

Chapter 9 Diagnostic testing for trisomy 18

9.1 Diagnostic procedures: amniocentesis

Introduction: An amniocentesis procedure involves the use of a small gauge needle inserted through the woman's abdomen to withdraw a small amount of amniotic fluid (about 10 mL) from the amniotic sac. This fluid contains fetal cells that can be used to diagnose aneuploidy (e.g., trisomy 18), along with other chromosomal disorders. The amniotic fluid contains additional analytes that can be used to diagnose other conditions. For example, measuring alpha-fetoprotein and acetylcholinesterase in the amniotic fluid is diagnostic for open neural tube and ventral wall defects (Brock and Sutcliffe, 1972). Historically, amniocentesis was occasionally performed in the 19th century in women with polyhydramnios, but it was not used for diagnostic purposes until the 1960s (Jacobson and Barter, 1967).

Indications: Because of the invasive nature, availability and cost of the procedure (currently several hundred dollars), amniocentesis is generally available only to women with pregnancies at risk of a diagnosable condition. Beginning in the 1960s, when amniocentesis became available, the main indications for the procedure were a previous history of aneuploidy (or other inherited chromosomal disorder) and advanced maternal age. As ultrasound improved and became widespread, abnormal anatomical findings were also considered an indication for diagnostic testing. Beginning in the mid 1970s, women with elevated maternal serum AFP test results were offered amniocentesis for open neural tube defects (Wald *et al.*, 1977; Haddow *et al.*, 1983). More recently, these women would not be offered invasive testing, as ultrasound visualization has improved and specific markers for open neural tube defects have been discovered. These findings included the lemon and banana sign in the fetal brain (Nicolaidis *et al.*, 1986).

Beginning in the mid 1980s, the association between serum AFP markers and Down syndrome was reported (Merkatz *et al.*, 1984) and soon implemented (Palomaki and Haddow, 1987). Women with Down syndrome risks above a certain threshold were offered amniocentesis and diagnostic testing. As serum and ultrasound markers improved, it became clear that the rationale behind offering diagnostic testing to all women of advanced maternal age was not justified, and, in 2007, the American College of Obstetricians and Gynecologists recommended offering screening to women of all ages (ACOG, 2007a; ACOG, 2007b). Currently, the indications for amniocentesis in

high risk centers around the world are, in rough order, women screen positive via serum screening (including ultrasound NT testing), advanced maternal age, abnormal ultrasound findings without serum test results, and a positive family history. According to anecdotal data from prenatal diagnostic centers, the proportion of women receiving amniocentesis in the early second trimester has fallen precipitously over the last five years, from 10% or more ten years ago, to a current level of 5% or fewer. This is most likely due to improvement in screening tests (higher detection rates with lower false positive rates), as well as the slow change away from routinely offering diagnostic testing women of advanced maternal age.

The procedure: Amniocentesis is generally performed for prenatal diagnosis between 15 and 22 weeks' gestation (Figure 9.1-1). Amniocentesis is performed later in pregnancy for other indications, such as determining lung maturity, determining Rh compatibility and diagnosing infection. Once the fluid is obtained, the cells are isolated for testing by one of several methods described in later sections of this chapter.

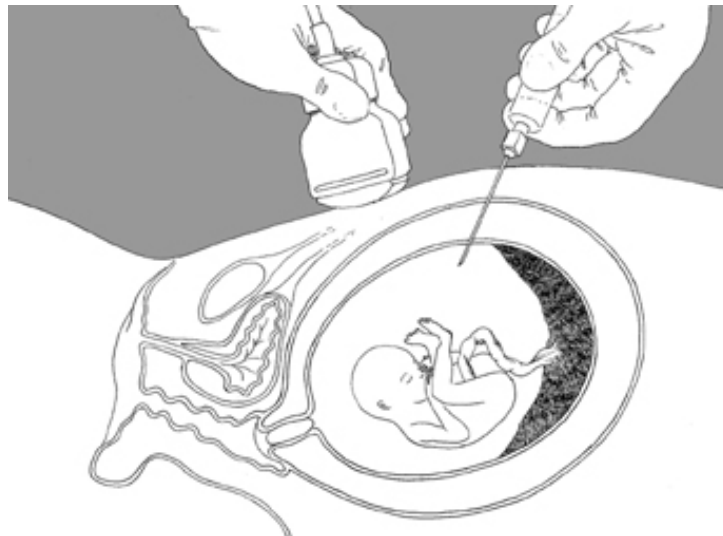


Figure 9.1-1. A diagram showing an ultrasound guided amniocentesis procedure. Ultrasound is initially used to identify the location of the fetus and placenta and then to guide the needle to obtain amniotic fluid containing fetal cells. Figure courtesy of Genetics and IVF Institute, Fairfax, Virginia.

Reliability: In experienced hands, the procedure will yield an acceptable sample more than 99% of the time. If oligohydramnios is present, it may be difficult to obtain the required amount of amniotic fluid and, subsequently, sufficient fetal cells for diagnostic testing. In a small percentage of procedures, the needle does not puncture the amniotic sac, but pushes the membrane aside (tenting). The result is that no amniotic fluid is obtained (a dry tap). Very occasionally the maternal bladder will be punctured and urine will be drawn instead of amniotic fluid. No fetal cells will be present and the procedure considered a failure.

Safety: The complications associated with the procedure include bleeding, rupture of the membranes and infection. In some instances, the fetus may be injured by the needle, but real time ultrasound guidance has reduced this risk considerably over the past two decades. Other fetal complications such as prematurity are also a concern. Higher rates of these complications are associated with multiple needle insertions, experience of the operator and position of the placenta. Only one randomized trial of the procedure-related loss associated with early second trimester amniocentesis has been performed (Tabor *et al.*, 1986) and it documented about a 1% extra fetal loss rate attributable to the procedure. The study has been criticized on several points (*e.g.*, needle size, younger population), but it remains the definitive report (Tabor *et al.*, 1988). The Cochrane Collaboration (Alfirevic, 2008) bases its conclusion of about 1% additional risk of fetal loss on this study, as well.

Early amniocentesis: One drawback to amniocentesis at 15 weeks and later is that the diagnostic test result (*e.g.*, karyotype) may not be available for at least a week, leaving less time for crucial decision-making by the couple. Another important factor is that women prefer the methods used in first trimester termination to those utilized later in pregnancy and there is more of a sense of privacy regarding their decision-making. In response to the perceived need to move the procedure earlier in pregnancy, some groups began performing amniocenteses at between 11 and 14 weeks' gestation. A Canadian collaborative study (CEMAT, 1998) definitively showed the increased risk associated with the procedure being performed this early in pregnancy. They not only found an increased rate of procedure-related loss (7.6% versus 5.9%, RR 1.29, 95% CI 1.03 to 1.63) but also an increased risk of talipes compared to CVS (another first trimester procedure, chorionic villus sampling) (RR 4.6). Based on these results, there are few, if any, amniocenteses being performed prior to 15 weeks, and virtually none prior to 14 weeks' gestation.

9.2 Diagnostic procedures: chorionic villus sampling (CVS)

Introduction: Amniocentesis is a relatively safe and widely available procedure, and the fetal cells collected are reliable for diagnostic testing for fetal aneuploidy. However, the procedure is relegated to 14 or 15 weeks' gestation or later. There is need for diagnostic procedures to collect appropriate samples earlier in pregnancy. Chorionic villus sampling can be performed late in the first trimester. One important difference between amniocentesis and CVS is that amniocentesis collects fetal cells from the amniotic fluid, while the CVS procedure aims at collecting placental tissue. In 98% or more of pregnancies, the karyotype of the fetus and placenta are the same. However, in 1 to 2% of pregnancies, they are different, and this can complicate the interpretation of the downstream diagnostic test.

Indications: The indications for CVS are similar to those for amniocentesis and include increased risk of Down syndrome or other aneuploidy and abnormal ultrasound findings. The costs for CVS are also similar to those for amniocentesis, but the procedure is considered to have a steeper learning curve than amniocentesis. Since it is less commonly performed in the United States, finding sufficient numbers of experienced providers can be difficult, especially outside of metropolitan areas.

The procedure: There are two separate routes for the CVS procedure; transabdominal and transcervical (Figure 9.2-1). In both, an ultrasound guided catheter is inserted and chorion villae are aspirated by an attached syringe. The choice is often the one the operator is most experienced and comfortable performing. Known venereal diseases or vaginal bleeding are contraindications for the transcervical route. Transabdominal CVS can be performed later in pregnancy, even into the second trimester. Transcervical CVS is generally considered more difficult to perform.

Reliability: Transcervical CVS is associated with more procedure failures than transabdominal CVS (Alfirevic, 2008). It is unclear whether sampling failures are more or less common for CVS versus amniocentesis. One study from Finland (Ammala *et al.*, 1993; Alfirevic, 2008) found transcervical CVS to have a somewhat lower procedure failure rate compared to amniocentesis (RR=0.55, $p=0.1$), but the overall rates for both procedures was quite high (2.5% for CVS and 4.5% for amniocentesis). In a much larger study (MRC, 1991), the opposite effect was found (RR 3.09, $p<0.001$), with important differences in the absolute rates at 4.8% and 1.6%, respectively. These differences may be due to operator skill and experience.

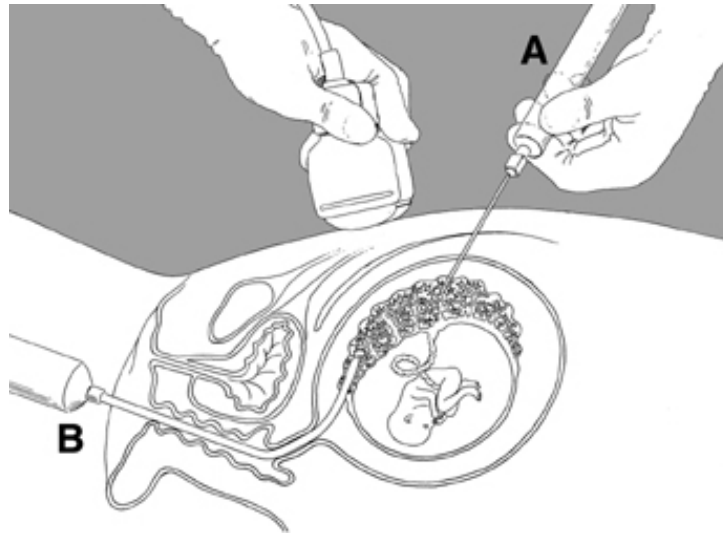


Figure 9.2-1 A diagram showing an ultrasound guided chorion villus sampling procedure. Ultrasound is initially used to identify the location of the fetus and placenta and then to guide the catheter to obtain placenta tissue. The procedure can be performed transabdominally (A) or transcervically (B). Figure courtesy of Genetics and IVF Institute, Fairfax, Virginia.

Safety: It has been difficult to directly compare CVS in the late first trimester to amniocentesis in the early second trimester. Two studies (Rhoads *et al.*, 1989; CEMAT, 1998) each found a slightly higher fetal loss rate for CVS than second trimester amniocentesis. A third study (MRC, 1991) found a 4.4% higher loss rate for CVS. There was some criticism of this latter study that the providers of CVS were not as well trained and experienced as those in the US and Canadian studies. Overall, CVS does appear to have a slightly higher procedure-related loss rate than amniocentesis. Several studies have compared the two approaches to CVS and tend to find little or no difference (Jackson *et al.*, 1992; Brambati *et al.*, 1990). However, a Danish study (Smidt-Jensen *et al.*, 1992) found a much higher loss rate for transcervical CVS. This finding has been criticized because the experience of the providers was mainly with the transabdominal route. The Cochrane Collaboration Review (Alfirevic, 2008) concludes that transabdominal is safer than transcervical, but that experience is an important factor in overall safety.

A second important issue with early CVS (prior to 10 weeks' gestation) was first reported in 1991 (Firth *et al.*, 1991) and involves severe limb reduction abnormalities. These CVS procedures were performed between 8 and 9 weeks' gestation and 5 of 289 fetuses

were associated with this relatively rare abnormality. Another large study in the US of 394 infants found another four instances of limb abnormalities (Burton *et al.*, 1992). Larger studies showed clear associations between the gestational age at the time of the procedure and the risk and severity of limb abnormalities (Mastroiacovo *et al.*, 1992). Large collections of CVS procedures did not necessarily confirm these findings made by single groups. For example, the WHO review of 216,381 CVS cases concluded that “CVS carries no increased risk for fetal loss or congenital malformation, including limb reduction defects” (WHO/PAHO, 1999). This issue of limb reductions associated with early CVS procedures has, in effect, set a lower limit of 10 completed weeks’ gestation.

9.3 Other invasive procedures

Introduction: Amniocentesis and chorionic villous sampling are, by far, the most common methods of obtaining fetal material for diagnostic testing. Occasionally, special circumstances would permit use of other invasive procedures (Shulman, 2008). These include:

percutaneous umbilical blood sampling (PUBS) involves the collection of fetal blood by ultrasound guided needle aspiration. This is a relatively specialized procedure that may be associated with higher fetal loss but is useful in at least two, and potentially, a third setting. As one example, if a late unscreened pregnancy (e.g., 20 weeks or later) is found to have specific ultrasound anomalies associated with chromosome abnormalities (e.g., heart defect and clenched hands/feet). In many centers, this is too late for CVS, and an amniocentesis/karyotype would take too long. Since there is a high chance of an affected pregnancy, the risk of procedure-related loss might be considered acceptable. The fetal blood can be directly observed for chromosome abnormalities. Another instance when PUBS might be considered would be an equivocal mosaic karyotype. A direct observation of the fetal blood might shed light on the expected fetal phenotype. A few trials have administered treatments directly into the fetus using the PUBS technique.

Fetal tissue biopsy involves ultrasound guided collection of tissues via forceps. In the past, skin biopsies were a common requirement for diagnosing certain severe skin disorders in the fetus. More recently, DNA analysis has replaced the need for biopsies. In certain circumstances, DNA testing for Duchene's muscular dystrophy would not be informative and a muscle biopsy required for definitive diagnosis. These procedures would generally not be used in diagnosing autosomal aneuploidy (the exception being exploring fetal mosaicism).

9.4 Diagnostic testing: karyotype

Introduction: In order for a karyotype to be produced, the cells must be in mitosis. This is accomplished by placing the cells collected from amniocentesis or CVS in a culture medium and growing them in specific conditions over one to two weeks. The cells are then stained (or banded), with darker staining indicating that the DNA has a higher concentration of adenine-thymine bonding. The most common G-banding (Giemsa) produces several hundred bands. The chromosomes are then identified based on size (e.g., chromosome 1 is the largest), the centromere position (e.g., chromosome 1 is metacentric with the centromere near the center of the chromosome) and the banding pattern. Prior to the mid 1990s, photomicrographs were cut by hand and arranged into a karyotype. Now this is done by computer recognition and visual verification. Both methods allow for counting of the number of chromosomes and identifying Down syndrome, trisomy 18 and trisomy 13.

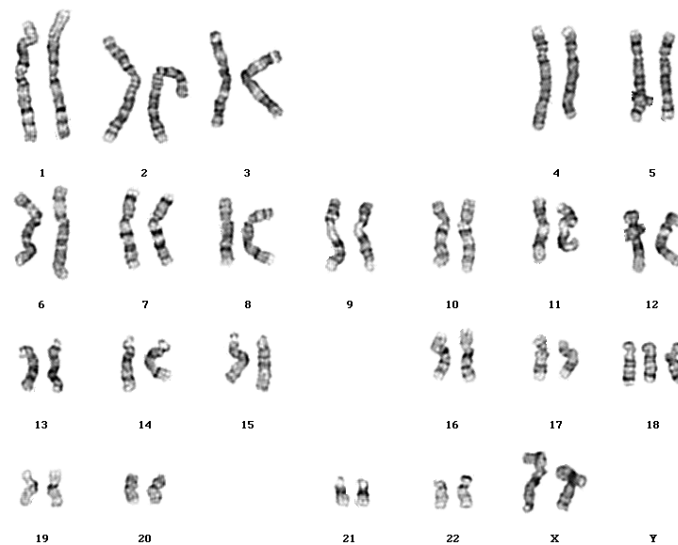


Figure 9.4-1. A karyotype of a fetus with trisomy 18. Note the three chromosome 18s at the end of the third row.

Other features such as large deletions, translocations or duplications can also be identified via karyotyping. Clinically, several colonies of cells are grown, and multiple cells from each colony are karyotyped. In some instances, there are discrepancies such as one or two cells from one colony being abnormal. This mosaicism may represent the true karyotype or be an artifact of the cell growth in culture. It can also be due to the

growth of maternal rather than fetal cells. This is most commonly seen in the karyotype of a male fetus that has a low level of female mosaicism considered to be maternal cell contamination. Specific technical/laboratory practice guidelines help define when the mosaicism is likely to be clinically relevant.

Reliability: When performed by experienced laboratories, karyotyping for aneuploidy is considered to be the gold standard of diagnostic tests. Except for sample mix-up, the detection rate approaches 100% with very few false positives. In the 1990s, the reported error rates were 0.01 to 0.02%, with the majority of errors being incorrect sex assignment due to maternal cell contamination (Benn, 2010). The few false negative cases of Down syndrome were karyotyped as 46, XX, again indicating maternal cell contamination. These could be considered errors of collection, rather than errors of karyotyping. Failures are infrequent. Using modern techniques with robotic harvesting of cells, failures could be as low as 1 in 500 samples (van Dyke, 2010).

9.5 Diagnostic testing: FISH

Introduction: The main limitation to karyotyping is the delay of 10 days to two weeks or more between sample collection and the availability of the diagnostic test result. This complicates decision-making and may cause problems with access to terminations, should an abnormal karyotype be identified. Fluorescent in situ hybridization (FISH) testing can be performed on uncultured whole cells in interphase, and results can be available in one or two days. In the 1990s, researchers investigate the use of DNA-based probes directed at unique sequences on specific chromosomes (Klinger *et al.*, 1992). These probes usually target only specific chromosomes responsible for the majority of major aneuploidies, namely chromosomes 21, 18, 13, X and Y. Each probe is attached to a specific phosphor that will emit a unique color when excited by a laser. Using this technique, each cell should have two 'dots' of light representing the normal two 21 chromosomes (perhaps red), while the two probes for 18 might fluoresce green. Not all probes need be used on each cell. Figure 9.5-1 shows two cells, one normal, and one trisomy 21 (Down syndrome).

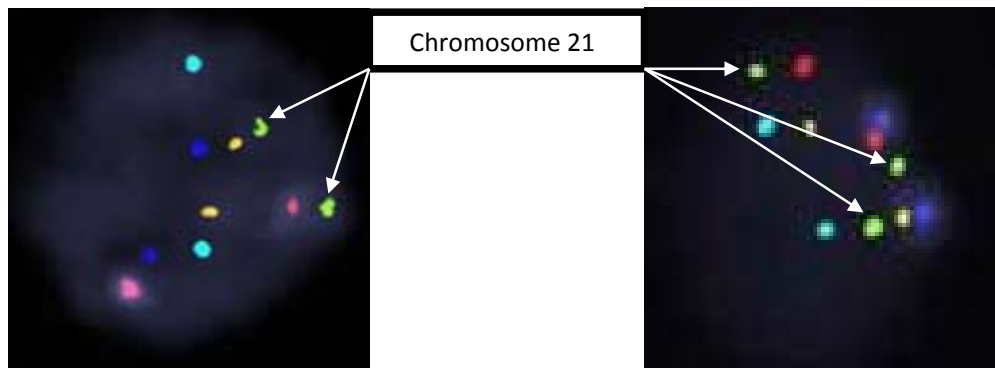


Figure 9.5-1. Two pictures of interphase cells tested with FISH probes. On the left is a normal cell with two 21 (green, with arrows), two 18 (blue), two 13 (red) and two 22 (gold) chromosomes. On the right is an abnormal cell with three 21 chromosomes, but a normal complement of 18, 13 and 22. Pictures courtesy of the University of Rochester Medical School (www.urmc.rochester.edu)

Reliability: By the late 1990s, commercial reagents were available allowing for widespread testing in diagnostic laboratories. In a 2007 summary paper (Shaffer and Bui, 2007), 19 publications of FISH testing for major aneuploidies in high risk cohorts had been published. Overall, over 26,000 FISH tests had been performed and validated against karyotyping. There was a 3.3% failure rate for the test, varying from 0% (even in some large studies) to up to 16%. Among the 1,640 detectable aneuploidies having successful FISH testing, 1,619 (98.7%) had positive test results. The 21 false negatives occurred in 9 of the 19 studies. Among the approximately 23,600 pregnancies without a detectable aneuploidy, four had false positive results (0.02%). These occurred in four different studies. The prior risk in these high risk cohorts was about 1:14. The odds among those with a positive test result were over 400:1 (1619:4), while the odds among those with a negative test result were about 1:1100 (23,600:21). While being close to diagnostic, many clinical FISH reports today suggest clinicians not take “irreversible clinical actions” until the results of the karyotype are available. FISH testing is not generally considered a replacement for karyotyping, but may be considered diagnostic in the presence of other indications of abnormalities (e.g., heart defect, clenched hands).

9.6 Diagnostic testing: qfPCR

Quantitative fluorescence polymerase chain reaction (qfPCR) measures the fluorescence intensity of highly polymorphic short tandem repeats (STRs) located on the chromosome(s) of interest. The first report of qfPCR for detecting aneuploidy during pregnancies was in the early 1990s (Mansfield, 1993). Figure 9.6-1 is a sample result from one STR (D13S252) that is located on chromosome 13. There are two clear peaks with equal height. One is 279 base pairs long, while the other (located on the second chromosome 13) is 303 bp. In this instance, the STR is heterozygous (2 different sizes) and the marker is said to be informative. If, for example, an STR is homozygous, only one peak would appear and that STR would be considered uninformative. Generally, 2 to 5 STRs will be targeted for each chromosome of interest, so that at least one would be informative.

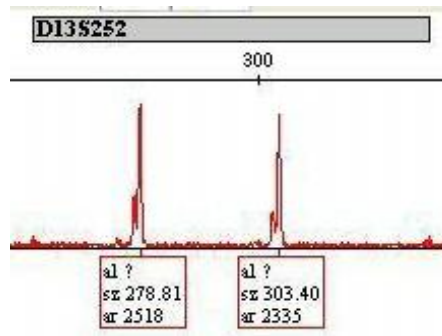


Figure 9.6-1. The result of a normal STR measurement on chromosome 13. In this electrophoretogram, the STR D13S252 is heterozygous. Since the two heights (fluorescence) are equal, there are two copies of chromosome 13 in the sample. Figure courtesy of the University of Leeds (www.leedsteachinghospitals.com).

When there are three chromosomes, the STR measurements may be homozygous/uninformative, may have three peaks (indicating varying STR lengths on all three chromosomes), or may have two peaks, but one is twice the height of the other. This later occurs when the STR on one pair of chromosomes is of equal length, but these are both different from the length on the third chromosome. Figure 9.6-2, shows examples of these abnormal qfPCR results.

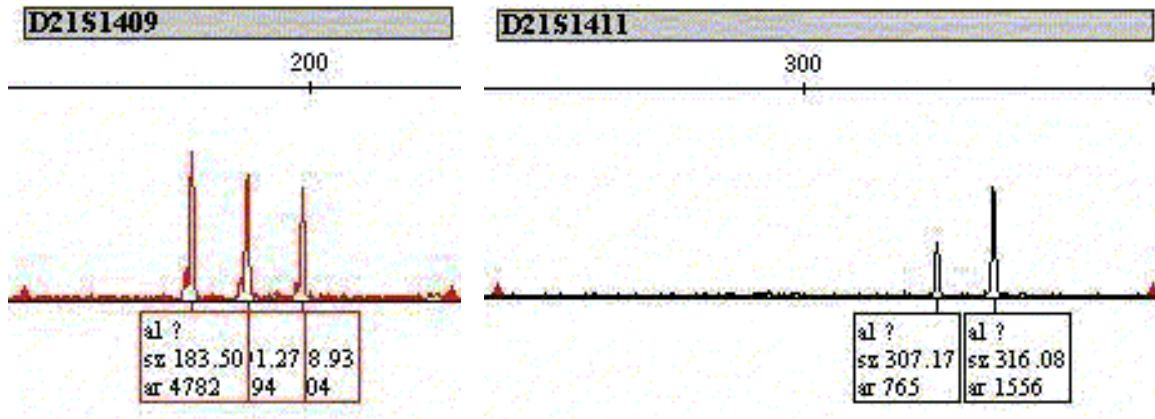


Figure 9.6-2. Abnormal qfPCR results. On the left are results from STR D21S1409 on chromosome 21. That STR is heterozygous over the three copies of the chromosome, indicated by relatively equal heights of the peaks. On the right are results from D21S1411 where one of the peaks (316 bps) is twice the height of the allele with 307 bps. Both results indicate the sample was from a Down syndrome fetus. Figure courtesy of the University of Leeds (www.leedsteachinghospitals.com).

Reliability The advantage of qfPCR is that testing is able to be done at much higher throughput levels, resulting in its being less expensive. It can also be performed on many sample types collected via amniocentesis, CVS, and fetal tissue. In a summary of 16 studies published between 1999 and 2006 (Shaffer and Bui, 2007), a total of nearly 42,000 samples have been tested via qfPCR and confirmed via karyotype. Among the 1,936 detectable abnormal karyotypes, 1,922 were found by qfPCR (99.3%). Among the 14 false negative results, more than half (8) occurred among the 310 fetuses with sex aneuploidies. Given the high performance, cost and throughput, qfPCR is often used in parallel with karyotyping, especially in Europe, to provide early indication of fetal abnormalities. In some countries, qfPCR alone is used for diagnostic testing after a screen positive test for Down syndrome.

9.7 Diagnostic testing: Other methods

Several other diagnostic testing methods exist, but are not generally targeted at identifying aneuploidy. All of these methods currently require the collection of fetal tissue via invasive procedures.

MLPA

Multiplex ligation–dependent probe amplification (MLPA) is a method used to identify gene dosage differences for multiple targeted segments of a patient's genome. In MLPA, the probes are amplified via PCR rather than the patient DNA. Each probe can be targeted for a specific region in the genome, and up to 45 probes can be combined in a single multiplex test. The probes hybridize to the target sequence and then amplified. The relative amount of the product is an indication of the number of copies of that target region. If probes were designed for chromosome 18, then the triple dose of targets on that chromosome would identify trisomy 18. Such probes are commercially available for the common aneuploidies (MLPA kit P095, MRC-Holland, Amsterdam, Holland).

Array CGH

Array-based comparative genomic hybridization (array CGH), or microarray analysis, is a method used to compare a patient's whole genome to a standard normal genome to identify chromosome imbalances. Findings include deletions, duplications, copy number variants and aneuploidy. These findings are the same as that of a karyotype, except the resolution of an array CGH is far higher. A karyotype can identify deletions that are 5 megabases or larger, while array CGH can find deletions that are under 100 kilobases (more than 50 times smaller). A simplest interpretation is that the array CGH is a 'super' karyotype. However, this high resolution also has disadvantages. However, an array CGH test cannot identify balanced translocations, but a karyotype can because each chromosome is visualized. Neither array CGH nor karyotyping can identify point mutation. Applying array CGH testing to apparently normal individuals finds that most carry 30 or more small copy number variants (CNVs), most of which do not result in any phenotypic changes.

At this time, insufficient data are available to clarify which of the CNVs might be associated with mild or even serious problems. This is an especially important issue when performing prenatal testing, when identifying these CNVs would result in difficult decision-making for virtually every patient. For this reason, targeted arrays have been

developed that can either focus on regions known to have clinical implications, or be designed in such a way that the small CNVs are not identified. Many commercial and academic laboratories are now offering prenatal array CGH testing. An expert in the field believes that targeted prenatal array CGH testing will replace karyotyping within the next few years because it is easier to perform, quicker and more informative than a karyotype (Petrone, 2010).

9.8 Fetal cells and free nucleic acids in maternal circulation

Introduction. Although karyotyping remains the gold standard, both FISH and qfPCR testing still require invasive procedures for sample collection. A test that can provide diagnostic, or near diagnostic, results early in pregnancy, without the need for invasive testing, has been sought for decades. By the late 1960s, fetal cells were known to be present in the maternal circulation (Walknowska *et al.*, 1969). However, successful isolation of these cells was not accomplished until the early 1990s (Simpson and Elias, 1993). These cells are rare, and difficult to find and test. In 1997, cell free fetal DNA and RNA was identified in the maternal circulation (Lo *et al.*, 1997). The advantage of cell free fetal nucleic acids over fetal cells is that the concentration relative to maternal free nucleic acids is much higher than for fetal cells. The disadvantage is that without the cells, it can be difficult to determine numerical chromosome abnormalities. The next two sections provide an overview of fetal cells in maternal circulation (9.8.1) and fetal nucleic acids in maternal circulation (9.8.1).

9.8.1 Fetal cells in maternal circulation

Several fetal cell types are present, but the one of most interest is nucleated erythrocytes, because they are present early in gestation, cross the placenta, and have characteristics that help make them distinguishable from maternal cells. There are several methods that can be used to identify fetal cells. After the blood is processed, the first step is to use a density gradient to concentrate the cell type of interest. Then, using fetal-specific antigens (e.g., CD71, HbF) as markers, magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) techniques are used to identify potential candidate cells. It is now estimated that fetal cells occur in less than one per million maternal cells (Simpson and Elias, 1993), so any successful methodology must be extremely accurate to both not miss the few fetal cells, and remove from consideration the vast number of maternal cells.

Rather than review the entire literature, a single larger experience from a national trial will be reviewed. The NIH-sponsored NIFTY trial (Bianchi *et al.*, 2002) began in 1995 and recruited patients for five years. Various changes in blood collection, processing and testing methodologies occurred over time, complicating the analysis. The two main outcomes of interest were the identification of male cells in the maternal circulation, and the identification of common aneuploidies (21, 18 and 13).

Confounding variables included the method of separation (MACS versus FACS), the

gestational age (<14 weeks versus \geq 14 weeks). Among the samples from women with singleton male fetuses, at least one male cell was detected 42% of the time. Among the women with singleton female fetuses, a male cell was identified 11% of the time (false positive rate). Testing for aneuploidy was performed prospectively in only 11 cases, and four (36%) were correctly identified. The associated false positive rate was 0.6%. When known cases were blindly submitted with a limited number of controls, the detection rate improved to 87.5%, but the false positive rate also increased to 4.1%.

Given the poor results and unresolved problems with the limited number of target cells and the difficulty and costs associated with testing, there has been a marked reduction in research in the area of fetal cells in maternal circulation.

9.8.2 Free fetal DNA/RNA in maternal circulation

Beginning in the late 1990s, several reports showed the presence of tumor-derived DNA in the circulation of patients with cancer (Chen *et al.*, 1996; Nawroz *et al.*, 1996; Gonzalez *et al.*, 2000). This line of research led to the finding that cell-free fetal DNA and RNA was found in the maternal circulation (Lo *et al.*, 1997). It was previously thought that these nucleic acids would have a short half-life in circulation, but they apparently can survive for appreciable periods of time due to protection by particulate matter (Ng *et al.*, 2002). This allows for the collection of nucleic acids from maternal circulation using existing plasma sampling methodologies. The fragments are relatively short, on average measuring 180 base pairs. These nucleic acids do not survive in the maternal circulation for more than one day after delivery. On average, about 5% to 10% of the free circulating nucleic acids are derived from the fetus. Most of the 'fetal' content is actually derived from the placenta. Maternal and fetal DNA are, in general, not distinguishable by their DNA or RNA sequence. Instead, epigenetic changes or differences can be utilized to determine aneuploidy status of the fetus. Lastly, the whole genome is represented by these fragments, in rough proportion to the size of each chromosome (*i.e.*, more fragments are expected for chromosome 1 than for chromosome 22). These circulating cell free fetal nucleic acids are already being used to determine fetal sex (Costa *et al.*, 2001; Mazza *et al.*, 2002) and to diagnose fetal Rh incompatibility (Lo, 2001; Legler *et al.*, 2002). However, detecting aneuploidy is more difficult, and more creative methods must be employed. Three of these are reviewed below. The last one discussed, massively parallel sequencing, is currently closest to becoming available as a clinical test.

RNA based SNP allelic ratios

The methodology relies on the premise that there are genes that are activated in the fetal/placental unit, but not in the mother (Lo *et al.*, 2007). Thus, circulating mRNA must be derived from the placenta and not the mother. If a highly polymorphic SNP (single nucleotide polymorphism) can be identified on a gene located on chromosome 21, then an information test for Down syndrome could be created for pregnancies in which the fetus is heterozygous for that SNP. Figure 9.8.2-1 shows how this might work.

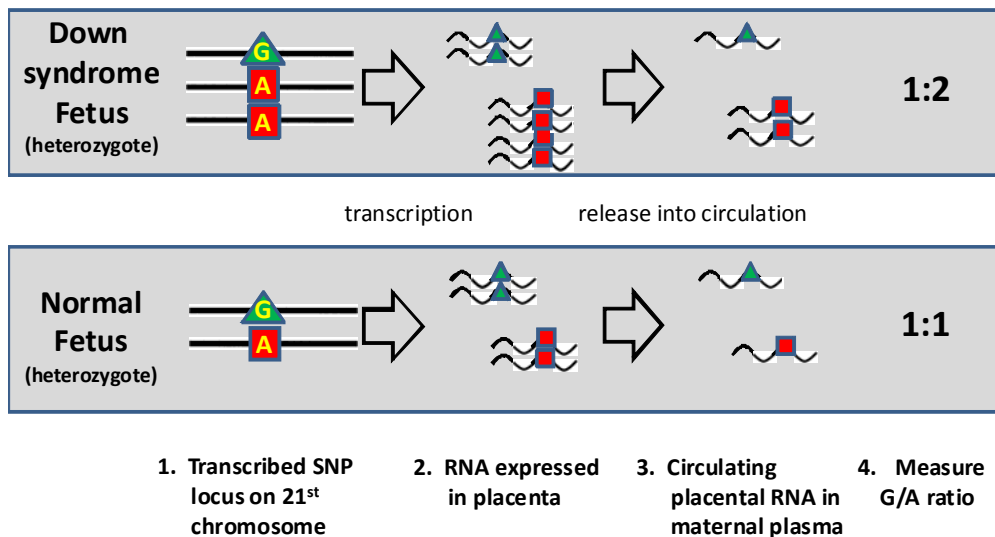


Figure 9.8.2-1. Schematic showing how the RNA SNP allelic ratio method of aneuploidy detection. In step 1, the DNA from a Down syndrome fetus (top gray rectangle) and from a normal fetus (bottom gray rectangle) are both heterozygous. This is indicated by the G and A single base pair difference. The Down syndrome fetus carries 3 copies of chromosome 21, while the normal carries only 2 copies. The RNA is transcribed (step 2) and enters the maternal circulation (step 3). At that point, a detection method would find equal numbers of each SNP allele (G/A ratio of 1.0) while the ratio in the Down syndrome sample would be 1:2. Alternatively, the Down syndrome sample could be 2:1, if the G allele were duplicated rather than the A allele.

In 2007, a proof-of-concept paper was published demonstrating the potential for this method to identify Down syndrome using free circulating RNA in maternal plasma

collected in the first and second trimester (Lo *et al.*, 2007). Using a single SNP (rs8130833) on the *PLAC4* gene (placental-specific protein 4), 10 of 21 cases and 57 of 98 control samples were found to be heterozygous (informative). Of these 57 informative control samples, two were outside of the 95% prediction limits (Figure 9.8.2-2) indicating a false positive rate of about 4%. Among the 10 informative cases, nine had ratios significantly higher, or lower, than that found in euploid pregnancies, give a detection rate of 90%. This figure is misleading, as only 9 of the 21 original cases were actually detected; 11 received no result as they were homozygous for the *PLAC4* SNP.

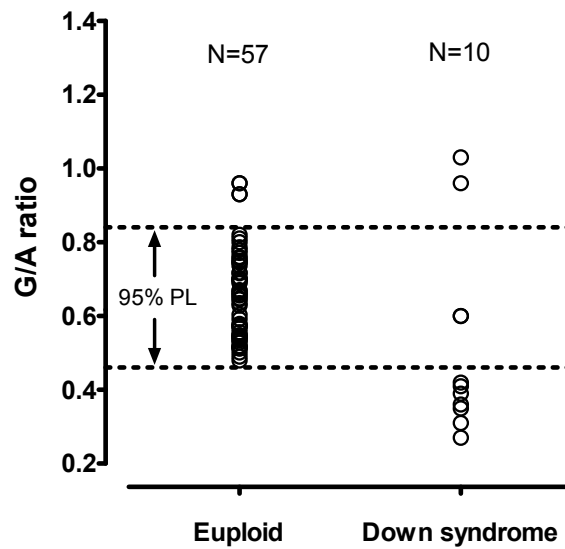


Figure 9.8.2-2. Scatterplot showing the results of an RNA-based SNP allelic ratio test for Down syndrome. A single SNP on the *PLAC4* gene was tested for the G to A allelic ratio. If the pregnancy was Down syndrome, the G/A ratio should be higher, or lower, than that found in euploid pregnancies.

For this test to be clinically useful, several important advances need to occur. There need to be more genes identified that are differentially active in the placenta. Alternatively, more SNPs might be identified on such genes. This would allow for an interpretation for all pregnancies tested. It was also found that the SNP allele frequencies differed dramatically by race/ethnicity. Thus, a set of SNPs that might provide coverage for 98% for Caucasian pregnancies might only provide informative results for 50% of Asian pregnancies. So far, it has not been possible to identify

multiple genes/SNPs that provide wide coverage for a diverse population while retaining high performance.

In addition, the processing required to retain the RNA for testing must be done in a short time frame (< 6 hours) and the samples must be frozen at -20°C or colder immediately after processing. That means that the samples would need to be shipped on dry ice. Such requirements could limit availability and adversely affect the cost of testing.

The platform used in the proof-of-concept report (Lo *et al.*, 2007) was the MassArray (Sequenom, Inc., San Diego, CA). Costs and turn-around time could, with sufficient numbers, make a methylation-based test competitive with current screening tests. The available data allow this test to be classified as being in the research and development phase.

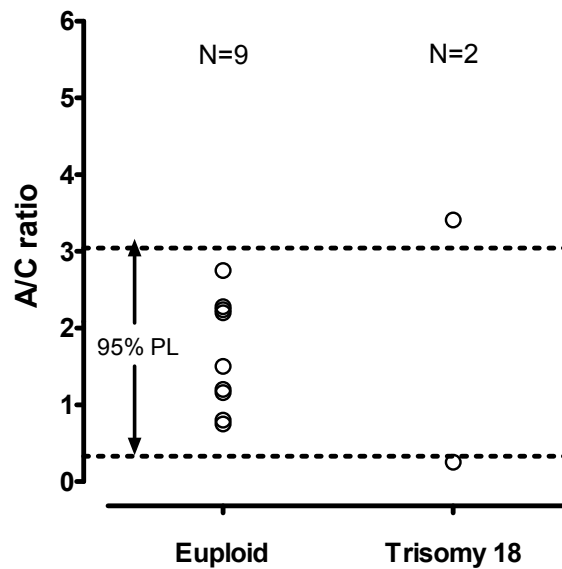
DNA methylation-based differences

The RNA method described above relies on differences in the production of a gene-specific RNA between the fetoplacental unit and the mother. This method relies on identifying differences in DNA methylation between the fetoplacental unit and the mother. Again, a gene must be found that is highly methylated in the mother and unmethylated in the fetus, or vice versa. In a proof of concept publication (Tong *et al.*, 2006), the *SERPINB5* gene promoter (maspin) was unmethylated in the placenta (turned on), but methylated (turned off) in the mother. *SERPINB5* is located on chromosome 18, so this testing would be directed at identifying trisomy 18. Using a methylation-specific PCR methodology, it is possible to measure a SNP allelic ratio, similar to the approach described in Figure 9.8.2-1. Figure 9.8.3-1 shows the results of such testing of maternal plasma from two second trimester trisomy 18 pregnancies. Controls consisted of one second trimester pool and eight near term euploid pregnancies.

Although the data are sparse, the methylation methodology has an advantage in a more stable sample type (DNA versus RNA). However, it still requires that heterozygous SNPs be present in the selected differentially methylated genes, so race/ethnic coverage remains an issue. It should be possible to identify differentially methylated genes on other chromosomes (*e.g.*, 21 and 13) in order that other common autosomal aneuploidies could also be identified. The platform used in the

proof-of-concept report (Tong *et al.*, 2006) was the MassArray (Sequenom, Inc., San Diego, CA). Costs and turn-around time could, with sufficient numbers, make a methylation-based test competitive with current screening tests. The available data allow this test to be classified as being in the research and development phase.

Figure 9.8.2-3. Scatterplot showing the results of a DNA-based methylation-specific SNP allelic ratio test for trisomy 18. A single SNP in the unmethylated



maspin region of the *SERPINB5* gene was tested for the G to A allelic ratio. The unmethylated *maspin* will be fetoplacental. The maternal DNA would be methylated and not included in the SNP ratio. If the pregnancy was trisomy 18, the A/C ratio should be higher, or lower, than that found in euploid pregnancies.

Massively parallel sequencing (MPS)

The third methodology takes on a more brute force approach to using circulating fetal DNA to identify Down syndrome. Two groups, almost simultaneously, published proof-of-concept papers demonstrating the use of third-generation sequencing as a viable methodology (Chiu *et al.*, 2008; Fan *et al.*, 2008). The underlying concepts to this test can be described in the following way. Consider a euploid pregnancy with a fixed proportion of fetal to maternal DNA in the mother's circulation. The number of DNA fragments originating from both the mother and the fetus are known. Now if this same pregnancy were to be affected with Down syndrome, there would be slightly more DNA contributed to the fetal DNA in circulation due to the extra 21

chromosome. The problem would then be to find a methodology to quantify this increase. Of course, this scenario can't exist, but it would still be possible if two further conditions were met. First, although the amount and proportion of circulating maternal and fetal DNA differs from woman to woman, the proportion of the DNA that derives from each chromosome (or at least chromosome 21) must be quantifiable and very constant. The increase in the proportion of trisomy 21 DNA must be different enough that it can be distinguished from that constant proportion seen in euploid pregnancies.

The following more detailed description of the theoretical basis behind MPS test relies on figures originally presented by the group from the Chinese University of Hong Kong (Chiu *et al.*, 2008). The bullets are linked to the highlighted text in Figures 9.8.2-4 through 9.8.2-6.

In Figure 9.8.2-4:

- DNA fragments in maternal plasma - the large rectangle represents the maternal circulation, with both maternal (thin) and fetal (thick) DNA fragments. The dashed box indicates the sample of plasma collected for testing. It is important to remember that there is no way (and no need) to distinguish fetal from maternal DNA.
- Sequence and align – for each of these fragments, 36 base pairs (bp) are sequenced, and that sequence is then aligned with the human genome to determine which chromosome was the source for the fragment. In some instances the fragment won't match anywhere in the genome, and if so, it is not considered further. In others, the fragment might match locations in two separate chromosomes and, if so, it would also not be considered further. Perfect and unique matches are sought. However, due to the presence of SNPs throughout the genome, a 36 bp sequence would only need to match on 35 of the bps to be considered a true match.

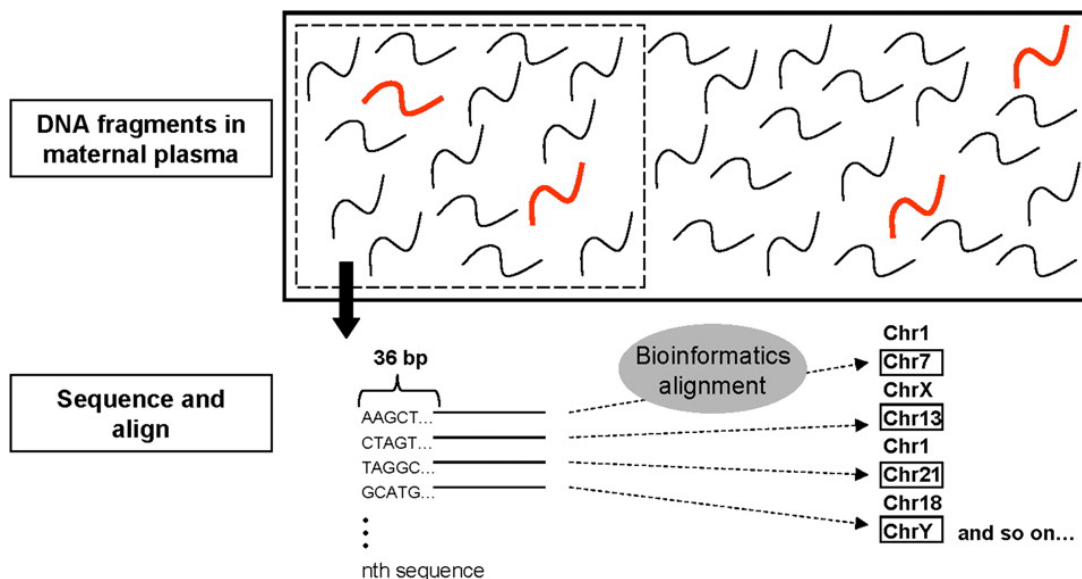


Figure 9.8.2-4. A cartoon showing the first two steps in massively parallel sequencing of free DNA in the maternal circulation. The content is described in the text.

In Figure 9.8.2-5:

- Sequence counting - for each matched sequence, the corresponding chromosome count is increased by one. For example, among all 24 chromosomes shown, there have been a total of 51 fragments already counted (each fragment indicated by a small circle. For chromosome 21, only 1 fragment has been found so far.
- % representation of unique sequences mapped to a chromosome – rather than representing the result as absolute counts of matches, the count for any given chromosome will be represented as a percentage. In this example, the proportion associated with chromosome 21 is 1/51 or about 1.96%. In practice, the X and Y chromosome counts are not included in the denominator, so the percentage could also be represented as 1/49 or 2.04.

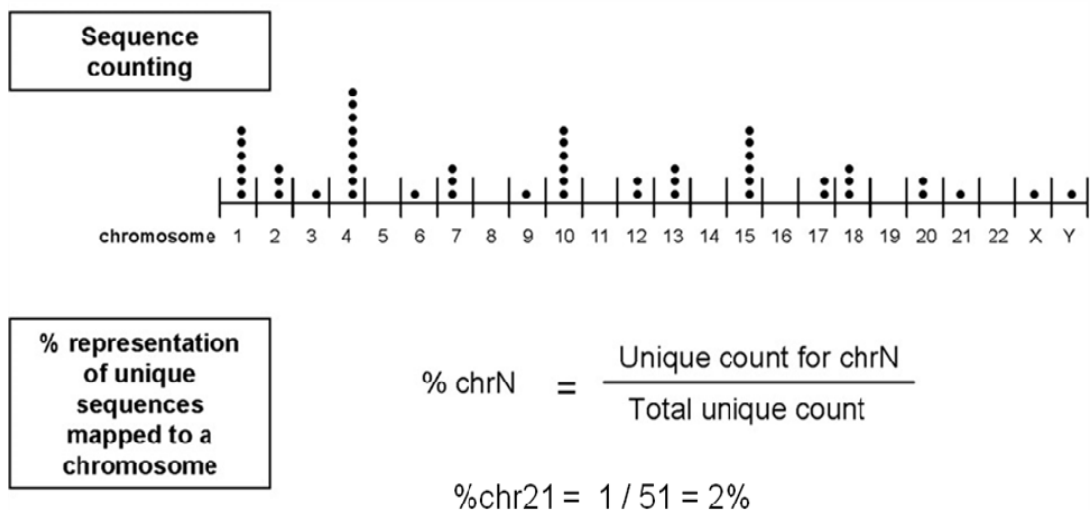


Figure 9.8.2-5. A cartoon showing the next two steps in massively parallel sequencing of free DNA in the maternal circulation. The content is described in the text.

In Figure 9.8.2-5:

- Disease status determination - once a large number of euploid pregnancies have been tested, it would be possible to compute the mean and standard deviation for the percentage of counts for any chromosome of interest, like chromosome 21. Assume, for example that the mean value for the percentage of chromosome 21 is, in fact 2.01%, and that this value is highly repeatable, with a standard deviation of 0.02%. If the next, unknown sample were to have a chromosome 21 percentage of 2.11%, this could be represented as a z-score of 5.0 (calculation shown in the figure).
- Bar chart graphic – given all of the earlier assumption, one would expect a series of test results to look something like the small bar in the figure. The small bars (A through D) that fall slightly above, or below, a z-score of 0 indicate results expected for euploid pregnancies. The larger positive bars (E through H) indicate results expected in Down syndrome pregnancies.

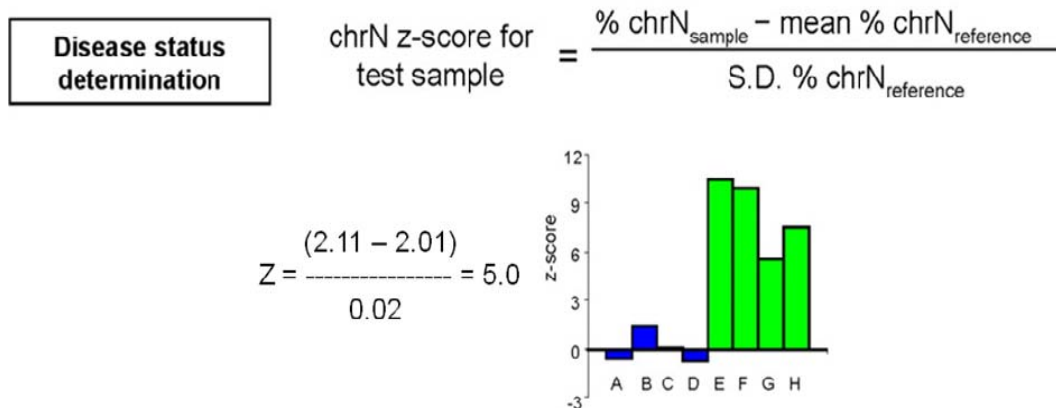


Figure 9.8.2-6. A cartoon showing the last step in massively parallel sequencing of free DNA in the maternal circulation. The content is described in the text.

Figure 9.8.2-7 shows the data collected as part of one of the proof-of-concept publication (Lo *et al.*, 2007). The MPS was performed on the Illumina platform (Illumina Inc, San Diego, CA). The observed detection rate was 100% (14/14) and the false positive rate was 0 (0/14). In this small, select dataset, MPS was able to correctly distinguish between Down syndrome and unaffected pregnancies.

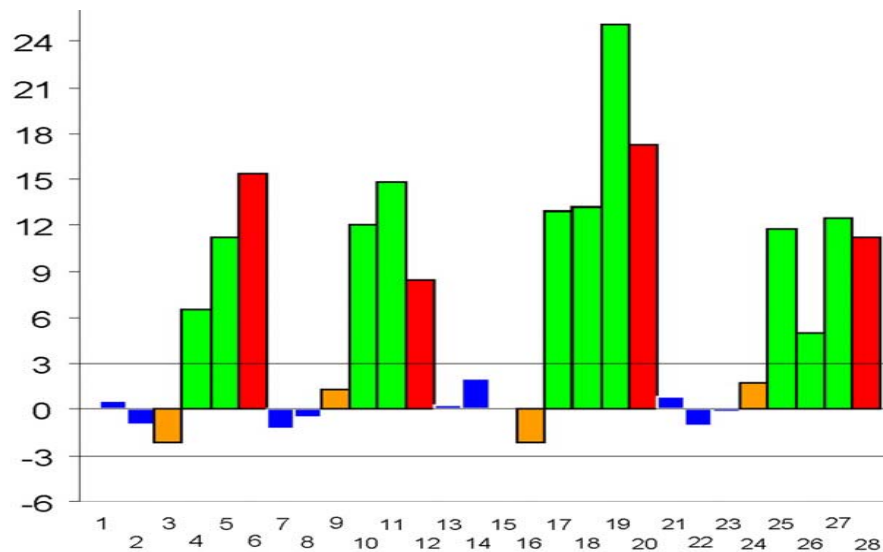


Figure 9.8.2-7. Chromosome 21 z-scores for 28 maternal plasma samples undergoing massively parallel sequencing. The horizontal lines indicate the normal range (± 3 standard deviations). Of the 28 samples, 14 were from Down syndrome pregnancies, and all had chromosome 21 z-scores above 3. Of the 14 samples from euploid pregnancies, none were outside the normal range.

Massively parallel sequencing has several advantages. It is based on the more stable DNA fragments and, unlike the previous methods, does not require heterozygous SNPs be identified for appropriate coverage. Essentially, all samples tested will get a result. It is also not limited to Down syndrome, as the collected could, in theory also identify trisomy 18 and trisomy 13 simply by performing another series of computations. However, no paper yet has shown it feasible to identify trisomy 18 or 13 using MPS.

On the other hand, massively parallel sequencing is extremely complex, requiring highly trained molecular technologists working on expensive equipment in tightly

controlled environments. This, along with reagent costs result in test that would be expected to cost \$1,000 or more per woman tested and take up to a week to complete. Other limitations exist. The amount of data to be collected and interpreted is staggering. Over 1 terabyte of data are collected for each flow cell. High performance computer clusters can take several days to complete the processing. Lastly, if the fetal fraction of the DNA were to become too low (*e.g.*, <5%), it would become difficult to resolve the small differences between a mother with a normal pregnancy with one having a Down syndrome pregnancy. Whether this limit can be lowered remains to be seen. Other improvements that could be anticipated to improve the potential for MPS would be multiplexed samples. Rather than running only one patient per lane, multiplexing allows running 2, 4 or even 8 or more patients mixed together in that same single lane. This improved throughput and reduces costs. Spin-offs from the \$1,000 genome initiative would likely reduce the resources required to run MPS and fourth generation sequencing platforms are beginning to emerge.

Several groups around the world are actively pursuing the development of MPS tests suitable for introduction into clinical practice. Whether or not this test can be translated from the bench to the clinical laboratory is not yet known. Even if it can be implemented clinically, it is not yet clear how MPS testing (or another free DNA/RNA test) might fit into the complex world of prenatal testing and diagnosis.