dhallan et al (2007) in the 2008 Brief. This approach is limited in that it can only be applied in male fetuses and that the detection of a paternally inherited polymorphism requires heterozygosity for the analysed SNP.
- the RNA-SNP allelic ratio method, as discussed by Lo et al (2007) in the 2008 Brief. It is thought that cell-free fetal RNA (cff-RNA) would be an ideal candidate to target as the level of RNA originating from the placenta in the maternal circulation is much higher than that of cff-DNA. Although this approach has demonstrated a high sensitivity and specificity for trisomy 21, it is limited by the variation in

[1] PCR = polymerase chain reaction
[2] SNP: single nucleotide polymorphism. DNA sequence variations that occur when a single nucleotide (A, T, C, or G) in the genome sequence is altered.

heterozygosity rates. The heterozygosity rate for mRNA coded by the *PLAC4* gene is 0.45, and would therefore only be of use in less than half of the population.

- epigenetic modifications have been proposed as a possible approach, in particular DNA methylation. Attempts have been made to identify DNA sequences on chromosome 21 that are differentially methylated between maternal and fetal DNA. However, current methods require a preparation step that degrades a high proportion of the sample DNA, which only constitutes approximately 3-10 per cent of the total amount of plasma DNA in the maternal circulation and therefore subsequent analysis may be limited by the amount of starting material. This may result in a higher than acceptable level of false negatives; and
- high-throughput shotgun sequencing, as described by Fan et al (2008) in the 2009 update. With the advent of next generation sequencing methods and the rapidly developing improvements in the technology, making it cheaper and more accessible, it would appear that this last method is more likely to be widely adopted.[2] Massively parallel sequencing (MPS) of maternal plasma can identify the chromosomal origin of each of the sequenced DNA molecules (fetal or maternal) and can detect chromosomal under- or over-representation.[3]

Two new MPS techniques have been recently described in the literature that are essentially single molecule counting techniques. These methods are capable of quantifying small increments in the total amount of DNA (maternal and fetal) from the aneuploid chromosome (eg trisomy 21) compared to normal pregnancies: [4]

- digital analysis of selected regions (DANSR) which quantifies non-polymorphic loci of interest on chromosomes simultaneously using targeted sequencing rather than shotgun sequencing methods. For example, the amount of plasma DNA molecules from chromosome 21 are compared to those from a normal chromosome, and will be elevated in cases of trisomy. To have a measure of precision, approximately 8,000 digital PCRs need to be performed to detect fetal trisomy in a maternal plasma sample.[4]
- parental support, where the allele distribution of the polymorphic loci on the chromosomes of interest are compared to the expected allele distribution based on the parental genotype.[3] However, measuring the proportional amounts of sequences from chromosomes with a high or low GC[3] content has been problematical. For this reason using MPS for the detection of trisomy 13 and 18 has been shown to be less precise and requires further validation.[4]

[3] GC = DNA bases guanine and cytosine

**Company or developer**

Natera Inc (California, USA) currently offer NIPT on a user-pays basis. This SNP microarray test, similar to that described by Fan et al (2008) in the 2009 Update, is now accessible via Victorian Clinical Genetics Services. Other commercially available NIPT tests for the determination of trisomy 21 include MaterniT21™ PLUS (Sequenom, San Diego, USA), PraenaTest® (LifeCodexx AG, Konstanz, Germany), verify® (Verinata Health Inc, CA, USA) and Harmony™ (Arisoa Diagnostics Inc, CA, USA). All of these products analyse circulating cell-free DNA extracted from a maternal blood sample that is analysed by massively parallel sequencing (MPS) techniques.[3]

**Reason for assessment**

Current mainstream methods for the definitive detection of fetal abnormalities such as trisomy 21 and 18 are associated with a risk of miscarriage, albeit small. These methods are costly, time-consuming and have a false positive rate of five per cent and a detection rate for true positives of 64-96 per cent.[5] There is a need to perform non-invasive fetal tests to allay anxiety in parents and to ensure the best outcomes for the fetus, especially in those women who have tested as a false positive by conventional testing methods.

**Stage of development in Australia**

| | | | |
|---|---|---|---|
| ☐ | Yet to emerge | ☐ | Established |
| ☐ | Experimental | ☐ | Established *but* changed indication or modification of technique |
| ☐ | Investigational | ☐ | Should be taken out of use |
| ☒ | Nearly established | | |

**Australian Therapeutic Goods Administration (TGA) approval**

| | | |
|---|---|---|
| ☐ | Yes | ARTG number (s) |
| ☐ | No | |
| ☒ | Not applicable | |

**Licensing, reimbursement and other approval**

As of 1 July 2010, the new TGA regulatory framework requires all new *in vitro* diagnostic medical devices (IVDs) to be listed on the Australian Register of Therapeutic Goods (ARTG). IVDs encompass pathology tests. A commercially available test for NIPT of congenital abnormalities would be classified as a Class III IVD and would be required to be registered on the ARTG, however IVDs developed "in-house" are only required to have approval and yearly validation from NATA[4]. Australian companies that collect samples to be sent to overseas companies for analysis do not have to be listed on the ARTG, however all

[4] National Association of Testing Authorities, Australia

equipment used to collect and transport samples have to be registered (personal communication TGA).

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| **Technology type** | **Diagnostic** |
| **Technology use** | **Diagnostic** |

## Patient Indication and Setting

### Disease description and associated mortality and morbidity

Although congenital anomalies are rare, they are a major cause of neonatal and infant mortality and are associated with a significant emotional and economic burden not only to affected families but to society. Several chromosomal abnormalities are more prevalent with advancing maternal age (>40 years), including trisomies[5] 21 (Down syndrome), 18 (Edwards syndrome) and 13 (Patau syndrome), however many affected pregnancies are detected and managed by early termination, which has resulted in a decline in children born with abnormalities.[6] Children with Down syndrome (DS) have delayed psychomotor development and have rates of congenital heart and gastrointestinal defects in addition to be being prone to coeliac disease, hypothyroidism and respiratory infections, resulting in an increased reliance on the health system. Individuals are now living longer with DS, with the median life expectancy increasing in the United States from 25 years in 1983 to 49 years in 1997.[7] This may result in care issues later in life with children outliving their parents or carers. Children born with trisomy 18 or trisomy 13 have severe mental retardation, as well as health problems involving nearly every organ system in the body. Approximately 90 per cent of babies born with trisomy 18 or 13 die during their first year of life, and although a small proportion of infants survive past the first year, few live into their teens.[8]

### Number of patients

The last full report produced by the National Perinatal Statistics Unit (NPSU) contains data collected on Australian birth anomalies during the period 2002-2003, however it should be noted that there is a great deal of variation in the manner in which the states and territories collect their individual data, and the definitions and classifications used, for example terminations of pregnancy at less than 20 weeks gestation are not included for Queensland, Tasmania and the Australian Capital Territory. Only South Australia and Western Australia have mandatory collection of termination of pregnancy data. As such, data between states cannot be compared.[6]

In 2003, for the whole of Australia, 252,584 mothers gave birth to 256,925 babies with an average gestational age of 38.9 weeks, with 7.9 per cent born pre-term. Using state and territory perinatal data, the fetal death rate was 7.1 per 1,000 births; the neonatal death rate was 3.0 per 1,000 live births; and the perinatal death rate was 10.1 per 1,000 births.

[5] Trisomy 21, 18 and 13 are congenital abnormalities associated with an extra chromosome

Half of all perinatal deaths were due to three factors: unexplained antepartum deaths, congenital abnormalities and spontaneous preterm births.[9] During the same period, 15,251 women or 3.1 per cent, gave birth to babies with one or more congenital anomalies, representing a rate of 308 per 10,000 births. Of the births with congenital anomalies 95 per cent were live born. The rate of congenital anomalies was slightly higher in babies born to Indigenous women at 3.5 per cent or 356 per 10,000 births.

Trisomy 21 is the second most commonly reported condition at birth, with a rate of 11.1 per 10,000 births, however approximately 64 per cent of fetuses diagnosed with trisomy 21 were managed by early termination of the pregnancy or were fetal deaths. When these terminations were included, the estimated rate for trisomy 21 was 26.3 per 10,000 pregnancies.[6]

For the period 2002-2003, the overall rate of trisomy 13 was 0.6 per 10,000 births. When the estimated number of births and terminations were included using data from the four states that can provide accurate pregnancy termination statistics, this rate increased to 2.6 per 10,000 pregnancies, indicating that 72 per cent of trisomy 13 affected pregnancies ended in termination. The highest rate of 1.3 per 10,000 births was observed in women aged over 40 years.[6]

During the same period, trisomy 18 was reported in 2.0 per 10,000 births, however using the available State termination data this rate increased to 7.1 per 10,000 pregnancies, with approximately 70 per cent of trisomy 18 pregnancies being terminated. The rate of trisomy 18 was 11.5 per 10,000 in women aged more than 40 years.[6]

It should be noted that a terminated fetus is not routinely tested to confirm the presence or absence of congenital abnormalities, and therefore the true accuracy or positive predictive value of amniocentesis or chorionic villus sampling (CVS) cannot be stated with any certainty.

Currently New Zealand does not have a nationally co-ordinated prenatal screening programme for trisomy 21, trisomy 18 or trisomy 13, however a programme is under development.[10] The New Zealand Birth Defects Registry has been operating a voluntary notification system for termination of pregnancy (TOPs) with birth defects since February 2011. However, a process of extending the scope of this registration programme to complete national coverage by the end of 2013-early 2014 is underway (NZ Birth Defects Monitoring Programme, personal communication). In 2010, 64,433 women in New Zealand completed 64,485 pregnancies resulting in 64,936 babies born live.[11] The number of live births with congenital abnormalities in New Zealand is unknown, however the number of TOPs with birth defects reported via voluntary notification were 92 in 2011 and 202 in 2012 (NZ Birth Defects Monitoring Programme, personal communication).

| | |
|---|---|
| **Speciality** | **Pregnancy and childbirth** |
| **Technology setting** | **Specialist hospital** |

**Impact**

**Alternative and/or complementary technology**

Alternative technology

**Current technology**

Two-dimensional ultrasonography (US) is used as a routine screening tool for all pregnancies in Australia and New Zealand and may be used to diagnose fetal abnormalities such as Down syndrome. Women considered to be at risk of carrying a DS fetus (>35 years) may be offered an US between 11 and 13 weeks gestation for the detection of nuchal translucency. A second trimester fetal morphology US scan is not recommended as the primary screening tool for Trisomy 21 and Trisomy 18, however it is recommended for women with an elevated serum α-fetoprotein.[10]

In addition, the Royal Australian and New Zealand College of Obstetricians and Gynaecologists recommend the detection of some fetal anomalies by the detection of analytes in the maternal serum at 9-14 weeks of gestation. Four analytes in maternal serum may be tested for: α-fetoprotein; free alpha subunit (α-hCG) and free beta subunit (β-hCG) of chorionic gonadotropin and unconjugated oestriol. These proteins are normally produced by, or in support of, a fetus and its placenta and have known values throughout each stage of pregnancy. A number of fetal abnormalities result in one or more of these analytes deviating (increasing or decreasing) from their normal range. If these biochemical values fall outside of the normal range then the fetus is deemed to be at an *increased risk* of having an abnormality and mothers are offered counselling along with further information.[10, 12]

Since 2010 New Zealand's National Screening Unit has provided antenatal screening for Down syndrome and other conditions. There are two screening options available, first trimester combined screening (maternal serum and a nuchal translucency US) and second trimester maternal serum screening.[13]

Pregnant women may be offered further invasive testing including amniocentesis and CVS, to confirm suspected fetal defects obtained either via an US or maternal biochemical testing. Some women may be offered these procedures based on maternal age alone. Both of these techniques carry a small increased risk of miscarriage (0.5-2.6%). Amniocentesis can only be carried out in the second trimester, or 15-17 weeks gestation, whereas CVS can be performed in the first trimester or 11-14 weeks. The time taken for cytogenic diagnosis can range from 1-3.[14] It should be noted that, unlike NIPT in its current form, amniocentesis can detect chromosomal abnormalities *other* than the trisomies 21, 18 and 13.

The timeline for prenatal screening and diagnostic tests is summarised in Figure 1. Further comparator information may be accessed in previous Briefs below.

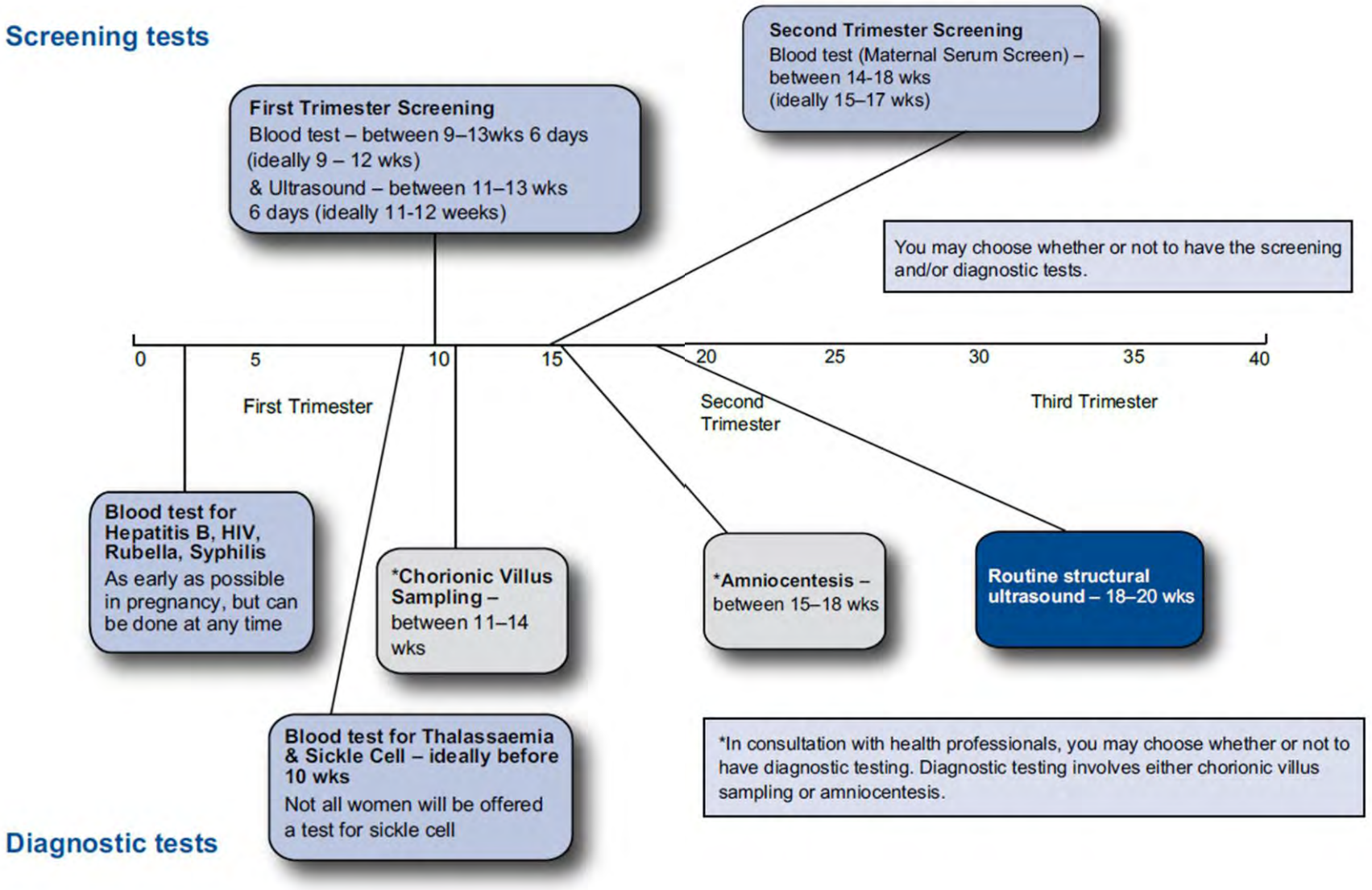


**Figure 1** **Screening and diagnostic test timeline and options** [15]

### Diffusion of technology in Australia

Victorian Clinical Genetics Services[6] (VCGS) was developing an in-house NIPT using a modification of a published study using massively parallel sequencing. Due to challenges with validation of the assay and hence accreditation with NATA, VCGS has abandoned this work and have opted to use the services of an overseas vendor, Natera Inc (San Carlos, California). Commencing in April 2013, VCGS have contracted Natera to test for trisomy 13, 18 and 21, in addition to X chromosome aneuploidies, using an SNP amplicon/MPS[7] strategy, whilst endeavouring to continue to establish a testing process in Australia. VCGS have processed approximately 60 samples in a research capacity (personal communication VCGS). In Western Australia, both private and public pathology laboratories offer NIPD testing on a fee-for-service basis with the analysis done by a number of overseas laboratories. Western Diagnostic Pathology use Inex (Singapore) / BGI (China) at a cost of $1,350 per sample, Clinipath (Sonic Health) use the US Company Verinata at an approximate cost of $1,250 and PathWest offered a service provided by Ariosa at a cost of US$575 (personal communication Western Diagnostic Pathology and PathWest). A business case has been written and submitted to the Health Department of WA for the provision of NIPD testing free of charge for high risk women who are under the care of obstetricians at King Edward Memorial Hospital in Perth. Virtus Health in Queensland, Victoria and New South Wales use the services of Natera Inc and HealthScope Pathology use Sequenom.

### International utilisation

| Country | Level of Use | | |
|---|---|---|---|
| | Trials underway or completed | Limited use | Widely diffused |
| United States | ✓ | ✓ | |
| United Kingdom | ✓ | | |
| Hong Kong | ✓ | | |
| The Netherlands | ✓ | | |
| Australia | ✓ | | |

### Cost infrastructure and economic consequences

The cost of sending a sample for NIPT to Natura Inc via the VCGS is currently A$1,200, which includes pre and post-test counselling services (personal communication VCGS). A positive result may have economic consequences for both the mother and health services depending on the chosen course of action, however the number of women likely to require this service is reasonably low at an approximate rate of 36 per 10,000 births and would therefore not have a large budgetary impact. This test would only be offered to women considered at

[6] VCGS is a wholly owned subsidiary of the Murdoch Childrens Research Institute and they provide screening and diagnostic laboratory services, including prenatal testing.
[7] MPS = massively parallel sequencing

high-risk of carrying a fetus with trisomy 13, 18 or 21 based on standard testing. Currently this test is available to women on a user-pays basis.

### Ethical, cultural or religious considerations

All women offered a prenatal screening test should be made aware of the consequences of a positive test. Women considered at high-risk of carrying a fetus with an abnormality on the basis of the results of a non-invasive screening test will be offered further diagnostic testing (ultrasound, chorionic villus sampling of amniocentesis). In the event of a positive diagnosis, the woman may choose to terminate or continue with the pregnancy. There are obvious ethical concerns surrounding the use of prenatal testing, the results of which may result in the termination of a pregnancy. Women need to be offered appropriate counselling and sufficient information on the advantages, disadvantages and accuracy of screening tests, the likelihood of a false positive or false negative result and the consequences of a positive result.[10]

Currently all NIPT tests available are supplied by commercial companies on a user pays basis, which limits access to a small proportion of women. Currently women from rural and remote locations considered to be at high-risk in Australia would be required to travel to a major metropolitan centre for invasive testing. However, commercially available NIPT tests will increase access to prenatal testing for rural and remote women in Australia. It may be feasible to provide counselling for these women via telemedicine.

## Evidence and Policy

### Safety and effectiveness

A recent systematic review identified 16 studies, published between 2007 and 2012, that evaluated the diagnostic accuracy of one or more NIPT methods for the detection of *trisomy 21* only in a high-risk population (level III-2 diagnostic evidence[8]).[3] Several of these studies have been discussed individually in previous Briefs. Of the 16 studies, five reported on the use of massively parallel sequencing (MPS) without pre-selection of chromosomes[16-20], and three with selection of chromosomes using the DANSR method.[21-23] Two studies used epigenetic methods[24, 25], three calculated the ratio of cff-RNA SNP alleles of the placental gene *PLAC4*[26-28], two studies calculated the ratio of chromosome 21 cff-DNA SNP alleles[1, 29] and one study reported on the amplification of the *HBB* gene by quantitative PCR.[30] The characteristics of the studies are summarised in Table 1. The quality of the included studies varied with many deemed to have a high risk of bias in terms of patient selection (12/16) and high concerns regarding the applicability in regard to the timing of NIPT during the pregnancy, with either a broad gestational age used or blood sampling taking place too late

[8] Although this was a systematic review, it was unclear from the methods if all of the included studies were blinded to the results of the reference standard.

in the pregnancy. Four studies did not include all samples in the analysis. An overall quality score was not reported for each individual study.

The diagnostic performance results of these methods are summarised in Table 2. Results were not pooled due to the number of different methods used and the small number of studies for each of these methods. Overall sensitivity of NIPT ranged from 59 to 100 per cent, with a higher specificity ranging between 83 and 100 per cent. Diagnostic accuracy appears to have improved with the use of DANSR and MPS, suggesting a maturing of the technology. Those studies published pre-2011 reported poorer sensitivities and specificities in addition to wide confidence intervals, which suggest a great deal of variation. Sensitivities of NIPT were good when DANSR (100%) and MPS (range 98.6-100%) techniques were used and these studies were also the largest. Sensitivity was slightly lower for cff-RNA SNP ratio (range 90-100%) and epigenetic methods (83-100%) with wide confidence intervals. In the earlier studies where cff-DNA SNP ratio and quantitative PCR were used, sensitivities were lower and more variable. Specificities for all methods were good, however less variation was observed when the DANSR and MPS methods were used.

In a diagnostic test such as this, a high sensitivity (the ability to correctly identify a true positive) and a high specificity (the ability to correctly identify a true negative) are of utmost importance when considering the potential consequences of a test result. To this end, the systematic review also went on to calculate the positive and negative predictive values[9] for all methods for different levels of prevalence: high-risk, where the odds of having a trisomy 21 fetus was 1:200, and two low-risk groups where the risks were 1:380 and 1:1500. Negative predictive values were excellent for all methods in all risk groups (approximately 100%). However, there was a great deal of variation in the positive predictive values for all methods in all risk groups, with overall values ranging from 19-100 per cent for the high risk of trisomy 21 group (1:200), from 11-100 per cent for the 1:380 group and from 3.1-100 per cent for the 1:1500 group. However, when considering DANSR alone, PPVs ranged from 93.6-100 per cent for the high risk of trisomy 21 group (1:200). The MPS technique resulted in a PPV ranging from 19.7-100 per cent in the same high-risk group.

[9] Positive predictive value is the proportion of all positive results that are true positives and the negative predictive value is the proportion of all negative results that are true negatives. Positive and negative predictive values are directly related to the prevalence of the disease in the population, with the PPV increasing and NPV decreasing with increasing prevalence. Trisomies are uncommon and therefore NPVs are likely to be high.

**Table 1 Characteristics of included studies describing the use of NIPT for trisomy 21[3]**

| NIPT technique | Trisomy 21 cases | Non-trisomy 21 samples | Gestational age (weeks, range or mean ± SD) | Indication | Consecutive sampling or matched T21: euploid | Sample taken before or after AC or CVS | Reference standard |
|---|---|---|---|---|---|---|---|
| DANSR[22] | 8 | 1,941 | 11-13 | 1st trimester & undergoing combined test | Consecutive | Before | KT or birth outcome |
| MPS[18] | 139 | 2,820 | 9-28 | High risk | Consecutive | Before | KT |
| DANSR[23] | 81 | 2,888 | 10-38.7 | Planned invasive procedure | Consecutive | Before | KT, FISH or QF-PCR after AC or CVS |
| DANSR[21] | 50 | 297 | 11-13 | High risk | Matched 1:3 | Before | KT after CVS |
| MPS 6-plex[16] | 92 | 404 | 10-23 | High risk | Matched 1:4 | Before | KT or FISH |
| qMSP UI[24] | 18 | 90 | ?, inter-quartile range 6-9 weeks | Later T21 detected by AC or CVS | Matched 1:5 | Before | KT or infant records |
| MPS 4-plex[20] | 212 | 1,471 | 11-20 | High risk | Matched 1:7 | Before | KT |
| MPS 4-plex[19] | 39 | 449 | 8-36 | High risk | Matched 1:11 | Before | KT, QF-PCR after AC or CVS |
| MeDiP[25] | 14 | 26 | 11.1-14.4 | ? | ? | ? | KT after CVS or AC |
| cff-RNA SNP ratio[26] | 25 | 85 | 9-20 | Unknown and high risk | Case-control | Before | KT after CVS or AC/ newborn reports |
| MPS 2-plex[17] | 86 | 146 | 13.1 ± ? | Indicated for AC or CVS | Matched 1:5 | Before | KT after CVS or AC |
| cff-DNA SNP ratio[29] | 7 | 20 | 9-36.1 | Indicated for AC or CVS | ? | Before and after | KT after CVS or AC |
| cff-RNA SNP ratio[28] | 4 | 58 | T21: 12.9 ± 0.5<br>Cont: 12.8 ± 0.7 | High risk | Consecutive | Before | KT after CVS |
| qPCR[30] | 17 | 30 | 12.23 ± 0.77 | ? | Matched 1:1 or 2 | ? | KT after CVS or AC |
| cff-DNA SNP ratio[1] | 3 | 57 | 8-38 | ? | ? | ? | KT after CVS or AC/ newborn reports |
| cff-RNA SNP ratio[27] | 10 | 57 | T21: 12.4 -20<br>Cont: 11.1- 14 | Based on KT result | Case-control | Before | KT after CVS |

NIPT = non-invasive prenatal testing, T21 = trisomy 21, DANSR = digital analysis of selected regions, MPS = massively parallel sequencing, CVS = chorionic villus sampling, AC = amniocentesis, KT = karyotyping, cff = cell free fetal RNA or DNA, SNP = single nucleotide polymorphism, QF-PCR = quantitative fluorescence PCR, qPCR = quantitative PCR, MeDiP = methylated DNA precipitation, qMSP = quantitative methylation specific PCR, UI = unmethylation index, FISH = fluorescence in situ hybridisation ,? = unclear

**Table 2 Diagnostic performance of included studies in systematic review of NIPT for Trisomy 21[3]**

| NIPT technique | Trisomy 21 cases | Non-trisomy 21 samples | True positive | True negative | Not testable or included in analysis | Sensitivity % [95% CI] | Specificity % [95% CI] |
|---|---|---|---|---|---|---|---|
| DANSR[22] | 8 | 1,941 | 8 | 1,941 | 100/2,049 | 100 [67.5, 100] | 100 [99.8, 100] |
| DANSR[23] | 81 | 2,888 | 81 | 2,887 | 370/3,228 | 100 [95.5, 100] | 99.97 [99.8, 100] |
| DANSR[21] | 50 | 297 | 50 | 297 | 3/350 | 100 [92.9, 100] | 100 [98.7, 100] |
| MPS[18] | 139 | 2,820 | 139 | 2,819 | 8,176/11,105 | 100 [97.3, 100] | 99.96 [99.8, 100] |
| MPS 6-plex[16] | 92 | 404 | 92 | 404 | 36/532 | 100 [96, 100] | 100 [99.1, 100] |
| MPS 4-plex[20] | 212 | 1,471 | 209 | 1,468 | 13/1,696 | 98.6 [95.9, 99.5] | 99.8 [99.4, 99.9] |
| MPS 4-plex[19] | 39 | 449 | 39 | 409 | 31/480 | 100 [91, 100] | 99.8 [98.6, 100] |
| MPS 2-plex[17] | 86 | 146 | 86 | 143 | 11/764 | 100 [95.7, 100] | 97.95 [94.1, 99.3] |
| qMSP UI[24] | 18 | 90 | 15 | 85 | 0/108 | 83.3 [60.8, 94.2] | 94.4 [87.6, 97.6] |
| MeDiP[25] | 14 | 26 | 14 | 26 | 0/40 | 100 [78.5, 100] | 100 [87.1, 100] |
| cff-RNA SNP ratio[26] | 25 | 85 | 23 | 87 | 10/121 | 95.8 [79.8, 99.3] | 100 [95.8, 100] |
| cff-RNA SNP ratio[28] | 4 | 58 | 4 | 52 | 91/153 | 100 [51, 100] | 89.7 [79.2, 95.2] |
| cff-RNA SNP ratio[27] | 10 | 57 | 9 | 55 | 0/67 | 90.0 [59.6, 98.2] | 96.5 [88.1, 99.0] |
| cff-DNA SNP ratio[29] | 7 | 20 | 7 | 20 | 13/40 | 100 [64.6, 100] | 100 [83.9, 100] |
| cff-DNA SNP ratio[1] | 3 | 57 | 2 | 56 | 0/60 | 66.7 [20.8, 93.9] | 98.3 [90.7, 99.7] |
| qPCR[30] | 17 | 30 | 10 | 25 | 0/47 | 58.8 [36, 78.4] | 83.3 [66.4, 92.7] |

NIPT = non-invasive prenatal diagnosis, DANSR = digital analysis of selected regions, MPS = massively parallel sequencing, cff = cell free fetal RNA or DNA, SNP = single nucleotide polymorphism, qPCR = quantitative PCR, MeDiP = methylated DNA precipitation, qMSP = quantitative methylation specific PCR, UI = unmethylation index

A recent comparative study by Sparks et al (2012) reported on the use of DANSR for the detection of trisomy 18 and 21 (level III-1 diagnostic evidence). Plasma samples taken from 171 women (gestational age ≥10 weeks, singleton pregnancy) with a known trisomy status were included as the training set. Of these, 127 were disomic, 36 trisomy 21 (T21) and eight were trisomy 18 (T18). The initial training set of samples were assayed against 576 non-polymorphic loci on both chromosomes 18 and 21. Sequence count data from these assays were normalised and the loci that were capable of discriminating T21 and T18 samples from normal samples using a z statistic were selected (384 out of the original 576 loci). An algorithm based on this information was developed, resulting in a FORTE[10] risk score. This individualised risk score takes into account age-related risks and the fetal fraction[11] of the sample. [31,32]

Six normal and one each of the T21 and T18 of the training samples did not meet sampling criteria (low fetal fraction or evidence of a non-singleton pregnancy). In the training samples, 120/121 (99.2%) of normal samples had a Z statistic <3 for chromosome 21 and 100 per cent of T21 and T18 samples had a Z statistic >3, yielding a sensitivity of 100 per cent for both T21 and T18 and a specificity of 99.2 and 100 per cent for T21 and T18, respectively. Plasma from 167 pregnant women (123 normal, 36 T21 and 8 T18) were then analysed to validate the DANSR and FORTE method. All samples were karyotyped, however, the trisomy status of the validation samples were blinded to researchers. There was no significant difference between the maternal age and gestational age between the training and validation sets. All samples met the sampling criteria. All trisomy samples were correctly identified and differentiated from disomy samples using the combined DANSR plus FORTE algorithm, regardless of fetal fraction (Figure 2). Although the authors were encouraged by these results, they acknowledged that the study was limited by the small sample size and recommend not only larger studies, but also the use of DANSR plus FORTE in mixed populations of high and low-risk pregnant women.

[10] FORTE = fetal-fraction optimised risk of trisomy evaluation

[11] Consideration of fetal fraction is important. The higher the fraction of cff-DNA, the greater the difference in the number of cff-DNA fragments originating from trisomic versus disomic chromosomes, which then makes it easier to detect trisomy.

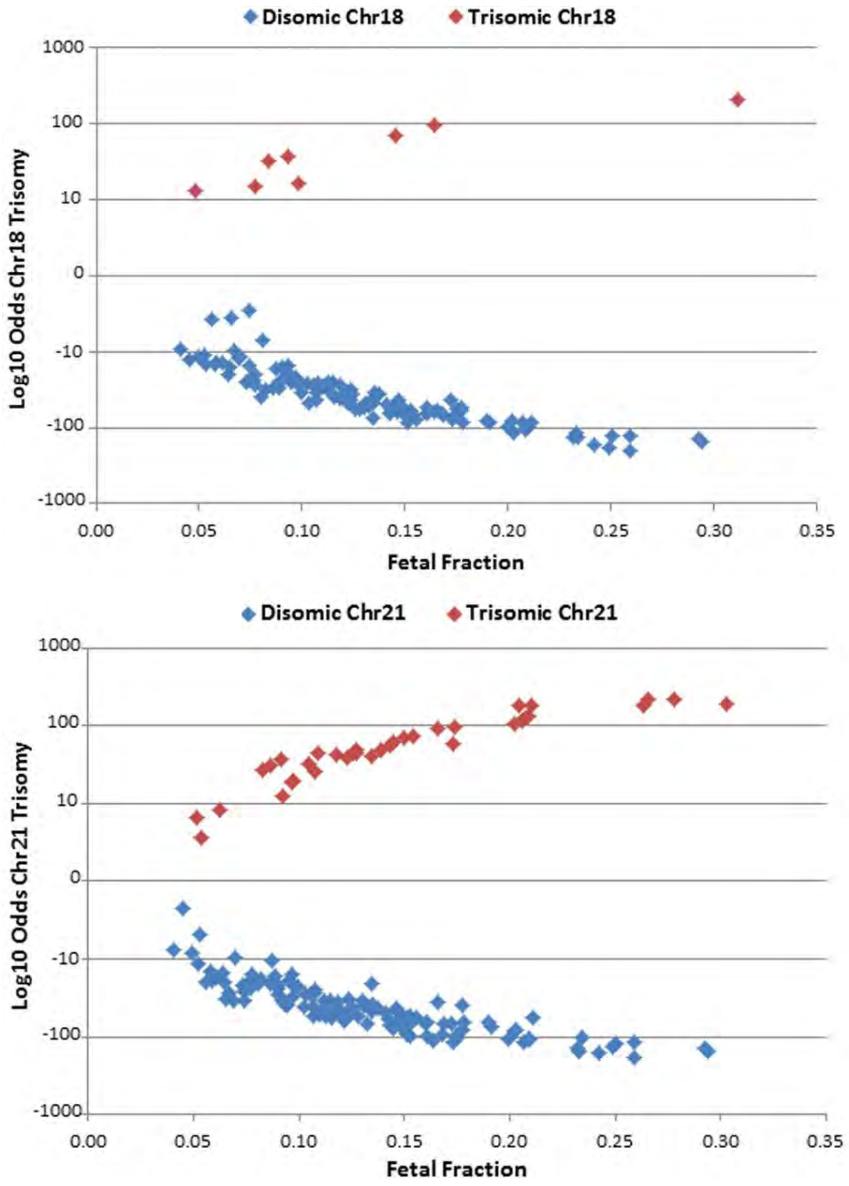


**Figure 2 FORTE computed odds for trisomy vs disomy for chromosome 18 and 21**[31]

A similar multi-centre study by Sehnert et al (2011) used an algorithm derived from chromosome ratios which were then normalised and applied to cff-DNA samples that had undergone massively parallel sequencing. As previously mentioned, the use of MPS on cff-DNA has been demonstrated to be effective for successfully identifying trisomy 21 but has been problematic in the identification of trisomy 18 and 13.[33]

Samples from 1,041 pregnant women (mean age 36.6 years, range 17-47; mean gestational age 15 weeks 4 days, range 6 weeks 1 day to 38 weeks) were taken and the majority of samples underwent karyotyping (98.7%). A training sample set of 71 women was selected from the initial 435 women to present, cff-DNA was extracted and sequenced using MPS. Six samples were excluded from analysis. A normalised chromosome value (NCV) was calculated for each chromosome of interest: 21, 18, 13, X and Y. Samples with a NCV >4.0 were classified as aneuploidy and those with a NCV<2.5 were classified as unaffected. [33] This

method was previously described by Chiu et al (2008) and is illustrated in Figure 5 in the 2009 Update.

The test sample set of 47 singleton pregnancies were selected from the next group of 575 women presenting to the clinic. Researchers were blinded to the karyotyping status of the samples (level III-1 diagnostic evidence). For chromosome 21, 13 samples were correctly identified as trisomy 21 with 34 unaffected individuals. There were eight correctly identified trisomy 18 samples, with 39 correctly identified as unaffected. For chromosome 13, 46 samples were correctly identified as unaffected; however one sample was classified as a no-call with a NCV falling in the range between 2.5 and 4.0. This sample was confirmed by karyotyping as trisomy 13 (Figure 3). As with the previous study by Sparks et al, this approach shows promise, however these results would need to be replicated in a larger study.

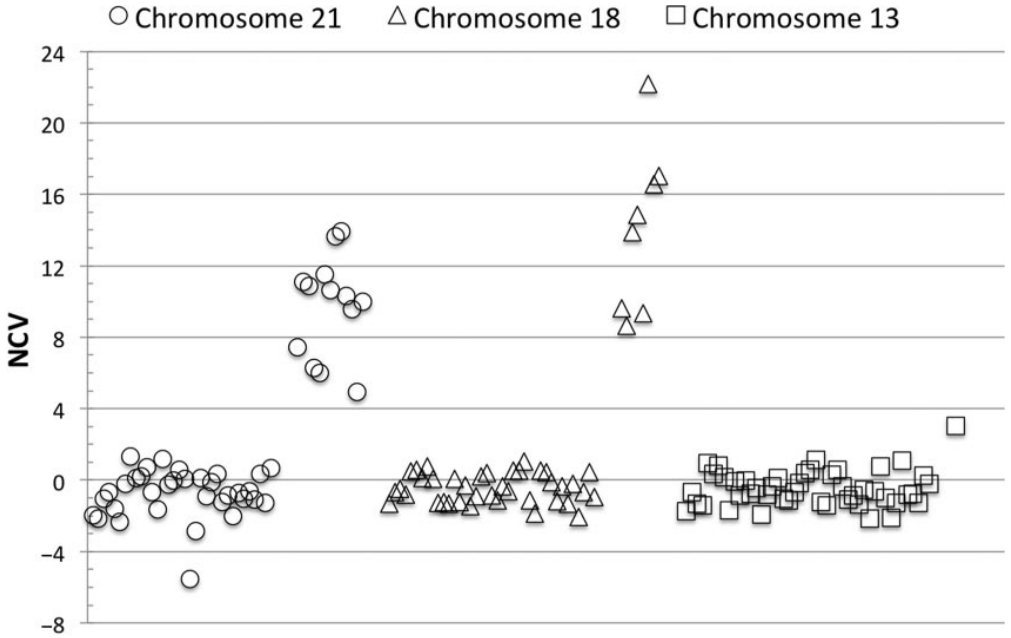


**Figure 3 Normalised chromosomal values for test sample chromosomes 21,18 and 13[33]**

### 2013 Economic evaluation

A clinical utility study conducted in the United States evaluated the cost-effectiveness of conventional screening methods, including serum markers, ultrasound and nuchal translucency, compared to NIPT used for screening high-risk pregnancies for *trisomy 21 only* using Markov modelling.[34] High-risk pregnancies were defined as women aged >35 years and those who returned a positive screen using conventional screening methods. A theoretical cohort of 4-million women was modelled based on the number of annual births in the United States. It was assumed that 70 per cent of these women would undergo conventional screening and that 14 per cent of the total cohort would be considered high-risk based on an age of ≥35 years. In addition, it was assumed that all women found to be positive with conventional methods would undergo NIPT, and that 75 and 99 per cent of

women found to be positive with conventional methods or NIPT would go on to request invasive testing. All costs were in 2012 US dollars and where possible the US Medicare 2012 Fee Schedule was used.

The cost of NIPT was estimated to be $795 (range $695-$995), based on the cheapest commercial test available on the current market (Harmony Prenatal Test, Arisoa Diagnostics), a test that uses cff-DNA analysed by MPS. This is considerably less than the A$1,200 quoted by the Victorian Clinical Genetics Services, however it is unclear whether or not this cost includes counselling as this is listed as a separate cost. The baseline cost for Down syndrome was estimated based on direct medical costs as well as indirect costs. Estimated costs used in the model are summarised in Table 3.

**Table 3 Estimated costs (USD) associated with screening for trisomy 21**

| Cost of variables US$ | Baseline estimate | Range |
|---|---|---|
| Office visit with counselling | $120 | $40-200 |
| First trimester serum screening | $42.66 | $30-100 |
| Second trimester serum screening | $144.07 | $75-300 |
| NIPT | $795 | $695-995 |
| First trimester ultrasound | $131.73 | $75-300 |
| Nuchal translucency | $127.98 | $75-200 |
| Invasive testing | $1300 | $500-2,000 |
| Elective termination | $600 | $350-1200 |
| Down syndrome | $677,000 | $400,000-800,000 |

Using first trimester screening (FTS) or integrated screening (INT) resulted in a high number of false positives who then went onto receive invasive procedures. The outcomes predicted by the model are summarised in Table 4. NIPT detected 28-43 per cent more cases of trisomy 21 compared to FTS and INT and reduced the number of invasive procedures performed by >95 per cent, in so doing, reduced the number of normal fetal losses due to having the invasive procedure by >99 per cent. The reduced cost of screening with NIPT is driven by the reduction in the number of invasive procedures performed but also the reduced number of missed cases of Down syndrome. A one-way sensitivity analysis was performed, with NIPT remaining dominant over an INT strategy in all analyses. NIPT was no longer a cost-saving compared to FTS only when costs for Down syndrome were less than $212,000. However, a two-way sensitivity analysis revealed that NIPT remained cost-saving at all costs of Down syndrome compared to FTS. An economic value was not given to the reduced number of euploid fetal losses due to less invasive procedures performed when NIPT was used.

**Table 4 Clinical outcomes predicted by Markov modelling on a theoretical cohort**

| Outcome | FTS | INT | NIPT |
|---|---|---|---|
| Number of invasive procedures | 108,364 | 108,760 | 5,330 |
| Number of trisomy 21 detected | 3,364 | 3,760 | 4,823 |
| Number of normal fetal losses | 525 | 525 | 3 |
| Cost per trisomy 21 detected | $1,125,314 | $1,042,417 | $705,528 |
| Screening strategy cost | $3.8 billion | $3.9 billion | $3.4 billion |
| Screening cost per pregnant woman | $946.42 | $979.84 | $850.71 |

FTS = first trimester screening including serum markers, ultrasound and nuchal translucency, INT = integrated screening, including FTS and plus Quad test serum markers (AFP: alpha-fetoprotein, hCG: human chorionic gonadotropin, uE3: unconjugated oestriol and inhibin A)

It should be noted that the baseline assumptions and costs may not reflect those of Australia or New Zealand. In addition, two of the three authors of this study are employed by Ariosa Diagnostics, with the remaining author being a clinical advisor to the company. A cost-effectiveness analysis using clinical data would be informative.

### 2013 Ongoing research

Several ongoing studies for non-invasive prenatal testing are registered on the clinical trials database.

The Prenatal Non-invasive Aneuploidy Test Utilizing SNPs Trial (PreNATUS) is a comparative study of pregnant women to be considered at moderate to high-risk of trisomy (NCT01545674). The primary outcome is the sensitivity and specificity of NIPT compared to amniocentesis or CVS when used to detect autosomal aneuploidy (chromosomes 13, 18, 21) and sex chromosome aneuploidy (X and Y). This prospective blinded study will assess the diagnostic capability of an informatics enhanced SNP-based technology to identify aneuploidies from free floating fetal DNA in the maternal blood. Recruitment of this multi-centre US study began in 2012 and aims to recruit 1,000 women by July 2013.

A similar, large scale study has recently been completed, that compared 4,664 women who had undergone testing either by amniocentesis or CVS to testing with a circulating cell-free fetal nucleic acid-based test (NCT00877292).

A non-comparative study sponsored by Natera Inc began recruiting 4,640 participants, including high-risk women, non-pregnant women, women who had miscarried, women who had given birth and male relatives of these women (NCT01574781). The assay used to detect free circulating fetal DNA in maternal blood would be used on all participants. A smaller (n=500), non-comparative NIPT study sponsored by Natera Inc is currently recruiting pregnant women who achieved pregnancy following in-vitro fertilisation (IVF) using Natera's pre-implantation aneuploidy screening (NCT01546324).

The Non Invasive Prenatal Diagnosis of Trisomy 21 by Genetic Analysis of Circulating Fetal Cells (ISETTRI21) study is currently recruiting French women (n=500) at high-risk of carrying a trisomy fetus, who are willing to undergo amniocentesis or CVS in addition to quantitative fluorescent PCR analysis of short tandem repeats, applied to single cells (NCT01725438).

**2013 Other issues**

There is the potential for this test to be offered by overseas companies via Australian private ultrasonographers on a direct to consumer basis, which may have implications for the health system if offered to women not considered to be at high-risk of carrying an affected fetus (personal communication VCGS).

In October 2011, the International Society for Prenatal Diagnosis (ISPD) issued a position statement on the use of massively parallel sequencing (MPS) for NIPT of trisomy 21. The ISPD recognised that MPS can detect a large proportion of pregnancies in high-risk populations affected by Down syndrome, with a low false-positive rate. However, although NIPT is being commercially offered to women in the United States and Europe, NIPT is not fully diagnostic and is considered an advanced screening test. Prior to the routine use of MPS-NIPT additional evidence should be provided by way of clinical trials that demonstrate:

- efficacy in low risk populations;
- that the test is suitable for the diverse sub-populations such as twins and IVF donor pregnancies;
- the test can be provided in a cost-effective, timely, and equitable manner; and
- when used in conjunction with other screening tests, the MPS-NIPT result can be combined to provide a composite risk estimate.[35]

The American College of Obstetricians and Gynecologists Committee on Genetics issued a position statement on non-invasive prenatal testing for fetal aneuploidy in December 2012: "*Noninvasive prenatal testing that uses cell free fetal DNA from the plasma of pregnant women offers tremendous potential as a screening tool for fetal aneuploidy. Cell free fetal DNA testing should be an informed patient choice after pretest counseling and should not be part of routine prenatal laboratory assessment. Cell free fetal DNA testing should not be offered to low-risk women or women with multiple gestations because it has not been sufficiently evaluated in these groups. A negative cell free fetal DNA test result does not ensure an unaffected pregnancy. A patient with a positive test result should be referred for genetic counseling and should be offered invasive prenatal diagnosis for confirmation of test results.*"[36]

In addition, the US National Society of Genetic Counselors (NSGC) issued a position statement on NIPT in January 2013. Whilst acknowledging that the introduction of these new non-invasive technologies into clinical practice will impact the current prenatal screening paradigm, the NSGC supports NIPT as an option for patients whose pregnancies

are considered to be at an increased risk for certain chromosome abnormalities. The NSGC urges that NIPT/NIPD only be offered in the context of informed consent, education, and counselling by a qualified provider. Patients whose NIPT results are abnormal should receive genetic counselling and be given the option of standard confirmatory diagnostic testing.[37]

**2013 Summary of findings**

All NIPT tests currently offered by commercial companies isolate cell-free fetal DNA from the maternal plasma, with the chromosomal content of these cells analysed using massively parallel sequencing techniques. These tests should only be offered to women carrying a singleton fetus and who are considered to be at high-risk of carrying a trisomy fetus. That is, women age 35 and older, women with a history of a pregnancy with a child with a trisomy, and women carrying a fetus with ultrasound findings indicating increased risk. This technique has been demonstrated to have a high sensitivity (98-100%) and specificity (98-100%) for the detection of trisomy 21 but has proven to be problematic for the detection of trisomy 18 and 13. Digital analysis of selected regions (DANSR) has also demonstrated high sensitivity (100%) and specificity (100%) for trisomy 21. Positive predictive values in women considered to be at high-risk of carrying a trisomy 21 fetus ranged from 20-100 per cent when MPS was used, however in the same group DANSR had PPVs that ranged from 93.6-100 per cent. Later DANSR studies that used algorithms applied to results have demonstrated an ability to discriminate almost 100 per cent of pregnancies carrying trisomy 18 and 13 from normal pregnancies. These latter techniques require further validation in larger populations of both low and high-risk pregnancies. Both MPS and DANSR have shown a maturing of the technology with later studies published demonstrating less variation in results, as evidenced by tighter confidence intervals.

Currently in Australia and New Zealand, NIPT testing is only available to women willing to pay to have their samples analysed by an overseas company.

Economic modelling indicates that NIPT, when used for all high-risk women (those aged >35 years *and* those who test positive with conventional screening methods), is cost-saving as it reduces the number of women who go on to require invasive procedures such as amniocentesis or chorionic villus sampling. This reduction in invasive procedures resulted in a reduction in normal fetal losses.

**2013 HealthPACT assessment**

Based on the good level of evidence demonstrating high sensitivity and specificity of NIPT tests, it is clear that there is a place for this testing in the clinical care pathway for pregnant women. At this stage in the development of NIPT it would not be considered as a stand-alone first line diagnostic test, but rather an advanced screening test, with women going on to receive more invasive CVS or AC testing if testing positive by NIPT. DANSR tests in development appear to be more promising than MPS techniques and may, in the long-term,

result in the reduction of amniocentesis procedures being performed. These tests are available commercially outside of the Australian health system and due to patent and commercialisation issues, this may remain the only means of access. Assistance in funding patients to have these tests or assisting the development of local facilities capable of performing these tests may be appropriate. HealthPACT recommend that the information in this Brief be disseminated to the Human Genetics Society of Australasia and to the Royal College of Pathologists of Australasia.

**Number of studies included**

All evidence included for assessment in this Technology Brief has been assessed according to the revised NHMRC levels of evidence. A document summarising these levels may be accessed via the HealthPACT web site.

| | |
|---|---|
| Total number of studies | 3 |
| Total number of level III-2 diagnostic evidence | 1 |
| Total number of level III-1 diagnostic evidence | 2 |

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**Search criteria to be used (MeSH terms)**

Aneuploidy
High-Throughput Nucleotide Sequencing
Humans
Prenatal Diagnosis
Sequence Analysis, DNA
Chromosomes, Human, Pair 18/*genetics
DNA/*blood/*genetics
Down Syndrome/*diagnosis
Trisomy/*diagnosis

## Prioritising Summary (update 2009)

### November 2009 – Background

A reliable method for the non-invasive prenatal diagnosis (NIPD) of fetal conditions including Down syndrome is required to reduce the risk of miscarriage that is associated with chorionic villus sampling or amniocentesis. It is also hoped that NIPD will enable an earlier diagnosis as cell-free fetal RNA and DNA can be detected at five weeks gestation. Chorionic villus sampling and amniocentesis are performed at 11-14 and 15 weeks gestation, respectively. A recent review of the field by Wright and Burton (2009) describes a number of potential clinical applications using cell-free fetal nucleic acids (cffNA) including:

* Sex determination by the detection of cffDNA sequences on the Y chromosome;
* Single gene disorders by the detection of a *paternally* inherited allele in cffDNA;
* Pregnancy-related disorders by the detection of the presence of a working copy of the Rhesus gene (RhD) or an elevation in the absolute concentration of cffDNA; or
* Aneuploidy, including syndromes such as Down, by the detection of an abnormal concentration of a particular chromosome.

Several methods were discussed in this review, including detection of paternally inherited SNP[12]s (discussed August 2008 by Dhallan et al), the RNA-SNP allelic ratio method (discussed August 2008 by Lo et al) and high-throughput shotgun sequencing (described below). However the authors concluded that only sex determination and RhD diagnosis are nearing translation into clinical practice for high-risk individuals. In the long-term, the authors felt that there was a place for an appropriate technology for the analysis of cffNA which may form part of a prenatal screening programme.

### November 2009 - Safety and Effectiveness Issues

After the publication of the papers by Dhallan et al and Lo et al in 2008, Fan et al (2008) proposed that it should be possible to use *digital* polymerase chain reaction[13]

[12] SNP: single nucleotide polymorphism. DNA sequence variations that occur when a single nucleotide (A, T, C, or G) in the genome sequence is altered.

[13] Digital Polymerase Chain Reaction is a refinement of conventional PCR methods that can be used to directly quantify and clonally amplify nucleic acids. Digital PCR is useful in the detection and quantification of low-level pathogens, rare genetic sequences, gene expression in single cells, and the clonal amplification of nucleic acids for the identification and sequencing of mixed nucleic acids samples such as fetal DNA in a maternal serum sample. http://en.wikipedia.org/wiki/Main_Page

(PCR) to create a test for fetal aneuploidy that was independent of polymorphisms. However, due to the low levels of fetal DNA (<10%) against the high maternal DNA background, this has to date proved technically challenging and unfeasible. As an alternative method, the Stanford group used direct shotgun sequencing, followed by mapping to the chromosome of origin to sequence cell free DNA from the plasma of pregnant women.

With shotgun sequencing large pieces of isolated target DNA (in this case DNA purified from maternal plasma) are sheared or physically broken into small fragments of varying size. Shearing is a random process and some of the fragments will overlap. Appropriate sized fragments (2,000 base-pairs or 2kb) are then isolated using gel purification. The 2kb fragments are then cloned into a vector and transformed into E coli for amplification of the clones, creating a sequencing library. The sequence of each clone in the library, of which there are 100s, is then determined by an automated DNA sequencer. Finally the sequenced fragments are assembled into one full length sequence using a computer algorithm which finds overlapping or continuous sequences of the fragments. Shotgun cloning will usually result in some gaps between contigs because some sequences are missing from the library by chance [38]. Follow this link for an animated overview of this technique.

Maternal plasma was obtained from a mixed population of 18 women, known to contain both normal and aneuploidy pregnancies (trisomy 13, 18 and 21) (level III-2 diagnostic evidence). The gestational age of the subjects at time of sampling ranged from 10 to 35 weeks. Blood samples were obtained immediately after amniocentesis or chorionic villus sampling. For each sample an average of 10 million 25-bp sequence tags were obtained and an average of 154,000, 135,000 and 65,700 sequence tags were mapped to chromosomes 13, 18 and 21 respectively. Shotgun sequencing successfully identified all nine women carrying a trisomy 21 fetus from the nine women who were disomy 21 (Figure 4). A 99 per cent confidence interval was constructed of the distribution of the sequence tag density of chromosome 21, with the dashed line in Figure 4 representing the upper boundary. Using the upper bound of the confidence interval as a threshold for detecting trisomy 21, the minimum fraction of fetal DNA that would be detected was approximately two per cent. The nine trisomy pregnancies outliers in the distribution were statistically significant compared to the disomy pregnancies ($p < 10^{-5}$). Plasma from two women carrying a trisomy 18 fetus and from one woman carrying a trisomy 13 fetus was also analysed. Over-representation of chromosomes 18 and 13 were observed, although the construction of a representative distribution was not possible due to the small number of cases. Despite the small numbers, both the trisomy 18 and 13 were

statistically significant outliers compared to the disomy samples, $p<10^{-7}$ and $p<0.05$ respectively.

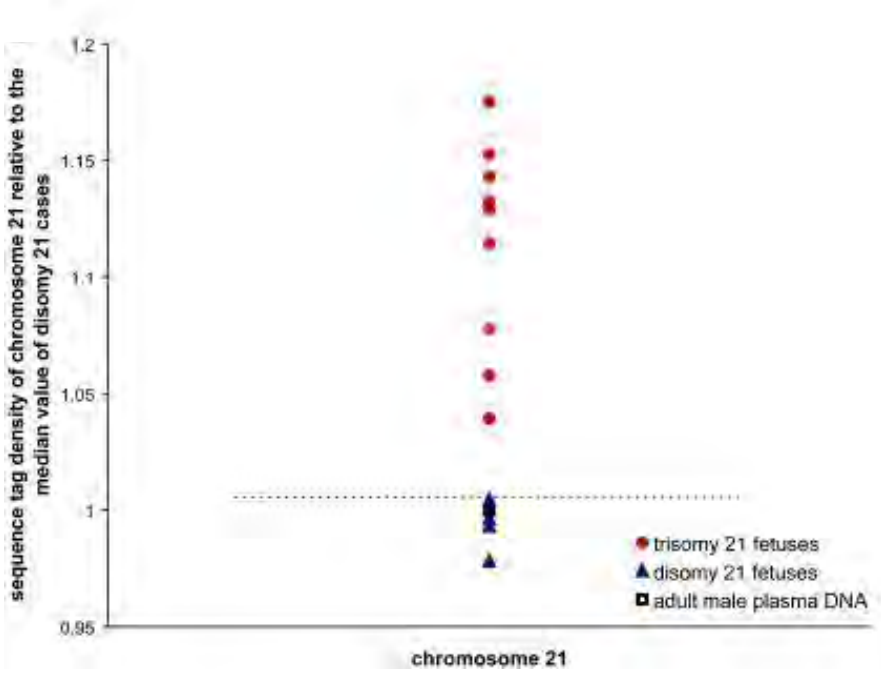


**Figure 4** **Chromosome 21 sequence tag density relative to the median chromosome 21 sequence tag density of normal cases (Fan et al 2008).**

Note: In the paper discussed below, Chiu et al (2008) reviewed the paper by Fan et al (2008). The trisomy 21 samples in the Fan et al study were taken at 18 weeks gestational age, compared to the disomy samples which were taken much earlier at 12 weeks. In addition, all samples were taken within 15-30 minutes of amniocentesis or chorionic villus sampling. Fetal DNA increases significantly in the maternal circulation immediately after invasive procedures such as amniocentesis and also with pregnancy progression, which may confound the results reported by Fan.

A similar technique was employed by Chiu et al (2008), however this research group referred to their technique as parallel genomic sequencing. As in the Fan et al (2008) assay, target DNA was obtained from maternal plasma containing small amounts of fetal DNA (red fragments) (Figure 5). One end of each DNA fragment was sequenced for 36 bp and the chromosomal origin was identified via mapping to the human reference genome. The number of unique sequences mapped to each chromosome was counted and expressed as a percentage of all unique sequences and z-scores were calculated. It is expected that pregnancies with an aneuploid fetus will have a higher z-score (those indicated by the green bars) compared to the normal fetus (shown in the blue bar).

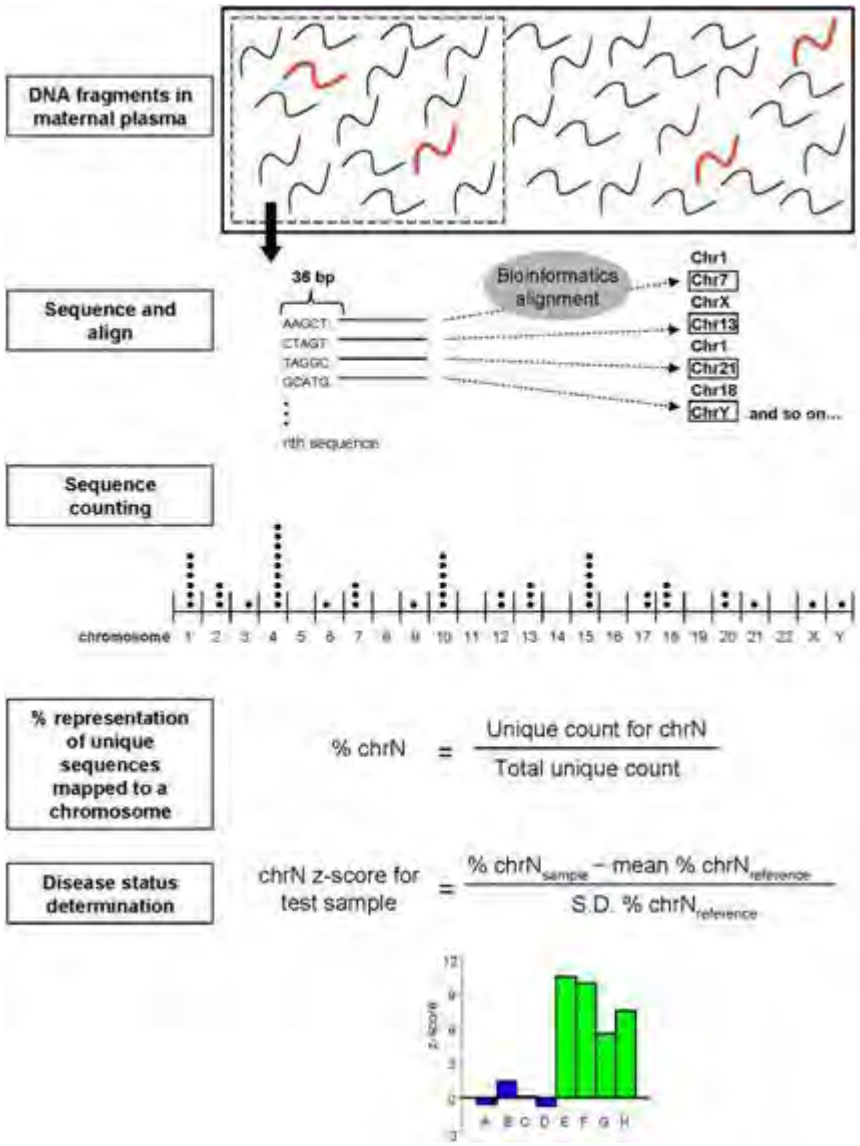


**Figure 5 Schematic illustrating the use of parallel genomic sequencing (Chiu et al 2008)**

Chiu et al obtained plasma samples from 14 women carrying a trisomy 21 fetus and 14 women pregnant with a normal fetus (mean gestational age 14.1 weeks). Chromosomal status was confirmed by full karyotyping (level III-2 diagnostic evidence). To objectively quantify the amount of over-representation in the chromosome 21 sequences of the trisomy fetuses, data from the normal male fetuses were used as a reference population. The z-scores for chromosome 21 and the X chromosome are summarised in Figure 6. For chromosome 21, all of the trisomy 21 fetuses had a z-score of >3 standard deviations above the normal reference sample (range 5.03-25.11). Therefore all trisomy 21 and disomy fetuses were correctly identified using this technique.

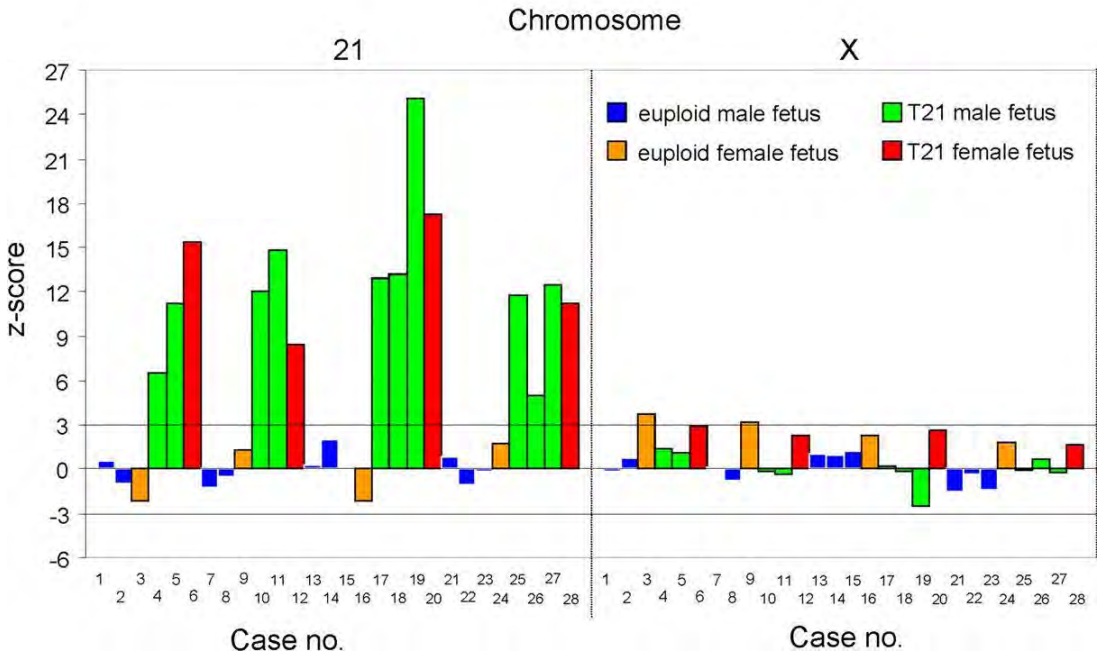


**Figure 6** **Plot of the z-scores for trisomy 21 fetuses (green and red) compared to normal fetuses (blue and yellow) for chromosome 21 and the X chromosome (Chui et al 2008)**

### November 2009 Cost Impact

Fan et al (2008) estimate the cost of their shotgun sequencing assay to be approximately US$700, with the cost of sequencing expected to decrease in the future. These technologies are technically challenging, complex molecular techniques which would require a great deal of expertise and a suitable molecular pathology laboratory.

### November 2009 Ethics

NIPD only requires a small sample of blood and can be performed relatively easily, raising ethical, social and legal issues. As the test can be performed very early on in gestation without risk to the mother or fetus it may become a more common practice than amniocentesis. Obtaining a true informed consent may become difficult over time if these techniques become routine practice, which may lead to undue pressure on the mother once test results are known.[39-44] Counselling measures may need to be updated if these diagnostic advances become routine practice. The incidence of Down syndrome may increase with increasing maternal age at conception. Many women reject the offer of prenatal screening and routine offers of screening without careful counselling may hinder informed decision making.[39-44]

**November 2009 Other issues**

The review by Wright and Chitty (2009) lists a number of sites with further information on NIPD:

* Special Non-Invasive Advances in Fetal and Neonatal Evaluation Network (www.safenoe.org) - European network of research.
* Reliable Accurate Non-Invasive Prenatal Diagnosis (www.rapid.nhs.uk).
* PHG Foundation (www.phgfoundation.org) - Report of the UK working group.

**November 2009 Recommendation:**

Several techniques for the non-invasive prenatal diagnosis of fetal chromosomal aneuploidy appear to be feasible and have promise. Whether or not the small-scale studies reviewed in the 2008 and 2009 prioritising summaries can be translated into routine clinical practice remains to be seen. Although there is an obvious and unmet need to perform non-invasive fetal tests at the earliest possible gestational time, these techniques are still in development. Therefore it is recommended that this summary be disseminated throughout the jurisdictions and that no further assessment is warranted at this time, as once the tests are suitable for routine clinical use they will be picked up by normal horizon scanning activity.

**November 2009 Number of included Studies**

| | |
|---|---|
| Level III-2 diagnostic evidence | 2 |

**November 2009 References:**

Chiu, R. W., Chan, K. C. et al (2008). 'Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma', Proc Natl Acad Sci U S A, 105 (51), 20458-20463.

Fan, H. C., Blumenfeld, Y. J. et al (2008). 'Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood', Proc Natl Acad Sci U S A, 105 (42), 16266-16271.

Mattheis, P. J., Hickey, F. et al (2008). 'Prenatal diagnosis: beyond decisions about termination', J Pediatr, 153 (5), 728; author reply 728-729.

Updegraff, E. (2009). What is shotgun sequencing? [Internet]. Conjecture Corporation. Available from: http://www.wisegeek.com/what-is-shotgun-sequencing.htm [Accessed 23rd September].

Wright, C. F. & Burton, H. (2009). 'The use of cell-free fetal nucleic acids in maternal blood for non-invasive prenatal diagnosis', Hum Reprod Update, 15 (1), 139-151.

Wright, C. F. & Chitty, L. S. (2009). 'Cell-free fetal DNA and RNA in maternal blood: implications for safer antenatal testing', BMJ, 339, b2451.

**Prioritising Summary (2008)**

**Stage of Development (In Australia)**

| | | | |
|---|---|---|---|
| ☒ | Yet to emerge | ☐ | Established |
| ☐ | Experimental | ☐ | Established *but* changed indication or modification of technique |
| ☐ | Investigational | ☐ | Should be taken out of use |
| ☐ | Nearly established | | |

**Australian Therapeutic Goods Administration Approval**

| | | |
|---|---|---|
| ☐ | Yes | ARTG number |
| ☐ | No | |
| ☒ | Not applicable | |

**International Utilisation**

| COUNTRY | LEVEL OF USE | | |
|---|---|---|---|
| | Trials Underway or Completed | Limited Use | Widely Diffused |
| Austria | ✓ | | |
| Hong Kong | ✓ | | |
| United States | ✓ | | |
| Canada | ✓ | | |

**2008 - Impact Summary:**

The use of free fetal DNA isolated from maternal plasma may be a non-invasive prenatal testing method to identify woman who may be carrying a fetus with trisomy-21, otherwise known as Down syndrome (DS).

**2008 - Background**

The majority of pregnant women are offered prenatal screening for fetal abnormalities such as Down syndrome in the form of an ultrasound for the detection of Nuchal translucency (thickness at the back of the neck in the fetus associated with DS) and markers in maternal serum. The results of these tests aim to identify women may be at risk of carrying a DS fetus, however a definitive diagnosis can only be made by invasive procedures, such as amniocentesis or chorionic villus sampling (see Comparators section), which both carry an inherent procedure-related risk to the

fetus (Saller & Canick 2008). In addition, these techniques are associated with a five per cent false positive rate and a detection rate for true positives of 64-96 per cent (Benachi & Costa 2007).

Several techniques are currently in development to enable the non-invasive prenatal diagnosis of DS. The 2007 Lancet paper by Dhallan et al presented the preliminary results of a study which isolated free fetal DNA from maternal serum. Diagnosis of fetal abnormalities using free fetal DNA is problematical due to the small volumes of free fetal DNA found in the maternal serum (approximately 3.4%) and the difficultly of distinguishing fetal DNA from maternal DNA. The use of formaldehyde during sample processing may increase the free fetal DNA yield to 25 per cent or more.

To distinguish fetal DNA from maternal DNA, Dhallan et al proposed using multiple single nucleotide polymorphisms (SNPs)[14] or mutations. For example, in Figure 7 only one SNP is considered. The green strand represents the maternal DNA which is homozygous for guanine (G/G). The blue strand represents paternal DNA which is homozygous at the same site for a different nucleotide, in this case thymine (T/T). The fetus must inherit one copy of each chromosome from each parent, therefore the fetal genome at that particular SNP site will be heterozygous (G/T). Therefore the presence of a thymine (Figure 7C) at the SNP site indicates the presence of a fetal signal.

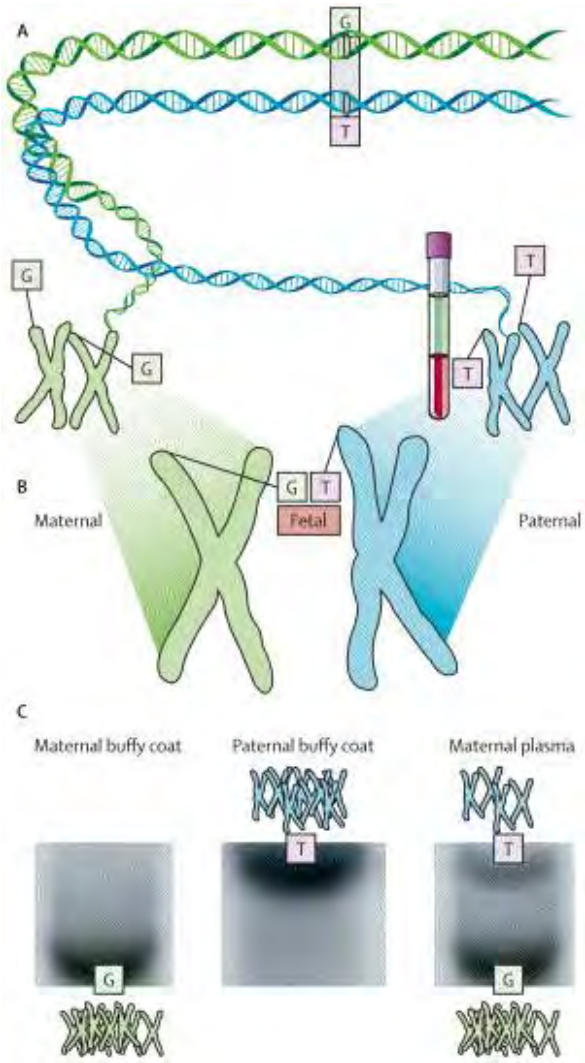


**Figure 7 Inheritance of a single nucleotide polymorphism**

1

[14] SNP: single nucleotide polymorphism. DNA sequence variations that occur when a single nucleotide (A, T, C, or G) in the genome sequence is altered.

When quantifying the allele[15] ratios for SNPs on chromosome 21, if there were twice as many SNPs in the fetal sample that match the paternal code than those matching the maternal code, then the disproportion would indicate that the fetus has an extra copy of chromosome 21 inherited from the father(Dhallan et al 2007).

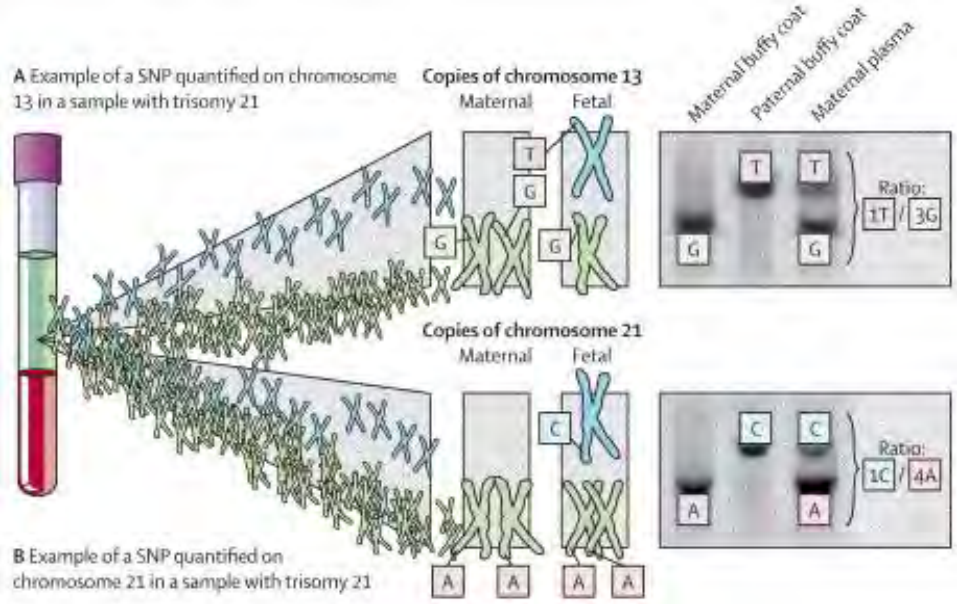


**Figure 8 Analysis of allele ratios in maternal plasma (Dhallan et al 2007)**

Figure 8 illustrates the quantification of SNPs on chromosome 13 in a sample with trisomy-21. In Figure 8A, the fetus inherits one maternal and one paternal copy of chromosome 13, therefore the ratio is 1T:3G. Over multiple SNP sites the mean ratio will approach 0.333. In Figure 8B with trisomy-21, the fetus inherits two maternal copies and one paternal copy of chromosome 21, and therefore the fetal allele ratio will approach 1:4 (0.25) over multiple SNPs. (Dhallan et al 2007)

Another technique which shows great promise is based on the Nature paper by Lo et al (2007) and is in commercial development (SEQureDx™) by the company Sequenom (Lo et al 2007; Sequenom 2008). This technique is based on the isolation of messenger RNA (mRNA) of fetal origin released into the maternal circulation, following some of the basic principles as outlined by Dhallan et al. Gene expression patterns from pre-and post-natal maternal blood samples were examined. Those genes expressed in pre-natal but not post-natal samples were determined to be of fetal origin. The chromosome 21 encoded gene, placenta specific 4 (*PLAC4*), was highly expressed and could be detected in all three trimesters of pregnancy. Figure 9 describes the basic principle of the RNA-SNP allelic ratio method. When an SNP is transcribed the fetus with trisomy-21 will have an extra copy of the gene (Figure 9a). The gene is then expressed in the placental tissue and the ratio of the two RNA

[15] Allele: Alternative form of a gene. One of a different form of a gene that exists at a single locus.

alleles in the trisomy-21 placenta will differ from the normal placenta (Figure 9b). When the transcripts are released into the maternal circulation, the difference in allelic ratios is reflected in the abundance of the transcripts, that is the level of circulating placental RNA will be greater in trisomy-21 samples (Figure 9c).

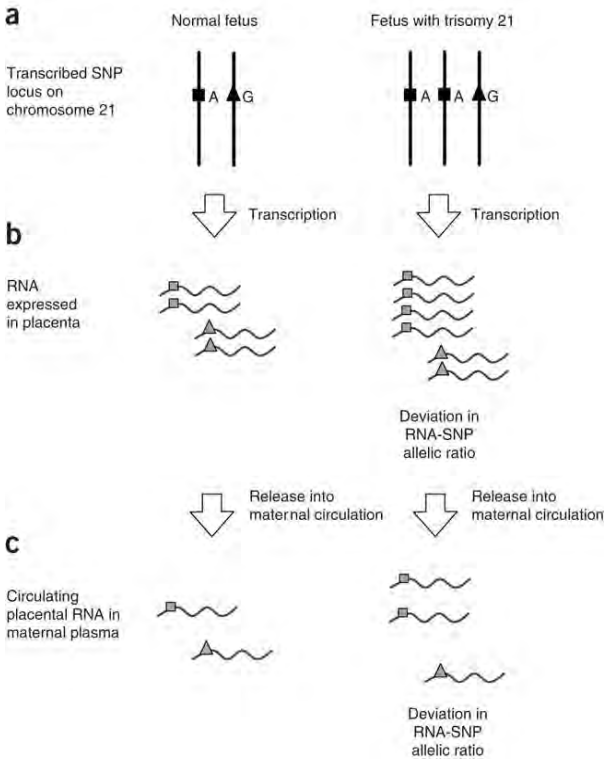


**Figure 9 The RNA-SNP allelic ratio method (Lo et al 2007)**

After RNA extraction from maternal blood, samples undergo RT-PCR followed by primer extension. Mass spectrometry is then conducted on these extension products.

### 2008 - Clinical Need and Burden of Disease

The last full report produced by the National Perinatal Statistics Unit (NPSU) contained data collected on birth anomalies during the period 1981-1997. In 1997, for the whole of Australia, there were 254,390 live births and 1,808 still births. During this same period there were a total of 4,489 congenital malformations (single and multiple), translating to a prevalence rate of 175 per 10,000 births[16]. The rate for chromosomal malformations was 22.5 per 10,000 births, with the majority of these being trisomy-21 or DS (13.0 per 10,000 births). (AIHW & UNSW 2001)

[16] Data includes congenital malformations diagnosed in liveborn infants in the first 28 days, or in still births of at least 20 weeks gestation or 400 gram birth weight. Terminations of pregnancies at ≥ 20 weeks gestation are included

More recent data are available from birth defects registers from the individual states (Victoria, South Australia and Western Australia). In South Australia during the period from 1986-2003, there was a significant increase in the prevalence of DS, associated with increasing maternal age. In 2003, 12,603 pregnancies (72% of all pregnancies) were screened for DS. During this period there were 41 notified cases of DS, translating to a rate of 1.8 per 1,000 total births. Ten cases were detected in the first trimester by maternal serum screening (MSS) and Nuchal translucency (NT), 10 cases were detected in the second trimester by MSS, nine cases were detected by amniocentesis without prior screening and three cases by ultrasound. Seven cases were not detected by screening. Two of these cases were missed by NT and MSS and seven did not undergo any form of screening. Of the 41 cases, 31 pregnancies were terminated and 10 (24%) went on to a live birth (Haan et al 2004).

Similarly the Western Australian Birth Defects Registry reported in 2006 there were 74 cases of DS translating to a total rate (livebirths, stillbirths and terminations of pregnancy) of 2.6 per 1,000 pregnancies. The rate for liveborn DS infants is approximately 1 per 1,000 births (Bower et al 2006).

In Victoria the prevalence of DS has also been reported to be increasing primarily due to increasing maternal age. During the period 1989-1992 there were 16.6 cases of DS per 10,000 total pregnancies compared to 28.6 per 10,000 total pregnancies for the period 2001-04. In 2003-04, the prevalence rate per 10,000 pregnancies for DS increased steadily from 8.5 for women aged 25-29 years, to 15.9 for those aged 30-34 years and 53.3 for woman aged 35-39. Woman aged >40 years had a prevalence rate of 203 per 10,000 pregnancies. During 2003-04, there were 360 cases of DS detected by screening. Of these 65 per cent of DS pregnancies were terminated at less than 20 weeks gestation with seven per cent terminated at greater than 20 weeks gestation. Over time, there has been no decline detected in Victoria of the number of babies liveborn with DS, with 27 per cent of DS pregnancies during 2003-04 going on to a live birth (Riley & Halliday 2006).

In New Zealand during 2003, there were 56,134 registered live births (New Zealand Health Information Service 2007). New Zealand data indicates in 2003, that of a total of 4,657 malformations, 55 were Down syndrome (NZ Birth Defects Monitoring Programme, personal communication).

**2008 - Diffusion**

These techniques are not currently in use in Australia or New Zealand for the diagnosis of DS. However, many molecular pathology laboratories would have the expertise to perform these techniques (DNA isolation, PCR etc). In addition, there are approximately 10 mass spectroscopy units in Australia capable of performing

nucleotide analysis with the Sequenom technology including the Victorian Clinical Genetics Services (VCGS) in the Murdoch Children's Research Institute. Sequenom estimate that the non-invasive diagnosis of DS will be commercially available in the United States by mid-2009 (personal communication Sequenom).

**2008 - Safety and Effectiveness Issues**

In a multi-centre study, Dhallan et al (2007) recruited 60 women (median age 34 years, range 18-43 years) with a singleton pregnancy (median gestation 17 weeks and 5 days, range 8 – 37 weeks). Approximately 35 ml (range 25-50 ml) of blood was taken and genomic DNA was isolated from both the mother and father of the baby. Amniocentesis or clinical assessment was used as the reference standard (level III-2 diagnostic evidence). Chromosome 13 was used as a reference standard as it is rarely associated with fetal abnormalities. SNPs were amplified from the isolated genomic DNA by polymerase chain reaction (PCR). The mean proportion of free fetal DNA isolated from the maternal plasma samples was 34.0 per cent (range 17.0-93.8%) The majority of the samples (51/60, 85%) had >25 per cent free fetal DNA. A mean of 22 (range 7-46) and 20 (range 8-43) SNPs were analysed on chromosome 13 and 21, respectively.

A significant difference in the ratio of fetal DNA to maternal DNA was observed in three samples (samples 4, 18 and 31) (Table 5). After amniocentesis or clinical postnatal clinical assessment, copy number was determined correctly in 58/60 (96.7%) of samples. The new diagnostic method correctly identified 56/57 (98%) of the normal samples and 2/3 (66.7%) of the trisomy-21 samples (samples 4 and 31). In sample four the mean ratio for fetal DNA for chromosome 21 was significantly higher than that for chromosome 13, indicating trisomy-21 and that the additional copy was inherited from the paternal genome. The mean ratio of fetal DNA for chromosome 21 was significantly lower in sample 31 compared to chromosome 13, indicating trisomy-21 in the fetus with the additional copy inherited from the maternal genome. The sensitivity of the new method was 66.7 per cent (95% CI [12.5, 98.2]) and the specificity was 98.2 per cent (95% CI [89.4, 99.9]). The false positive rate was 1.8 per cent, compared to the five per cent for conventional diagnostic methods. Sample 18 was a negative result by amniocentesis and was therefore considered to be a false positive. Amniocentesis identified sample 55 as trisomy-21, however the new method classified this as a normal sample and therefore a false negative.

**Table 5 Comparison of mean fetal DNA ratios for chromosomes 13 and 21**

| Sample | Chromosome | Number of SNPs quantified | Ratio of fetal to maternal DNA | Difference in fetal DNA ratio (13 vs 21) | p value |
|---|---|---|---|---|---|
| 4 | 13 | 46 | 0.8826 | -0.3621 | 0.04 |
| | 21 | 35 | 1.2446 | | |
| 18 | 13 | 11 | 0.1218 | -0.0772 | 0.05 |
| | 21 | 10 | 0.1990 | | |
| 31 | 13 | 34 | 0.2704 | 0.0877 | 0.04 |
| | 21 | 23 | 0.1827 | | |
| 55 | 13 | 25 | 0.1887 | -0.0310 | 0.34 |
| | 21 | 23 | 0.2197 | | |

Initial analysis by Lo et al (2007) was conducted on 42 normal and 12 trisomy-21 placental tissue samples. All trisomy-21 samples had RNA-SNP allelic ratios which deviated from the normal samples and a reference interval was determined (mean RNA-SNP ratio for normal samples ± 1.96 standard deviations). Only one trisomy-21 sample fell inside this reference interval, translating to a sensitivity and specificity of 91.7 and 100 per cent, respectively. This method was then applied to 57 maternal blood samples from women known to be carrying a normal fetus (mean gestation 13 weeks, range 11.1-14 weeks) and 10 women carrying a trisomy-21 fetus (mean gestation 14.7 weeks, range 12.4-20 weeks). Two of the normal samples fell outside of the normal reference interval and one of the trisomy-21 samples fell inside the normal reference interval, translating to a sensitivity and specificity of 90 and 96.5 per cent, respectively (level III-3 diagnostic evidence) (Lo et al 2007).

Several other authors have reported on non-invasive prenatal diagnostic techniques (Bauer et al 2006; Chim et al 2008; Go et al 2008; Krabchi et al 2006; Puszyk et al 2008).

### 2008 - Cost Impact

To date there has been no costing information published on this technology. It has been estimated that these methods will be cheaper and more rapid than sequencing. The cost of a basic mass spectrometry unit capable of performing the techniques described in this summary is approximately AUD$350-375,000 (personal communication Sequenom).

### 2008 - Ethical, Cultural or Religious Considerations

There are obvious ethical concerns surrounding the use of prenatal testing, the results of which may result in the termination of a pregnancy. Woman need to be offered appropriate counselling and sufficient information as to the likelihood of a false positive or false negative result.

**2008 - Summary of findings**

The isolation of fetal RNA and DNA from maternal blood appears to be feasible for the non-invasive diagnosis of Down syndrome. Current diagnostic techniques have detection rates for true positives ranging from 64-96 per cent and false positive rates of five per cent. The DNA technique proposed by Dhallan et al resulted in a low false positive rate of only 1.8 per cent, however the true positive detection rate was also low at 67 per cent. Although the preliminary results reported by Lo et al using circulating fetal RNA resulted in an improved true positive detection rate of 90 per cent, the false positive rate also increased to 3.5 per cent. However, these results should be treated with caution as the number of women enrolled in each study was low.

**2008 - HealthPACT action:**

Fetal DNA and RNA isolation from maternal blood samples appears to be a feasible technique for the non-invasive determination of trisomy-21. Both tests need to be optimised for their routine use in prenatal testing and large-scale studies should be conducted. Therefore HealthPACT recommended that this technology be monitored for further information in 12-months.

**Number of included Studies**

Total number of studies

| | |
|---|---|
| Level III-3 diagnostic evidence | 1 |
| Level III-2 diagnostic evidence | 1 |

**2008 - References:**

AIHW & UNSW (2001). Congenital Malformations, Australia 1997, AIHW National Perinatal Statistics Unit, Sydney. http://www.npsu.unsw.edu.au/NPSUweb.nsf/resources/CM/$file/cm97.pdf.

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Riley, M. & Halliday, J. (2006). Birth Defects in Victoria 2003-2004, Victorian Perinatal Data Collection Unit, Melbourne. http://www.health.vic.gov.au/perinatal/downloads/bdr_report0304.pdf.

Saller, D. N., Jr. & Canick, J. A. (2008). 'Current methods of prenatal screening for Down syndrome and other fetal abnormalities', Clin Obstet Gynecol, 51 (1), 24-36.

SAMSAS (2005). Information for Health Professionals about Maternal Serum Screening [Internet]. South Australian Maternal Serum Antenatal Screening Programme, Women's and Children's Hospital. Available from: http://www.sadi.org.au/activities/documents/articles/SAMSAS%20for%20Health%20Professionals%20Maternal%20Serum%20Screening.pdf [Accessed 10th September, 2007].

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