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METHYLOME AND TRANSCRIPTOME- Towards Non Invasive Prenatal Diagnosis

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The present doctoral dissertation was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy of the University of Cyprus. It is a product of original work of my own, unless otherwise mentioned through references, notes, or any other statements.

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Περίληψη

Η μη-επεμβατική προγεννητική διάγνωση αποτέλεσε ένα από τους πιο απαιτητικούς ερευνητικούς τομείς των δύο τελευταίων δεκαετιών. Η ανακάλυψη της παρουσίας ελεύθερου εμβρυϊκού DNA στην μητρική κυκλοφορία αποτέλεσε κινητήριο μοχλό για πολλές ομάδες που στόχευαν στην ανίχνευση εμβρυοειδικών βιοδεικτών. Η ανίχνευση του εμβρυϊκού DNA (~10%) παρουσία μητρικού DNA (90%) απαιτεί την εφαρμογή μεθόδων εμπλουτισμού υψηλής ευαισθησίας και ακρίβειας.

Μέσω της επιγενετικής, η ομάδα μας ταυτοποίησε διαφορετικά μεθυλιωμένες περιοχές (DMRs) του εμβρύου και της μητέρας στα χρωμοσώματα 21, 18, 13, X και Y, και ανέπτυξε μια μη επεμβατική προγεννητική μέθοδο για την ανίχνευση της τρισωμίας 21.

Ο πρωταρχικός σκοπός της μελέτης αυτής αφορά στη διερεύνηση της πιθανότητας η μεταβλητότητα του εμβρυϊκού ποσοστού να επηρεάσει το σωστό προσδιορισμό της τρισωμίας 21, χρησιμοποιώντας την υπάρχουσα μέθοδο MeDIP. Με τη χρήση qPCR, πραγματοποιήθηκε ποσοτικοποίηση 224 μητρικών δειγμάτων αίματος και 124 μητρικών δειγμάτων πλάσματος, για την συσχέτιση των εμβρυϊκών ποσοστών και της διαγνωστικής αξίας της μεθοδολογίας. Τα αποτελέσματα έδειξαν ότι η μεταβλητότητα των εμβρυϊκών ποσοστών δεν επηρεάζει τον σωστό καθορισμό της τρισωμίας 21.

Ο δεύτερος στόχος αποσκοπεί στην ανάπτυξη μιας μεθόδου εμβρυϊκού εμπλουτισμού, για την αύξηση του ποσοστού του εμβρυϊκού DNA που βρίσκεται στη μητρική κυκλοφορία κατά τη διάρκεια της εγκυμοσύνης και το σωστό προσδιορισμό των εμβρύων με τρισωμία 21. Χρησιμοποιώντας δείγματα χοριονικών λαχνών (CVS) και δείγματα πλάσματος από μη εγκυμονούσες γυναίκες, ετοιμάστηκαν δείγματα που μιμούνται τα ποσοστά DNA (μητρικό και εμβρυϊκό) όπως εντοπίζονται στο πλάσμα της μητέρας κατά την κύηση. Τα συγκεκριμένα δείγματα, χρησιμοποιήθηκαν για ανοσοκατακρήμνιση ακολουθούμενη από digital PCR, επιτυγχάνοντας τη σωστή ταξινόμηση των τρισωμικών δειγμάτων από τα φυσιολογικά, και καθιστώντας τη νέα μέθοδο έτοιμη για επικύρωση.

Ο τρίτος σκοπός αυτής της μελέτης, αφορά στην επέκταση του πίνακα των εμβρυϊκών βιοδεικτών μελετώντας το εμβρυϊκό RNA. Αυτό θα προσδιορίσει, διαφορικά εκφραζόμενα γονίδια (DEGs) μεταξύ των φυσιολογικών εμβρύων και των εμβρύων με τρισωμία 21. Μικροσυστοιχίες έκφρασης που καλύπτουν όλο το RNA, εφαρμόστηκαν σε RNA δείγματα από φυσιολογικές και τρισωμικές χοριονικές λάχνες (CVS), και συγκρίθηκαν με τα αντίστοιχα RNA δείγματα από τη μητέρα. Πραγματοποιήθηκαν μελέτες συσχέτισης, των DEGs σε μεταγραφικό επίπεδο σε σχέση με το πρότυπο μεθυλίωσης και τον φαινότυπο του συνδρόμου Down. Ανιχνεύθηκαν πολλαπλά DEGs ειδικά για την τρισωμία 21, πολλά από τα οποία βρέθηκαν να σχετίζονται με το φαινότυπο του συνδρόμου Down. Τα μεθυλιωμένα πρότυπα των DEGs δεν έδειξαν να συσχετίζονται με το μεταγραφικό επίπεδο. Λόγω της μεταβλητότητας του μεταγραφικού επιπέδου που παρουσιάζεται μεταξύ διαφορετικών ατόμων, απαιτείται περαιτέρω διερεύνηση σε μεγαλύτερο αριθμό δειγμάτων προκειμένου να επικυρωθούν τα ευρήματα αυτής της μελέτης.

Συμπερασματικά η μελέτη αυτή χρησιμοποιεί υπάρχουσες διαφορές μεταξύ της μητέρας και του εμβρύου, και πέτυχε την ανάπτυξη μιας μη επεμβατικής προγεννητικής μεθοδολογίας για τη διάκριση της τρισωμίας 21 από φυσιολογικά έμβρυα. Επιπλέον, επέκτεινε τον πίνακα των εμβρυϊκών βιοδεικτών με τον εντοπισμό νέων διαφορών ανάμεσα στη μητέρα και το έμβρυο που θα μπορούσαν στο μέλλον να χρησιμοποιηθούν για την μη επεμβατική προγεννητική διάγνωση.

ABSTRACT

Non-invasive prenatal diagnosis has been one of the most challenging fields in the past two decades. The discovery of fetal nucleic acids in the maternal circulation encouraged several groups to work on the identification of fetal specific biomarkers. Recovery of fetal DNA fragments (~10%) in the presence of maternal DNA (90%) requires high sensitivity and specificity enrichment methods. Our group has successfully used epigenetics to identify differentially methylated regions (DMRs) between the fetus and the mother on chromosomes 21, 18, 13, X and Y, and developed a non-invasive prenatal methodology (MeDIP-qPCR) for the detection of trisomy 21.

The first objective of this study aims to investigate whether the variability of fetal percentage among individuals affects the correct classification of trisomy 21 using the existing MeDIP methodology. Quantification of 224 maternal whole blood and 124 maternal plasma samples was carried out, by qPCR, followed by correlation studies between the fetal percentages and the diagnostic value of the methodology. Results showed that the variability of fetal amount among individuals does not interfere with the correct classification of trisomy 21.

The second objective targets the development of a robust fetal epigenetic enrichment method which will increase the proportion of fetal DNA in maternal circulation during pregnancy and correctly classify trisomy 21 fetuses. Spike-in samples were prepared using chorionic villi samplings (CVS) DNA and non-pregnant female plasma DNA which are used to imitate the maternal DNA. These samples underwent immunoprecipitation followed by digital PCR for quantification. Correct classification of trisomy 21 spike-in samples from normal ones was achieved. This newly developed method is now ready for validation.

The third objective of this study aims to expand the panel of fetal specific biomarkers by uncovering the fetal transcriptome. This will identify differentially expressed genes (DEGs) among the trisomy 21 and normal fetuses from their mothers. Expression microarrays covering the whole transcriptome were applied to normal and trisomy 21 CVS RNA samples together with their matching maternal RNA. Association studies of identified DEGs transcription level with their methylation patterns and Down syndrome phenotype were performed. Multiple trisomy 21 specific

DEGs were identified and were found to be associated with the Down syndrome phenotype. Methylation patterns of DEGs showed no association with the transcription level. Due to the transcription level variability among individuals, identified DEGs must be further investigated in a large-scale study in order to confirm our findings.

As a conclusion this work utilised already existing differences between the mother and the fetus, DMRs, and succeeded the development of a non-invasive prenatal methodology for the discrimination of trisomy 21 from normal fetuses. In addition, it expanded the panel of fetal specific biomarkers by identifying new differences between the mother and the fetus which can potentially be used for non-invasive prenatal diagnosis.

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Finally, I thank my parents and brother for supporting me throughout all my studies, for their love and encouragement.

And most of all I offer my thankfulness to my loving, encouraging, and patient husband whose faithful support during the final stages of this Ph.D. is so appreciated. Thank you.

Dedication

This thesis is dedicated to my husband and my parents for their love, endless support and encouragement.

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Abbreviations

5mC	5-methylcytosine
ADAM12	ADAM metallopeptidase domain 12
AFP	Alpha fetoprotein
cDNA	Complementary DNA
cffDNA	Cell free fetal DNA
CGA	Glycoprotein hormones, alpha polypeptide
CGB	Chorionic gonadotropin, beta polypeptide
COL6A1	Collagen, type VI, alpha 1
cRNA	Complementary RNA
CVS	Chorionic villi sampling
DEG	Differentially expressed gene
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
dPCR	Digital polymerase chain reaction
D-value	Diagnostic value
FISH	Fluorescent in situ hybridization
GE	Genome equivalent
hCG	Human chorionic gonadotropin
hPL	Human placental lactogen
IGF2	Insulin growth factor
INHA	Inhibin alpha
MeDIP	Methylated DNA immunoprecipitation
miRNA	micro RNA
mRNA	messenger RNA
ncRNA	Non-coding RNA
NIPD	Non-invasive prenatal diagnosis
NRBC	Nucleated red blood cells
NT	Nuchal transluency
PAPP-A	Pregnancy-associated plasma protein A, pappalysin 1
PGF	placental growth factor
PLAC4	Placenta specific 4
PSG1	pregnancy specific beta-1-glycoprotein 1
QF PCR	Quantitative fluorescent polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
Rh	Rhesus
RNA	Ribonucleic acid
SERPINB5	Serpin Peptidase Inhibitor, Clade B (Ovalbumin), Member 5
SNP	Single nucleotide polymorphism
STS	Steroid sulfatase (microsomal), isozyme S
T21	Trisomy 21
ZFY	Zinc finger protein, Y-linked

1. Introduction

About 2 to 3% of fetuses born worldwide have some type of major birth defect due to the abnormal separation of genetic material. These types of abnormalities cause about 48% of the cases, Down syndrome, 16% and 6% Edwards and Patau syndromes (Alimilo et al., 2013). Many chromosomal abnormalities result in severely affected offspring's or fetal death. Prenatal diagnosis is used to determine whether the fetus has a genetic abnormality before birth. Techniques that are currently available for prenatal diagnosis are mainly invasive with a significant risk of miscarriages (0.5-1%) (Tabor, et al., 2010). The introduction of first and second trimester screenings increase significantly the chances to detect the fetal abnormalities. The first trimester screening involves the combination of fetal ultrasound and maternal blood testing, whereas the second trimester screening includes several blood tests. An abnormal result during these screening tests indicates the need for invasive procedures. Thus the necessity of non-invasive techniques became a challenging goal for many scientists. The discovery of minor fractions of fetal nucleic acids among the huge maternal background in plasma was an innovative step in the field of non-invasive prenatal diagnosis (NIPD).

1.1 Techniques Currently Used In Prenatal Diagnosis

1.1.1 Screening Tests

First trimester screening tests refer to the combination of fetal ultrasound and maternal blood testing performed during the first trimester of pregnancy, 10^{th} to 13^{th} gestational weeks, supporting the determination of fetal birth defects.
The ultrasound test involves the measurement of nuchal translucency (NT) which examines the area of the fetal neck for increased fluid. Maternal blood test accompanies the ultrasound with the measurement of pregnancy-associated plasma protein (PAPP-A) and human chorionic gonadotropin (hCG), which are produced by the placenta in early pregnancy.

Abnormal levels of these two proteins are associated with an increased risk for chromosomal abnormalities. The combination of the two tests, nuchal translucency screening and maternal blood test, gives a risk factor for fetal birth defects, such as Down syndrome, trisomy 18, or trisomy 13. In case of high risk factor further testing such as chorionic villus sampling or amniocentesis is necessary for accurate diagnosis.

Second trimester prenatal screening involves several blood tests measuring multiple markers. This screening is usually performed between the 15th and 20th gestational weeks and involves the following hormones: alpha-fetoprotein screening (AFP), produced by the fetal liver, human chorionic gonadotropin hormone (hCG) and estriol. This blood test is usually called the triple or quadruple screen test where the only difference is that the latter measures an additional hormone, the inhibin. Abnormal levels of these hormones may signal open neural tube defects, Down syndrome, other chromosomal abnormalities and defects in the abdominal wall of the fetus. This test is not a diagnostic test but it only indicates the necessity for additional testing. Usually an ultrasound is performed to examine the fetal spine and other body parts for defects. In case of further testing, an amniocentesis should be performed to evaluate the fetal condition. All different prenatal screening options in the different trimesters are collectively shown in figure 1-1.

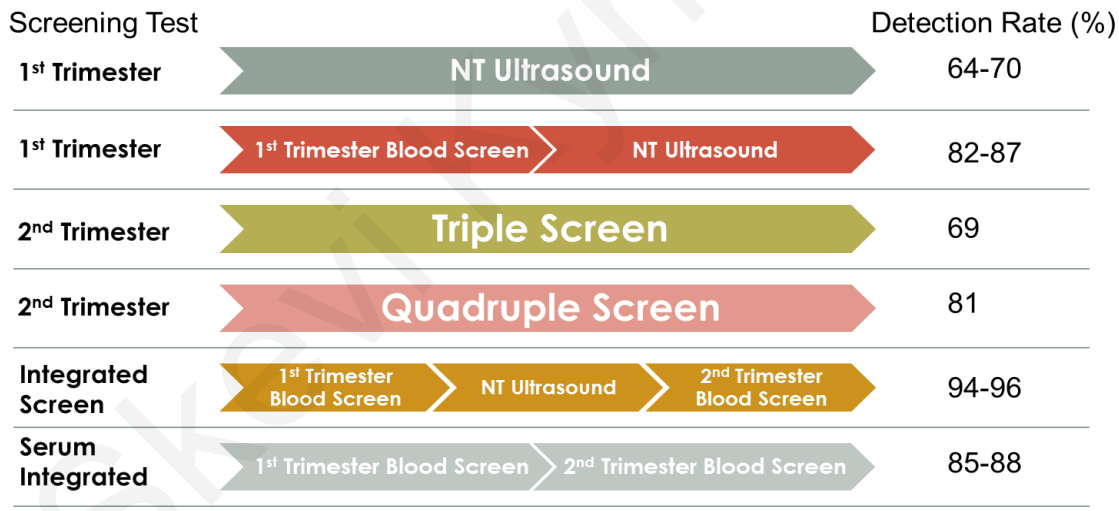


Figure 1-1: Prenatal Screening Options and Detection Rates. All first and second trimester screening tests are shown above with their detection rates. (ACOG Practice Bulletin No. 77, January 2007)

1.1.2 Invasive Prenatal Diagnostic Tests

1.1.2.1 Chorionic Villus Sampling (CVS)

Chorionic Villus Sampling is an invasive prenatal diagnostic test used to determine whether a fetus has any genetic abnormalities. CVS is performed as early as 10 weeks of gestation (typically 10-13 weeks). Chorionic villus sampling is executed by first visualizing the fetus and placenta using ultrasound scan, ensuring that the fetus and placenta are not in any danger of injury. The Obstetrician then passes a catheter through the vagina and cervix into the placenta (fig.1-2) and a small amount of tissue is sent to the lab for karyotype or Fluorescent In Situ Hybridization (FISH) analysis. Analysis takes about 2 weeks. Alternatively, a long, thin needle is placed through the maternal abdominal wall and reaches the placenta. CVS has the disadvantage of being an invasive procedure, and it has a significant rate of fetal morbidity of about 1%. The likelihood of maternal Rh sensitization is also present. There is also the risk of maternal contamination due to the transfer of maternal blood cells along with the placenta sample and this may lead to misleading results interpretation.

1.1.2.2 Amniocentesis

This is an alternative invasive procedure that involves a needle passing through the mother's lower abdomen into the amniotic cavity (fig. 1-2). For prenatal diagnosis, amniocentesis is performed between 14 and 20 weeks gestation. An ultrasound examination is always applied prior amniocentesis in order to confirm gestational age, determine the position of the fetus and placenta, and the quantity of amniotic fluid present. Fetal skin cells are found in amniotic fluid and can be grown in culture for chromosomal analysis such as karyotyping or FISH. It takes about 2 weeks for the analysis to be completed. Drawback of amniocentesis associates with the risk for fetal loss and maternal Rh sensitization in Rh negative mothers. The increased risk for fetal mortality is about 0.5%. Upon completion of the procedure the amniotic sac replenishes the liquid over the next 24–48 hours.

The most common abnormalities detected, by both techniques, are Down syndrome (trisomy 21), Edwards syndrome (trisomy 18), and Turner syndrome (monosomy X).

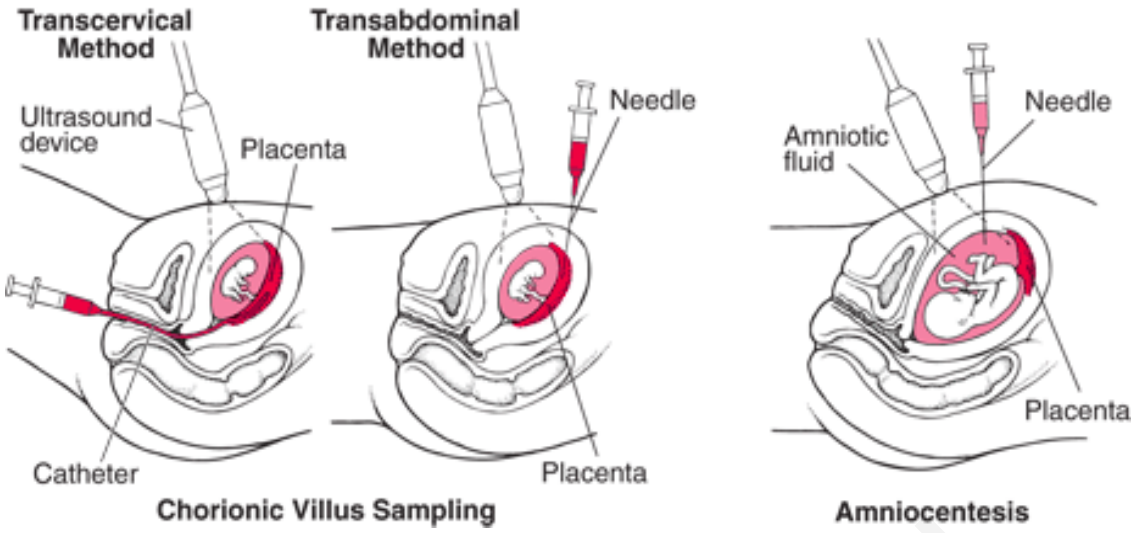


Figure 1-2: Illustration of the current invasive prenatal techniques. (Prenatal Diagnostic Testing)

1.2 Common Chromosomal Abnormalities

Trisomy 21: Also known as Down syndrome, it is a meiotic nondisjunction disorder caused by the full or partial extra chromosome 21. Usually the nondisjunction is coming from the maternal gamete. Rarely, 1-2%, of the Down syndrome cases can be mosaic and 2-3% of the cases may be caused by Roberstonian translocation. The incidence of Trisomy 21 is 1 in 830 live births and is associated with increasing maternal age. The most commonly seen congenital abnormalities include: cystic hygroma (abnormal fluid accumulation around the neck area), nuchal-fold thickness (skin on the back of the neck is thicker than normal), hydrops (abnormal fluid accumulation in the body such as around the heart, the lungs, the abdomen or under the skin), cardiac defects, renal hydronephrosis (part of the kidney has abnormal collection of fluid) and skeletal abnormalities. It is related with intellectual disability, hypotonia during infancy and specific facial characteristics. Affected individuals usually develop medical conditions involving the gastrointestinal system, speech, vision and hearing in some cases leukaemia and Alzheimer disease (Down syndrome, 2012).

Trisomy 18: Also known as Edward syndrome, it is a genetic disorder caused by the presence of an extra chromosome 18. It is the second most common autosomal abnormality with an incidence of 1 in 5000 live births. This chromosomal defect is associated with major congenital anomalies. The most commonly associated abnormalities include: intrauterine growth restriction, cardiac defects, club foot/feet or

rocker bottom feet, and omphalocele. These abnormalities include an unusually shaped head with a wide occipitoparietal and narrow frontal diameter. Most foetuses with Trisomy 18 die before 6 months of infancy but there is a small percentage that will survive beyond infancy. This disorder is seen more often in females than males (Trisomy 18, 2012).

Trisomy 13: Also known as Patau syndrome, it is a chromosomal abnormality caused by an extra chromosome 13 due to nondisjunction of chromosomes during meiosis. The risk of Patau syndrome increases as the maternal age increases. Trisomy 13 has a reported incidence of 1 in 16000 live births. This chromosomal defect is associated with major congenital anomalies. The most common of which include: holoprosencephaly (the two cerebral hemispheres are fused) or other central nervous system abnormalities, abnormal midface development including clefting, and congenital heart defect. Most fetuses with Trisomy 13 die before they reach term and are miscarried. Some foetuses with Trisomy 13 are born alive but die by the age of 1 month or 6 months. Rarely, they may survive to adulthood (Trisomy 13, 2013).

Klinefelter syndrome: Is a condition in which human males have an extra X chromosome and is the most common sex chromosome disorder in males. The condition exists in roughly 1 out of every 500-650 newborn males but many of these may not show symptoms. Fetuses are typically identified during amniocentesis performed for advanced maternal age. Major effects are hypogonadism, reduced fertility, gynecomastia and reduced body hair (Klinefelter, 2013).

Turner syndrome: A chromosomal disorder which involves the present of only one X chromosome in females instead of the usual two sex chromosomes. It affects 1 in 2,500 newborn girls but is more common in miscarriages and stillbirths. Affected individuals have webbed neck, swelling of the hands and feet, kidney and heart defects which can be lethal. The majority of affected girls show normal intelligence. In most cases, Turner syndrome is not inherited but is a random effect (Turner, 2012).

The frequency in percentage of the abovementioned chromosomal abnormalities is shown in figure 1-3.

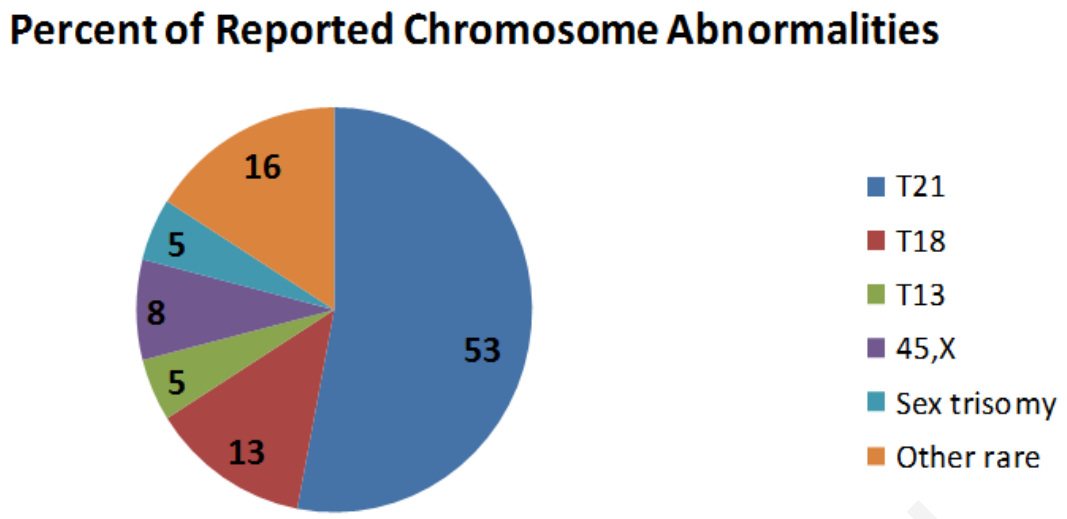
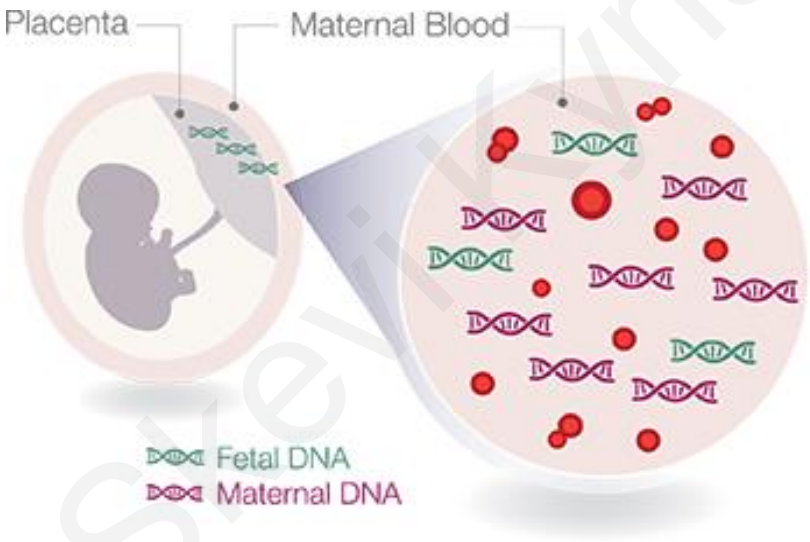


Figure 1-3: Prevalence chromosomal aneuploidies (Wellesley D, et al., 2012).

1.3 Feto-maternal Circulation - Origin of Fetal Nucleic Acids in Maternal Circulation

The placenta is defined as an organ that associates the fetus to the uterine wall allowing nutrient uptake, gas exchange and waste elimination through maternal blood supply. Deoxygenated fetal blood passes to placenta via umbilical arteries which eventually form an arterio-capillary-venous system. This system brings the fetal blood very close to maternal blood but no intermix of fetal and maternal blood occurs forming a placental barrier. Thus, the discovery of fetal DNA in maternal circulation, by Lo et al., 1997, introduced queries on the possible source of this fetal DNA (fig. 1-4). There are reports showing the presence of nucleated fetal red blood cells in maternal circulation (Bianchi et al, 1997), which could be the source of the fetal DNA. In addition, Lo et.al in 1997, reported the presence of fetal DNA in maternal plasma where only free nucleic acids are found. Thus, indicating that fetal nucleated red blood cells are not the source of fetal DNA in maternal circulation or are not the only source. In plasma there is free circulating DNA which associates with circulating nucleosomes, a characteristic of apoptotic by-products. Therefore, the initial hypothesis was that nucleated fetal red blood cells (NRBCs) undergo apoptosis liberating their DNA in the plasma (Sekizaawa A et al, 2000; Van Wijk et al, in 2000). According to Leung TN et.al, in 1998, fetal DNA concentration shows an increase in women that undergo preterm labor, in contrast with the number of fetal NRBCs in maternal circulation that shows no elevation (Hoesli I et.al, 2002). Thus, apoptotic fetal cells are not the major source of fetal DNA found in maternal plasma.

A possible primary source of fetal DNA could be the trophoblasts which develop into a large part of the placenta. Trophoblasts are specialised cells of the placenta that interact with the maternal uterus. They come in direct contact with the maternal circulation, facilitating the exchange of nutrients, wastes and gases between the maternal and fetal systems. Apoptotic trophoblasts were detected in the maternal circulation of normal pregnancies and in women with pre-eclampsia (Ishihara N et.al, 2002). Direct association of apoptotic trophoblasts and concentrations of fetal DNA in maternal plasma showed significant correlation (Ariga H et.al, 2001; Smith and Baker, 1999). Consequently, evidence supports that it is most likely that the majority of fetal DNA in plasma originates from apoptotic trophoblasts. Overall, the majority of free fetal DNA found in plasma of a pregnant woman originates form the apoptosis of trophoblasts and a minor concentration from NRBCs and direct transfer.



<u>Figure 1-4:</u> Cell free fetal DNA in maternal circulation. (About Noninvasive Prenatal Testing)

1.4 Non-Invasive Prenatal Approaches For The Diagnosis Of Aneuploidies

1.4.1 Targeting Fetal DNA

In 1948, Mandel and Metais reported the existence of nucleic acids in plasma of healthy and sick individuals. This study was the start point towards research on circulating cell-free nucleic acids in plasma.

Lo et al., was the first to investigate the cell-free fetal DNA in maternal plasma and serum, in 1997, by targeting a Y-chromosome specific sequence in pregnancies carrying male fetuses. In 1998, the same group (Lo et al., 1998), quantified the concentration of fetal DNA in maternal plasma and serum resulting in high concentrations in maternal plasma, which corresponds to a mean value of 25.4 genome equivalents/mL (mean 3.4%) in early pregnancy and a mean value of 292.2 genome equivalent/mL (mean 6.2%) in late pregnancy. A more recent study reports a fetal mean percentage of 10% in early pregnancy and 20% in late pregnancy using digital PCR and Next generation sequencing (Lun M.F. et al., 2008). Free fetal DNA in maternal plasma is fragmented at its majority at 162bp (Fan et al., 2010) and can be detected by 5^{th} gestational week. The fetal amount increases as gestation proceeds and is readily cleared after delivery (Lo et al., 1997; Wright and Burton, 2009).

1.4.1.1 Epigenetic Approaches

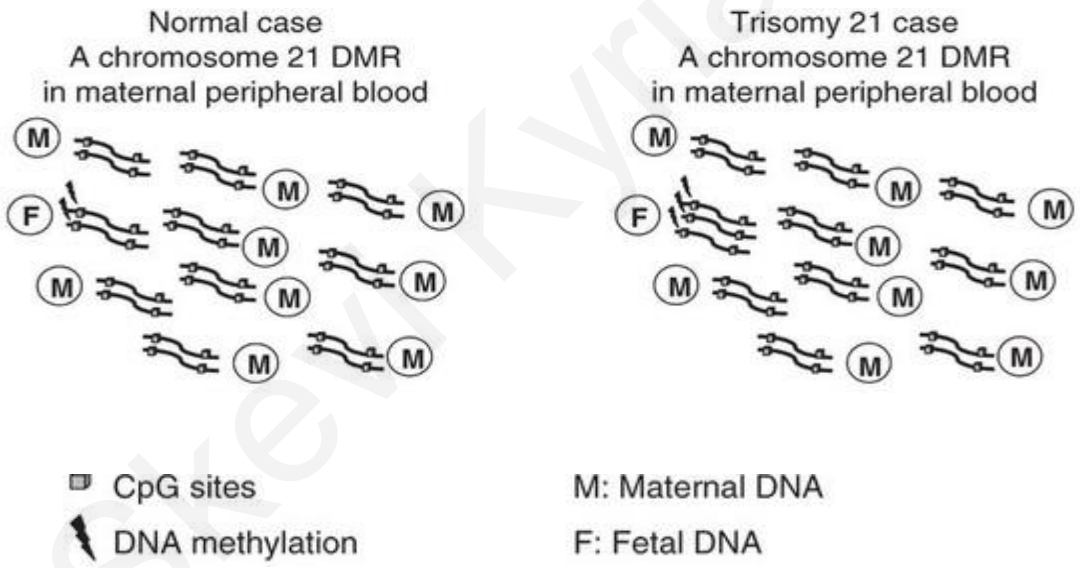
Epigenetic modifications are heritable changes that do not involve any change on the DNA sequence but cause alterations in gene expression. Subsequently, the phenotype can change but not the genotype. These modifications can be part of a physiologic function of a cell or may have damaging effects resulting in cancer. DNA methylation, which is the addition of a methyl group to a cytosine nucleotide, can initiate an epigenetic change. It is capable for alterations in gene expression and the resulting modification is permanent.

The fetus inherits half its DNA from the mother, thus the discrimination of cell free fetal DNA (cffDNA) from maternal DNA in maternal circulation is very challenging. To overcome this challenge, one of the approaches that have been implemented is to target epigenetic differences between the fetus and the mother. Differential DNA methylation profiles between placenta and maternal DNA have first been reported in 2002 by Poon et al. They targeted the human IGF2-H19 locus, on chromosome 11, in which the paternal allele is methylated and the maternal allele is un-methylated. The fetal DNA present in maternal plasma, can be distinguished through the paternal methylated allele which derives from the father and can be used to differentiate the fetal DNA from the maternal DNA. Such regions are known as differentially methylated region (DMR). Bisulphite conversion followed by methylation-specific PCR can successfully detect the fetal specific DMR. The detection of fetal DNA in maternal plasma using DMRs suggested the possible introduction of a prenatal diagnostic tool.

In 2005, Chim et al., reported a DMR on chromosome 18 (SERPINB5) which was found to be hypomethylated in placenta and hypermethylated in maternal blood cells. Using bisulphite treatment, unmethylated cytosine is converted to uracil leaving methylated cytosine unchanged. Thus, the same team saw the possibility of trisomy 18 detection and tested the sensitivity (100%) and specificity (false positive rate 9.7%) of their method a year after (Tong et al., 2006). The next step was to focus on trisomy 21 which is the most frequent compared to other aneuploidies. They tried to develop a similar method for the non-invasive detection of trisomy 21 (Chim et al., 2008). This study resulted in the detection of multiple DMRs on chromosome 21 which was promising at the beginning, but later they realised their method, bisulphite conversion, was their major drawback since it destroys a large proportion of DNA introducing variability (Chim et al., 2008).

In 2009, Papageorgiou et al., proposed a new approach, an antibody-based approach (Methylated DNA immunoprecipitation, MeDIP) for targeting the methylated DNA coupled with quantitative polymerase chain reaction (qPCR). New methylation profiles of chromosome 21, 18, 13, X and Y were identified using high resolution tiling oligonucleotide array analysis succeeding in the identification of new DMRs. In 2010, the same team proceeded to the evaluation of the method using multiple DMRs on chromosome 21 together with a diagnostic formula and provided correct diagnosis of 40 trisomy 21 and 40 normal cases (Papageorgiou et al., 2010). A further larger validation study followed in 2012, where 175 samples were tested showing 100% sensitivity and 99.2% specificity (Tsaliki et al., 2012).

Although all the methods mentioned above are gender and polymorphic independent, their clinical value must be proven prior proceeding to the implementation of a non-invasive diagnostic test.



<u>Figure 1-5:</u> Fetal differentially methylated regions among the huge maternal background. (Papageorgiou et al., 2010)

1.4.1.2 Single Nucleotide Polymorphism - based Approaches

The single nucleotide polymorphisms (SNP)-based approach in the field of non-invasive prenatal diagnosis were first introduced by Dhallan et al., in 2007. Paternal, maternal cells and maternal plasma (maternal and fetal) genotypes were evaluated by Sanger sequencing. To estimate the chromosome dosage, intensity of maternal plasma bands were compared to maternal only and paternal bands. The

sensitivity and specificity of this approach were 66.7% and 98.2% respectively after running 60 samples. In 2010, Ghanta et al., used tandem SNP sequences on chromosome 21 and analysed them on cycling temperature capillary electrophoresis. This method is polymorphism dependent, thus to be informative the mother must be heterozygous to permit fetal chromosomal dosage by calculating haplotype ratio. The sensitivity and specificity of the method is 100% but is limited to maternal heterozygosity and the need of high DNA yields. Almost one third of the samples tested were excluded due to low DNA yield or lack of maternal heterozygosity (fig. 1-6).

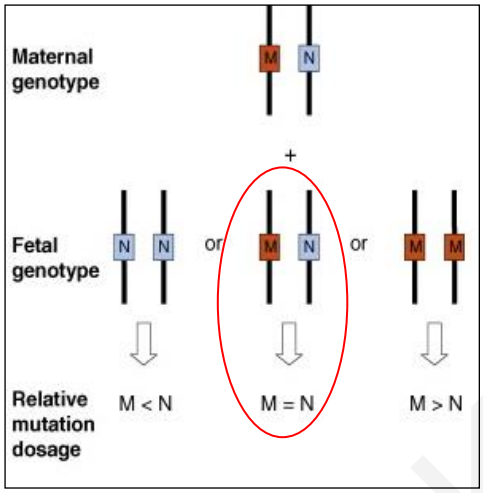


Figure 1-6: Maternal heterozygosity and possible fetal genotypes. The second fetal genotype (red circle) is identical to maternal genotype therefore is not informative and will be excluded from any polymorphism dependent test. (Chiu W.K. et al., 2009)

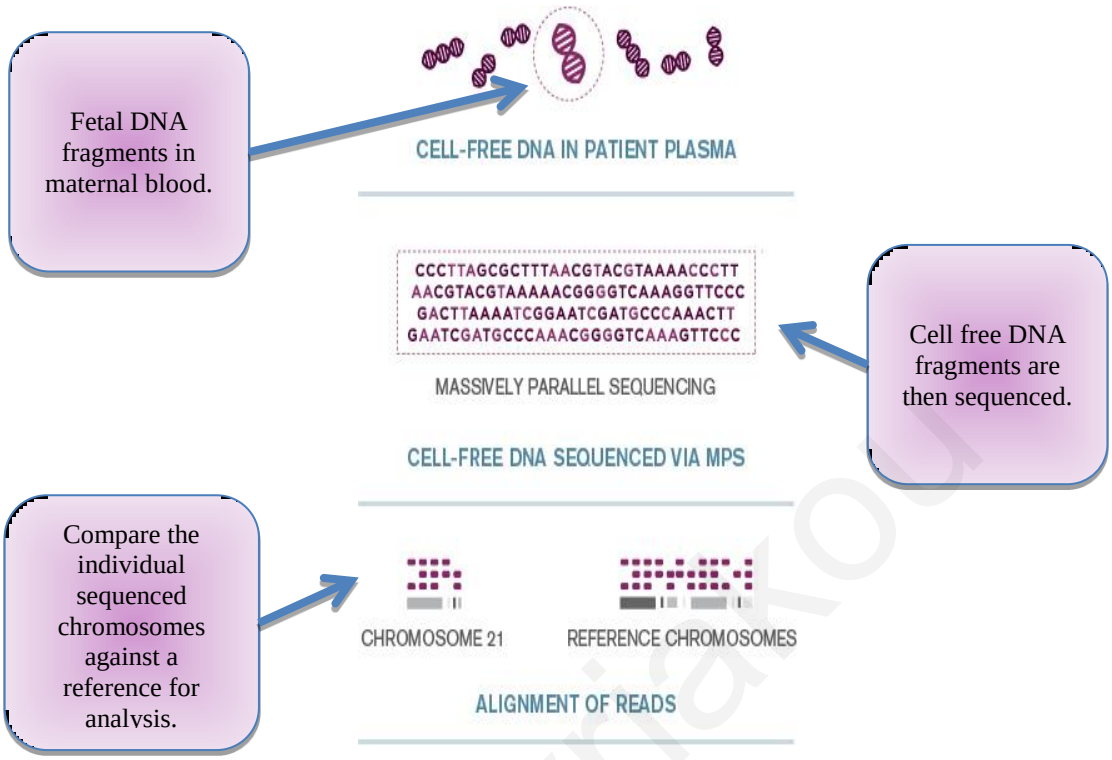
1.4.1.3 Next Generation Sequencing Approaches

With the evolution of Next Generation Sequencing in the last few years, scientists in the field of prenatal diagnosis are convinced they have found a powerful technology that will lead them to a robust non-invasive prenatal diagnostic test. Indeed in 2008, two studies reported the successful non-invasive prenatal diagnosis of fetal aneuploidy by high-throughput shotgun sequencing of cffDNA in maternal plasma (Fan et al., 2008; Chiu et al., 2008). By sequencing maternal plasma, counts represent both maternal and fetal genome. Thus, first counts are mapped to their corresponding chromosome which is known from the human genome map. Then mapped counts from the chromosome of interest are compared to a known euploid case within the same run. The counts from the chromosome of interest in the known euploid case are

normalized with the other disomic chromosomes within the same run or are compared against counts seen from known euploid cases. The result is calculated in a z-score value which represents the probability of a pregnant woman to be at high risk. This approach depends on the percentage of fetal DNA found in maternal plasma since it measures counts from both mother and fetus. The differences between aneuploidies and euploidies are very small in term of counts, a range of ~0.04 to ~ 0.2 depending on the percentage of fetal DNA. Thus, cases with less than 4% are excluded from the beginning (Fan et al., 2010).The major limitations of this approach are the high cost and the need for a bio-informatician for the interpretation of the results.

Some groups, in order to reduce the cost, used targeted massively parallel sequencing where they target the chromosomes of interest (chromosome 21, 18 and 13) (Sparks et al., 2012). The proof of principle is the same, counts are measured, mapped and compared to known euploid cases. They combine the results from sequencing with maternal age and provide a risk factor, thus this is a probability test.

In 2012, some groups turn their interest to single nucleotide polymorphisms (SNP) coupled with sequencing. Zimmermann et al., amplified 11 000 SNPs on chromosome 21, 18, 13, X and Y of maternal plasma in a single multiplex PCR reaction and then sequenced. Using an algorithm, they managed to correctly classify 145 samples with an accuracy of 99.92%. In 2013, more groups focus on SNP-sequencing approach. Nicolaides et al., reported a SNP-based method where 19 488 SNPs on chromosomes 21, 18, 13, X and Y are amplified in a single multiplex reaction and sequenced. The analysis of this method uses maternal genotype and recombination frequencies to construct an in silico panel of possible fetal genotypes. Then compares the counts from the maternal plasma with the possible fetal genotypes and calculates a relative likehood for each hypothesis. With this approach, this group managed to classify correctly all the samples tested but the sample size was very small therefore needs further validation in a larger population. A basic workflow of next-generation sequencing methodology is shown in figure 1-7).



<u>Figure 1-7:</u> Basic work flow of Next Generation Sequencing approaches.

1.4.2 Targeting Free Fetal RNA

Cell free RNA was initially detected in plasma and serum of cancer patients by Lo et al., in 1999. A major concern in using RNA as a target was the existence of RNases in the circulation where they degrade RNA and lead to instability. This issue was answered in 2000, by Halicka HD et al, who demonstrated that free fetal RNA is protected from RNases within apoptotic (fig. 1-8) vesicles and is very stable. The initial studies performed on circulating RNA markers were particularly promising since it increased the rate of tumor detection compared to the detection rate obtained when using DNA markers (Silva J et al., 1999; Chen Q et al., 2000). This encouraged researchers to work with RNA towards NIPD. In 2000 Poon et al. reported the presence of fetal specific mRNA (ZFY), Y-chromosome encoded transcript. This study showed the detection rates in early and late pregnancies which are 22% and 63% respectively. Placenta expressed genes (hPL and hCG) are detected in maternal plasma with a detection rate of 100% in all trimester, confirming the stability of RNA, and rapid clearance after delivery (Ng et al., 2003). These findings provide an additional source of potential fetal diagnostic biomarkers for non-invasive prenatal aneuploidy detection. Still the technical difficulties in isolation of RNA and the variation in the expression level is a challenge.

Studies for the identification of placental mRNA markers detectable in maternal plasma, aiming for a non-invasive prenatal test, were reported in the last decade. Starting with the work done by Oudejans et al., in 2003, where a specific mRNA named C21 orf 105, encoded by chromosome 21 genes, was detectable in maternal plasma. Although, in theory this transcript could lead to the discrimination of trisomy 21 from euploid cases, the high variability among individuals made it impossible. A study by Tsui et al., reported in 2004, using oligonucleotide microarrays, gene expression profiles of placental tissues (1^{st} and 3^{rd} trimester) and maternal whole blood were screened targeting differentially expressed genes between the tissues. Placental specific transcripts in first and third trimester showing increased expression level compared to whole blood were identified constructing a panel of multiple transcripts. The same group, developed a method to determine the dosage of chromosome 21 using a 21 –encoded transcript, PLAC4 (placenta-specific mRNA), which was detectable in maternal plasma. This was an RNA-SNP ratio approach using mass spectrometry. In case of trisomy 21, the RNA-SNP allelic ratio should be 1:2 or 2:1.

This strategy has a diagnostic sensitivity of 90% and specificity 96.5% (Lo et al., 2007). The same approach was tested in trisomy 18 cases using SERPINB2 mRNA and the results were similar and promising. A major drawback of this approach is the limitation to the population with a heterozygosity of the SNP used (a polymorphism depended approach). In the same year, an effort was made to overcome this limitation by coupling RNA-SNP approach with total PLAC4 mRNA concentration (Tsui et al., 2010). In cases of heterozygosity, they applied the SNP-allelic ratio and in cases of homozygosity they measure the total PLAC4 cDNA concentration by real time qPCR and digital PCR. Independent of the method used (qPCR or digital PCR), they obtained low sensitivity and specificity (91.7%, 81.7% for qPCR and 83.3%, 83.5% for digital PCR) thus still there is a need for optimization in order to be performed as a diagnostic test.

MicroRNAs (miRNAs) are short single stranded nucleotides, 20-25 base pairs (Lee and Ambros, 2001; Lagos-Quintana et al., 2001), that regulate gene expression by binding to the 3' untranslated region of mRNAs or to the gene promoter regions (Portnoy, V. et al., 2011). Currently there are more than 2000 known miRNAs according to the miRBase database and can be used as tissue specific biomarkers. The discovery of placenta-specific miRNAs biomarkers can be potentially used for the detection of fetal aneuploidies since miRNAs are highly conserved (Kim, V.N, 2005). Chim et al, in 2008, studied 157 known placental miRNAs in maternal plasma using real-time quantitative RT-PCR and compared them with termed placental tissue and maternal cells. Few, 17, were found to have higher expression in placenta than maternal cells and only 4 miRNAs were detectable in maternal plasma. No more studies were published on miRNA biomarkers discovery and remain unknown whether miRNAs can be used in non-invasive prenatal aneuploidy detection.

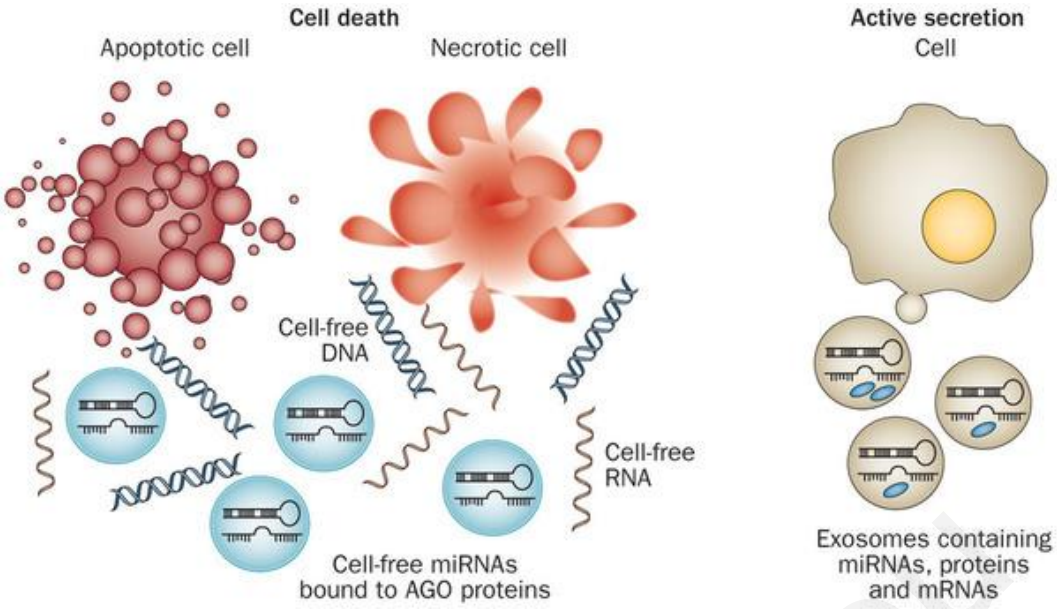


Figure 1-8: Free Fetal RNA origin and its circulation in maternal plasma. (Schwarzenbach H, et al., 2014)

1.5 Methylation and Gene Expression during Embryonic Development

The embryonic development of mammals is controlled by a series of steps which are organized by a wide system in order to accomplish cellular differentiation. These steps are organized by the activation and repression of progressive genes which are regulated by DNA methylation. DNA methylation is one of the main epigenetic modifications in mammals targeting the CpG dinucleotides of the mammalian genome (Robertson and Wolffe, 2000). CpG dinucleotides are mostly found close to promoter regions, usually upstream, within gene introns and exons (Bird A, 2002). These gene-related targets are usually demethylated with some exceptions such as imprinted genes and X-inactivation genes. Around 40% of the mammalian genome is made of retrotransposons which can induce mutations when inserted near or within a gene (Lander et al., 2001) thus one of the main functions of DNA methylation is the regulation of these regions. Fetal growth and development is based on the expression or repression of critical regulatory genes which have been shown to influence postnatal biological processes such as suckling behaviour and even cognition (Plagge et al., 2004). Epigenetic reprogramming in mammalian development is separated into two key stages, one during preimplantation development and one during gametogenesis (Reik and Walter, 2001). The methylation initiates on E 7.5 and ends on E 12.5 (fig. 1-9) and is related with the primordial germ cell development

(Ginsburg et al., 1990). By E 12.5, almost all sequences are de-methylated. In males, methylation re-occurs on E 15.5 till birth (Davis et al., 1999).

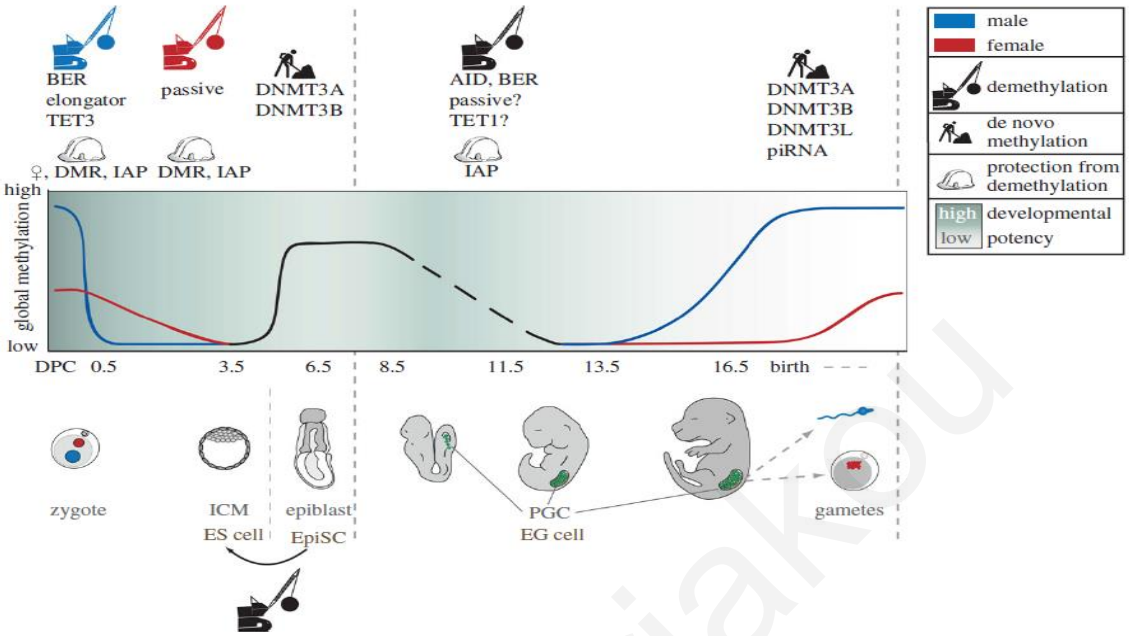


Figure 1-9: Epigenetic regulation during embryonic development. (Seisenberger S, et al., 2013)

Epigenetic errors during development can result in gene misregulation and thus disease and syndromes without any change in the genetic code. Mammalian embryonic development is a complex process where major developmental changes are taking place due to epigenetic alterations which are linked to changes in gene expression during pregnancy. DNA methylation is well known for its impact on the X inactivation process in females. One of the two copies of X chromosome is silenced by DNA methylation. This prevents females having twice as many X chromosome genes than males which only carry one X chromosome. This is a random process occurring in the placenta during pregnancy (TM Nafee, et al., 2007).

The nanog and Oct4 genes have critical functions during embryonic development. They are responsible for the maintenance and establishment of pluripotency and are regulated through DNA methylation. They are negatively regulated through methylation of their promoters in normal pregnancy (Farthing, et al., 2008). The nanog promoter is found non-methylated in oocytes and methylated in sperm. Demethylation of the paternal allele occurs after fertilization. The Oct4 promoter is partially methylated in sperm and non- methylated in oocytes. Controlling these key pluripotency genes is crucial for normal embryo development (Farthing, et al., 2008).

The mechanism that generates patterns of different cell types during embryonic

development is not only based on DNA methylation but also on the regulatory transcription factors that have the ability to control gene expression. For example, Sp1 is a well-known transcription factor that its expression changes during embryonic development. It interacts directly with TATA-box protein factors, promoters and regulatory elements regulating many genes (Marin, et al., 1997). Transcription factors are responsible for cell differentiation. Differentiation of hematopoietic cells requires regulation through transcription factors such as GATA-1 which has an instructive role in granulocyte and monocyte differentiation (Nakajima H., 2011).

Genes are silenced or activated in order to promote cell differentiation and cell growth. Gene regulation is therefore a crucial mechanism for the normal development of the embryo.

2. Specific Aims, Importance and Innovation

2.1 SPECIFIC AIMS

The discovery of fetal nucleic acids in maternal plasma stretches the potential for non-invasive prenatal diagnosis. Investigations conducted to date lead to the detection of differences between the mother and the fetus called fetal specific biomarkers. This study first focused on the usability of known fetal specific biomarkers, methylation based, and then on the discovery of new fetal specific biomarkers, expression based.

Methylation differences between the fetal and maternal DNA have been reported in the past few years. Our team has successfully identified several DMRs on chromosomes 21, 18, 13, X and Y using oligonucleotide microarrays and introduced a MeDIP-qPCR methodology for the identification of trisomy 21 (Papageorgiou et al., 2010, Tsaliki et al., 2012). The main limitation in non-invasive prenatal diagnosis is the very low free fetal amount in maternal circulation. The huge maternal background may introduce major drawbacks in the NIPD tests decreasing the sensitivity and specificity.

The current study is separated into three main stages. The first stage investigated the variability of fetal percentage among individuals and whether it influenced the correct classification of trisomy 21 using the existing MeDIP-qPCR methodology

The second stage involved the development of a robust method based on the epigenetic differences between fetus and the mother which further enriched the fetal DNA and distinguished trisomy 21 from normal cases.

The third stage of the study aimed to expand the panel of fetal specific biomarkers based on expression differences among trisomy 21 and normal fetuses from their mothers. Association studies of identified DEGs transcription level with their methylation patterns were investigated. Differentially expressed genes were categorized according to their function and associated with the phenotypic characteristics of Down syndrome.

2.2 *IMPORTANCE AND INNOVATION*

A simple method was proposed, from our group, for the enrichment of fetal specific differentially methylated regions, where the maternal whole blood is immunoprecipitated (MeDIP) and then undergoes real-time qPCR. With the introduction of a robust diagnostic formula, which considers the diagnostic power of each DMR, the MeDIP-qPCR methodology classifies correctly the normal from the trisomy 21 pregnancies. Compare to other methodologies MeDIP-qPCR is cheaper, faster and simpler. The main difference of the MeDIP-qPCR methodology with the other enrichment methods is that it uses whole blood instead of maternal plasma and that the variability of fetal percentage among individuals is not considered in the normalization steps. The first part of this study investigates the correlation of fetal percentage with the diagnostic value of the existing methodology in order to assess the need of an extra normalization step. In order to expand our diagnostic test to other aneuploidies on chromosomes 18, 13, X and Y this study suggests the implementation of this methodology in maternal plasma where the fetal amount is more compared to whole blood.

Part of this study aims the development of MeDIP-qPCR methodology in maternal plasma aiming a high sensitivity and specificity method to be used in the discrimination of aneuploidies from euploidies. The major drawback on using plasma as a starting material in MeDIP is the very low DNA concentration. There is no commercial kit that applies MeDIP in such low concentrations. Thus the implementation of MeDIP methodology in maternal plasma will not only aid in the development of a diagnostic test that potentially will cover all aneuploidies but also the development of a MeDIP assay from limited quantities of DNA. This could be used by other groups in other fields that manipulate very low DNA quantities.

The identification of new fetal biomarkers based on differences between the normal CVS, abnormal CVS (trisomy 21) and the maternal cells expression levels will expand the panel of fetal biomarkers. There are several studies investigating the differentially expressed genes on fetal trisomy 21 tissues and only one study on expression differences between normal CVS and maternal whole blood. No reports are found on expression differences between normal and trisomy 21 CVS tissues and their mothers. The majority of these studies focused on biomarkers located on chromosome 21 but the phenotypic characteristics of this aneuploidy suggest that can

be related to genes located on other chromosomes as well. Therefore this study will further expanded the panel of fetal specific biomarkers by adding trisomy 21 CVS specific biomarkers that cover the whole genome. Identifying these markers may lead to the development of a new non-invasive prenatal diagnostic test which will target expression differences between normal and trisomy cases.

To further understand how epigenetics influence the expression levels of genes, this work associated the methylation status of DEGs with their expression characteristics. This will expand our understanding on how epigenetics is related to pathology through expression levels of disease-associated genes.

3. Methods

The work is separated into three stages, each one with the following distinct methodology and experimental procedure:

A. Quantification of ffDNA in maternal plasma and correlation of total and fetal amount with the diagnostic D-value.

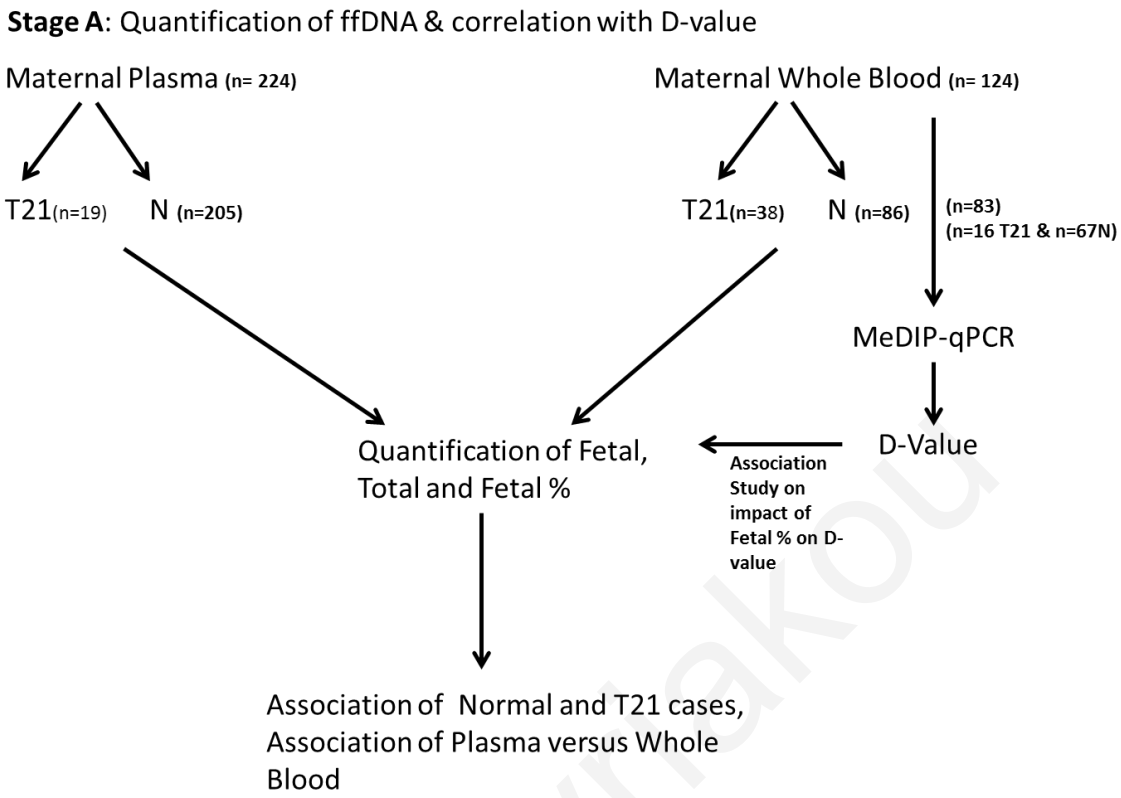
1. Sample collection and DNA extraction

2. Absolute quantification of total and fetal DNA found in maternal plasma and whole blood

3. Correlation of total and fetal amount between normal and T21 cases

4. Correlation of total amount, fetal amount and 'fetal fraction' found in maternal plasma with D-value obtained from MeDIP-qPCR of T21 methodology in maternal whole blood (Tsaliki et al.,2012)

Schematic diagram of Stage A



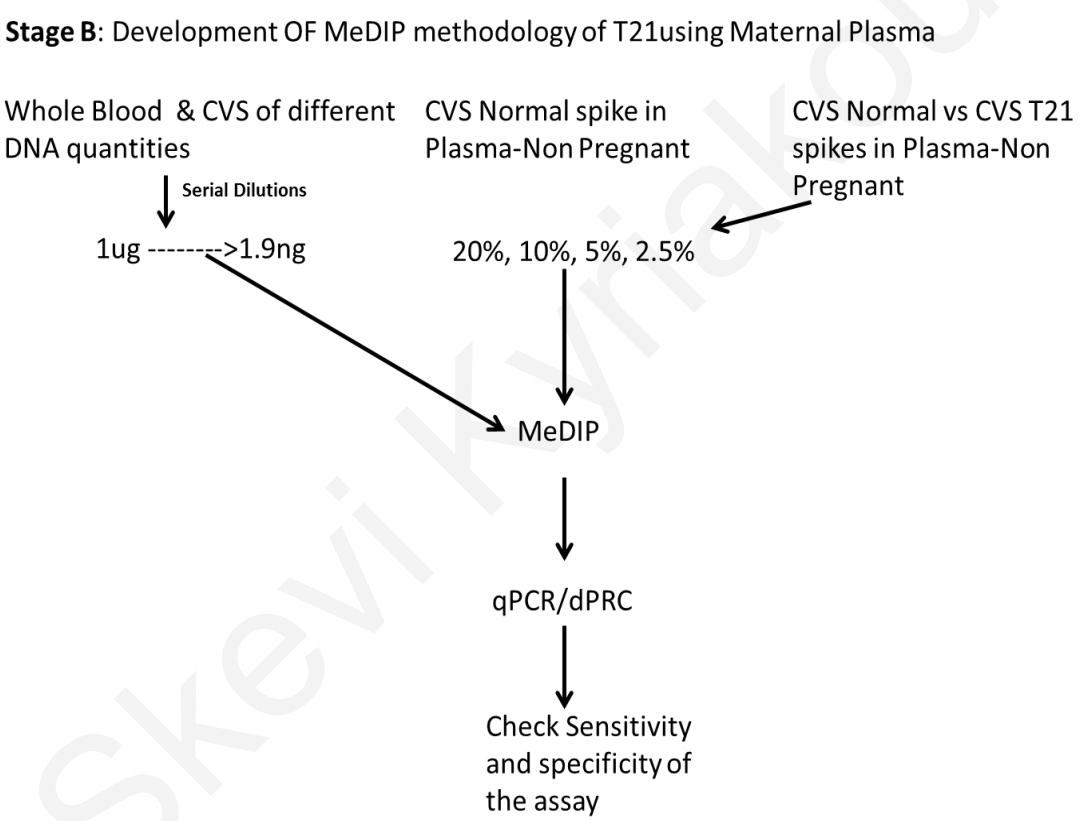
B. Development of MeDIP-qPCR of T21 using maternal plasma

1. Sample collection and DNA extraction

2. Methylated DNA Immunoprecipitation

3. Classification of T21 from normal spike in samples using MeDIP methodology

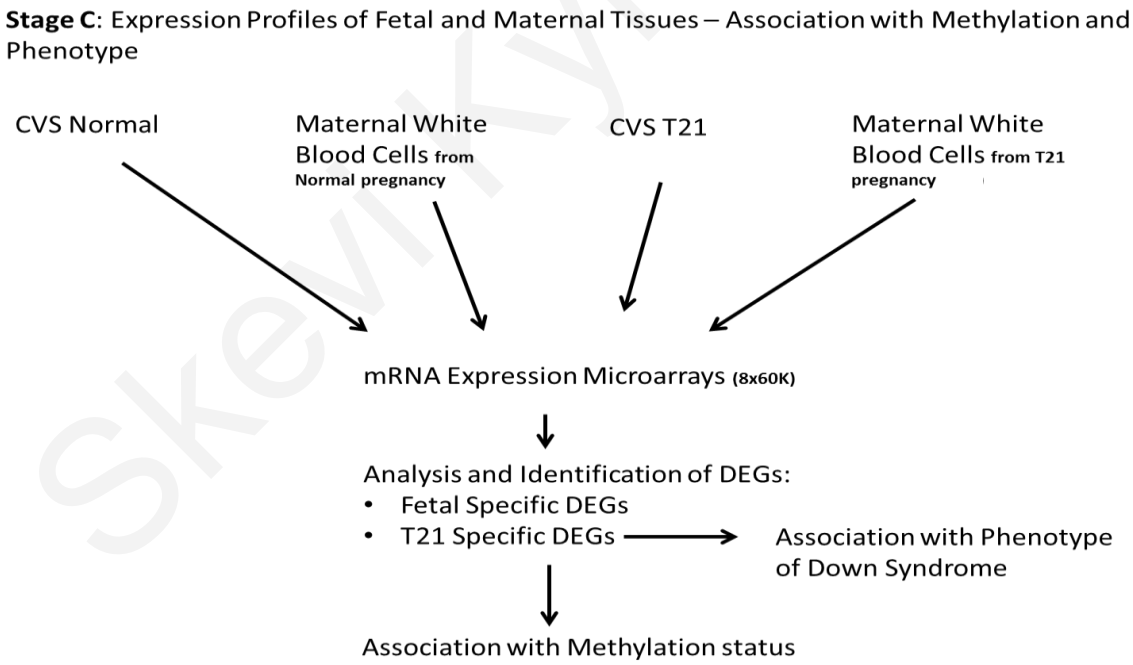
Schematic diagram of Stage B



C. Expression Profiles of Fetal and Maternal tissues – Association with Methylation profiles and Phenotype

1. Sample collection and RNA extraction
2. mRNA expression microarrays
3. Identification of Differentially Expressed Genes
4. Correlation of DEGs identified with their methylation status
5. Categorization of DEGs according to their involvement in diseases related to Down Syndrome
6. Identify common phenotypes of DEGs selected

Schematic diagram of Stage C



3.1 Sample Selection and Nucleic Acids Extraction (Common for all stages)

For the purposes of the first stage of the study maternal plasma and maternal whole blood were collected from 224 and 124 individuals respectively. Nineteen out of 224 maternal plasma samples were carrying T21 foetuses (14 male and 5 female foetuses) and the remaining normal foetuses (141 male and 64 female foetuses). Thirty eight out of 124 maternal whole blood samples are carrying T21 foetuses (21 male and 17 female foetuses) and the remaining normal foetuses (43 male and 43 female foetuses). For the second stage, five whole blood non pregnant samples, five normal 1^{st} trimester placentas, pool of plasma non pregnant, ten normal 1^{st} trimester and ten T21 1^{st} trimester were collected. For the third stage of the study four 1^{st} trimester normal CVS and two 1^{st} trimester T21 CVS with their matching maternal white blood cells were collected.

The peripheral blood samples were obtained from pregnant and non-pregnant women between 20-40 years of age. Peripheral blood samples, 20ml, were collected from Cypriot volunteers and temporary stored at 4^{o}C for maximum 4 hours. Then, 4ml of peripheral blood was aliquoted in 1.5ml Eppendorf tubes. The remaining blood underwent double centrifugation for plasma and buffy coat collection. Upon completion of this process samples were stored at - 80^{o}C until needed for DNA extraction. For the first two stages, first trimester chorionic villi samples (CVS) were collected from the department of Cytogenetics and Genomics, Cyprus Institute of Neurology and Genetics (Nicosia, Cyprus). First trimester CVS collected from Cyprus were cleaned, separated from maternal tissues, embedded in PBS medium and stored at - 80^{o}C until needed for DNA extraction. Chorionic Villi and maternal white blood cells for the third stage of the study were collected from Mother and Child Hospital University in Spain. Chorionic villi were cleaned, separated from the maternal tissue and immediately fully embedded in trizol. In case of white blood cells collection, transfer of blood in 1.5ml Eppendorf tubes and immediate addition of erythrocyte lysis buffer was required. Incubation and centrifugation steps follow to remove all red blood cells. When these steps were completed, addition of trizol followed and all samples were immediately stored at - 80^{o}C until needed for RNA extraction.

All participants signed a consent form and informed in detail of the specific aims and confidentiality of the study. The consent form included in detail all the information regarding the study and approved by bioethics committee.

DNA from whole blood was extracted via spin column method using the Qiagen Blood Midi kit (QIAGEN, Hilden, Germany), whereas female and maternal plasma using the QIAamp Circulating Nucleic Acid kit (QIAGEN, Hilden, Germany), for the 2nd stage of the study, or the iPrep™ PureLink® Virus Kit (Invitrogen), for the 1st stage of the study, according to the manufacturer's directions. DNA from chorionic villi from 1st trimester, this applies for the first two stages only, was extracted using the QIAamp DNA mini kit according to the manufacturer's directions (QIAGEN, Hilden, Germany). For the third stage of the project RNA was extracted from chorionic villi and white blood cells using the miRNeasy mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Karyotype or QF PCR was performed for chromosomal analysis to confirm the status of CVS and placentas and to avoid any maternal contamination. The quantity and quality of the DNA was determined using the Nanodrop (Thermo Scientific) or Qupit Fluorometer and stored in -20°C freezers. All RNA samples were measured with Qubit Fluorometer for their quantity, with Nanodrop (Thermo Scientific) for their quality, and with Tapestaion 2200 (Agilent) for their degree of degradation. Subsequently, all RNA samples were treated with DNase I to avoid DNA contamination and stored at -80°C. A DNA and RNA bank established consisting of all necessary information pertaining to the subjects.

3.2 Absolute Quantification

Real-time quantitative PCR was performed using Applied Biosystems 7900HT real time system with the baseline set automatically. Quantification of cffDNA was achieved using the DYS14 gene and the β-globin was used as a housekeeping gene. In addition to the two amplification primers, a dual-labelled fluorogenic TaqMan probe was used for each gene. DYS14 primers and probe were as follows: GGGCCAATGTTGTATCCTTCTC (forward) (Zimmermann et al., 2005), GCCCATCGGTCACTTACACTTC (reverse) (Zimmermann et al., 2005), TCTAGTGGAGAGGTGCTC (TaqMan probe) (Zimmermann et al., 2005). The β-globin primers and probe were as follows: GTGCACCTGACTCCTGAGGAGA

(forward)(Sedrak et al.), CCTTGATACCAACCTGCCCAG (reverse) (Sedrak et al.) and AAGGTGAACGTGGATGAAGTTGGTGG (TaqMan probe) (Sedrak et al.). Cyclic conditions used were 2 minutes incubation at 50 C°, 10 minutes denaturation 95 C°, 45 cycles of 95 C° for 15 seconds and 60 C° for 1 minute.

For absolute quantification, standard curves were generated using a human male genomic DNA (Promega) with five serial 10-fold dilutions. Using absolute quantification, the quantity of DYS14 and β-globin was measured and subsequently the concentration in Genome Equivalents/PCR reaction (GE/PCR), Genome Equivalents/mL (GE/mL) was obtained. By comparing the concentrations of the two genes the percentage of cffDNA for each sample was estimated. In every experiment, positive and negative controls were used, namely genomic DNA from a male (positive) and a female (negative) including a female pregnancy (negative) and a male pregnancy (positive).

3.3 Methylated DNA Immunoprecipitation Assay (MeDIP)

(second stage)

Methylated DNA Immunoprecipitation (MeDIP) technique aims the enrichment and therefore the increase of methylated DNA sequences in your testing sample. The technology relies on the isolation of methylated DNA fragments using an antibody against 5-methylcytosine (5mC). For the implementation of the MeDIP approach, the extracted DNA was randomly fragmented using sonication. Sonication process was preferable due to its random shearing of DNA, its fast and simple procedure. The DNA fragments must be in the range of 300 to 1000 base pairs in length. This process was important in obtaining and improving the efficiency of the subsequent immunoprecipitation steps. The antibody's efficiency also depended on the number of 5mC. The presence of more 5mC led to a more efficient enrichment through a better binding of the antibody. Gel electrophoresis (2%) performed to confirm the size fragments of the DNA when applicable. At this stage, a small volume of DNA was removed, called input DNA. The so called "input DNA" represented the genomic DNA of the sample under investigation after being fragmented and was used as reference material during the analysis pipeline. Next, the remaining DNA was denatured at 100˚C to produce single–stranded DNA and was incubated with monoclonal 5mC antibodies. The antibody-DNA was then exposed to magnetic beads

conjugated to anti-mouse-IgG. These magnetic beads were used to bind to anti-5mC antibodies. The unbound DNA was washed away and the DNA was released via pH shift from the complex DNA-antibody-beads. After DNA released, DNA of interest was ready for further analysis. The collected DNA was cleaned up using silica beads and stored at -20˚C if not used immediately.

3.4 Quantification of Immunoprecipitated product (2nd stage)

Real time qPCR was used to evaluate the performance of immunoprecipitation method. SYBR Green dye was used for the detection of the product. Standards curves were generated using a genomic DNA and an absolute measure of the product was obtained in Ct value. Positive and negative control markers were used together with four DMRs located on chromosome 21. The positive control marker used is hypermethylated whereas negative control is hypomethylated in both the fetus and the mother.

Digital PCR was introduced in this study to measure the immunoprecipitated product with higher sensitivity and specificity than real time qPCR. Digital PCR is a new approach that quantifies nucleic acids using molecular counting and is an alternate method to real time qPCR (Whale A.S, et al., 2012). A single molecule can be amplified a million fold or more. TaqMan dye-labelled probes were used in this study to detect the targets. The targets were four DMRs on chromosome 21 and the controls. An absolute count of the target molecule was generated, without the need of standards, in copies per microliter.

3.5 Correlation Study - Statistical Analysis (first stage)

Normal and trisomy 21 samples were compared in terms of the DYS14 and β-globin quantities and the 'fetal fraction' (fetal DNA/total DNA X 100) using the Mann-Whitney U test (Mann and Whitney, 1947) to determine whether there was significant evidence that there is an increase of these quantities in trisomy 21 samples.

The enrichment ratios for each Differentially Methylated Region (DMR) and the diagnostic D-value were calculated as previously described (Papageorgiou et al., 2010; Tsaliki et al., 2012) The enrichment ratios correspond to the amount of DNA that was enriched via the MeDIP process in comparison to known euploid samples used as controls and was calculated for each DMR as shown below.

Enrichment Ratio = Normalized Ct value of a Normal case or Trisomy 21 /Median of normalized Ct of normal controls

The diagnostic D-value combined these enrichment ratios for all the DMRs to give the diagnosis of trisomy 21 as previously described (Papageorgiou et al., 2010).

$$D = -6{,}331 + 0{,}959\ X_{EP4} + 1{,}188\ X_{EP5} + 0{,}424\ X_{EP6} + 0{,}621\ X_{EP7} + 0{,}028\ X_{EP8} + 0{,}387\ X_{EP10} - 0{,}683\ X_{EP11} + 0{,}897\ X_{EP12}$$

where XEPn = enrichment ratio Sample, EPn = DMR, and D is the diagnostic value.

The coefficient in front of each enrichment value shows the diagnostic power of each DMR as defined by Papageorgiou et al in 2010. A D-value greater than 0.798 classifies a sample as trisomy 21 and as euploid otherwise.

The Pearson product-moment correlation coefficient (Pearson, 1896) was used to test whether there was an association between the evidence of trisomy 21 (enrichment ratios and D-value) and the amount of cffDNA, total DNA and fetal fraction present in the sample. The one-sided p-value cut-off of 0.05, derived from a t-distribution with (number of samples - 2) degrees of freedom, was used to decide on whether there was significant evidence of the presence of an association between these quantities.

3.6 Expression Profile using Microarrays (3rd stage only)

Gene expression microarray studies allow the study of genes and their associated functions, enabling the determination of transcript levels for every known gene in the genome. This is achieved by high quality probes which are able to characterize high and low abundance transcripts using only a few nano grams of RNA. In this study, expression microarrays analysis of mRNA, which includes protein coding and long non coding genes, applied using SurePrint G3 Human Gene Expression 8x60K v2 Microarray kit (Agilent, UK). The number of samples for this part of the project is 12, out of which 4 were normal CVS, 2 T21 CVS with their matching maternal white blood cells. A custom design platform which covers 50 599 biological features designed based on Refuses build 50, Ensemble Release 52, Uni Gene Build 216, Genbank, Broad Institute Human ncRNA catalog and Broad Institute TUCP transcripts catalog. The array platforms included probes (Agilent Technologies) with a

median length 60 base pairs. To achieve the construction of the transcriptome of known genes, 1 slide per sample was needed. Samples were shipped to Oxford Gene Technologies (UK) to perform the mRNA assays. Total RNA was reverse transcribed to cDNA and then in vitro transcribed to cRNA and label with single dye. The labelled cRNA complex was then fragmented and hybridized on the array slide. The slides were scanned with Microarray Scanner (Agilent Technologies) and raw data was analysed using the GeneSpring software.

3.7 Identification of Differentially Expressed Genes

In order to identify differentially expressed genes between the trisomy and normal populations, GeneSpring software was used which first normalizes the raw data using the average of the probe replicates. Next, a fold change was calculated as the ratio of one sample compared to the other. The selected fold change in this study was 2. This is the default fold change of GeneSpring Agilent software which indicates significant difference between two samples. To identify differentially expressed genes that can be used later as biomarkers for the classification of T21 cases using maternal plasma, two distinct forms of analysis were applied. First analysis (Analysis 1) aimed to identify DEGs between trisomy 21 CVSs and normal CVSs with a fold change of 2. The second analysis (Analysis 2) identified DEGs between mothers carrying T21 fetuses and mothers carrying normal foetuses with a fold change of 2. The DEGs from the first analysis underwent further filtering aiming the identification of DEGs that are differential expressed not only against normal CVS but also against the maternal tissues. This led to the construction of two groups of DEGs. The first group of DEGS were T21 CVS against normal CVS and the mothers (Analysis 1A), and the second group were the remaining DEGs (Analysis 1B).

3.8 Association of Differentially Expressed Genes with Methylation patterns

Whole methylome of CVS normal and T21 together with maternal cells were scanned by others in the lab at high resolution, using next generation sequencing, where for the purposes of this study underwent further investigation aiming their association with the differentially expressed genes, protein coding and long non coding genes, obtained

from this study. All DEGs found within promoter regions, close to promoter regions, transcription start sites, regulatory elements, first exon, and repetitive regions were investigated. For this to be achieved, Eukaryotic promoter database was used to ensure the location of the promoters in the DMRs and UCSC browser for repetitive regions and other transcription regulatory elements. Subsequently, these regions were cross reference with the expression data from microarrays in order to study their level of transcription.

3.9 Correlation of Differentially Expressed Genes with Down syndrome Phenotype

All differentially expressed genes were categorized in groups according to the phenotypes linked. DEGs where their known biological function is linked to phenotypic characteristics of Down syndrome were reported and emphasized. Cognitively, a large number of Down syndrome children shows intellectual disability and are linked to other developmental delays such as thinking and learning. Overall Down syndrome phenotype involves cognitive, social, linguistic and motor malfunctions as well as cardiac issues such as arrhythmia, chest pain and symptoms of apnea. Physical characteristics of Down syndrome are numerous including flat occiput, flat nasal bridge, small nose and mouth, shortened limbs and others. Knowing the phenotypic characteristics of the syndrome, DEGs were grouped aiming the association of different levels of transcript in Down syndrome cases with the phenotype and finally with disorders. For this Human Gene Database were used.

4. Results

4.1 Quantification of cell free fetal DNA in maternal plasma and whole blood

Maternal plasma and maternal peripheral whole blood were quantified using Y-chromosome specific marker, DYS14, and a housekeeping gene, B-Globin. DYS14 was used to classify the fetal sex, present in male foetuses and absence in female foetuses, and to quantify the fetal amount in maternal circulation. In case of maternal plasma, all 224 pregnancies were classified correctly in terms of fetal sex and quantified successfully (Appendix, table 1). In case of maternal whole blood, 24 (male cases) out of 124 (64 male cases) were not classified correctly in terms of fetal gender (Appendix, table 2). Thus, fetal quantification failed in these samples.

Fetal amount in maternal plasma was reported to be higher in T21 pregnancies compared to normal pregnancies according to the literature. In the current study, 83 maternal plasma, 68 normal (57 male and 11 female) and 15 T21 pregnancies (13 male and 2 female) were quantified and compared in term of total DNA (indicated by B-Globin), fetal DNA (indicated by DYS14) and 'fetal fraction' (DYS14/B-GLOBIN * 100) (Table 4-1, Figure 4-1). Although the number of T21 pregnancies is small compared to normal ones, results seems to agree with literature giving a median fetal amount 17.56 GE/mL in normal and 28.31 GE/mL in T21 cases (p-value = 0.002). Total amount of DNA was found to be 666.72 GE/mL in normal and 946.85 GE/mL in T21 cases (p-value = 0.019). This data suggests a significant difference between normal and T21 fetuses in terms of total and fetal amount but there is high variability among individuals and overlap between the two groups. In contrary, 'fetal fraction' in normal cases is 2.92% and 3.94% in T21 cases (p-value = 0.071) showing no significant difference between the two groups (Table 4-1, Figure 4-1).

	Total DNA Range (GE/mL)	Median Total DNA (GE/mL)	ffDNA Range (GE/mL) (only male pregnancies)	Median ffDNA (GE/mL) (only male pregnancies)	Fetal Fraction Range (%) (only male pregnancies)	Median fetal fraction (%) (only male pregnancies)
Normals (n=57 Male) (n=11 Female)	215.42 - 1871.67	666.72	1.54 – 57.52	17.56	0.53 - 11.68	2.8
Trisomy 21 (n=13 Male) (n=2 Female)	415.35 - 2659.16	946.85	16.56 – 111.18	28.31	0.63 – 14.49	3.69
Total (n=83)	215.42 - 2659.16	739.74	1.54 – 111.18	20.9	0.53 – 14.49	3.04

Table 4-1: **Quantification of ffDNA (DYS14), total DNA (β-globin) and "fetal fraction" analyzed in GE/ml in a total of 83 samples.**

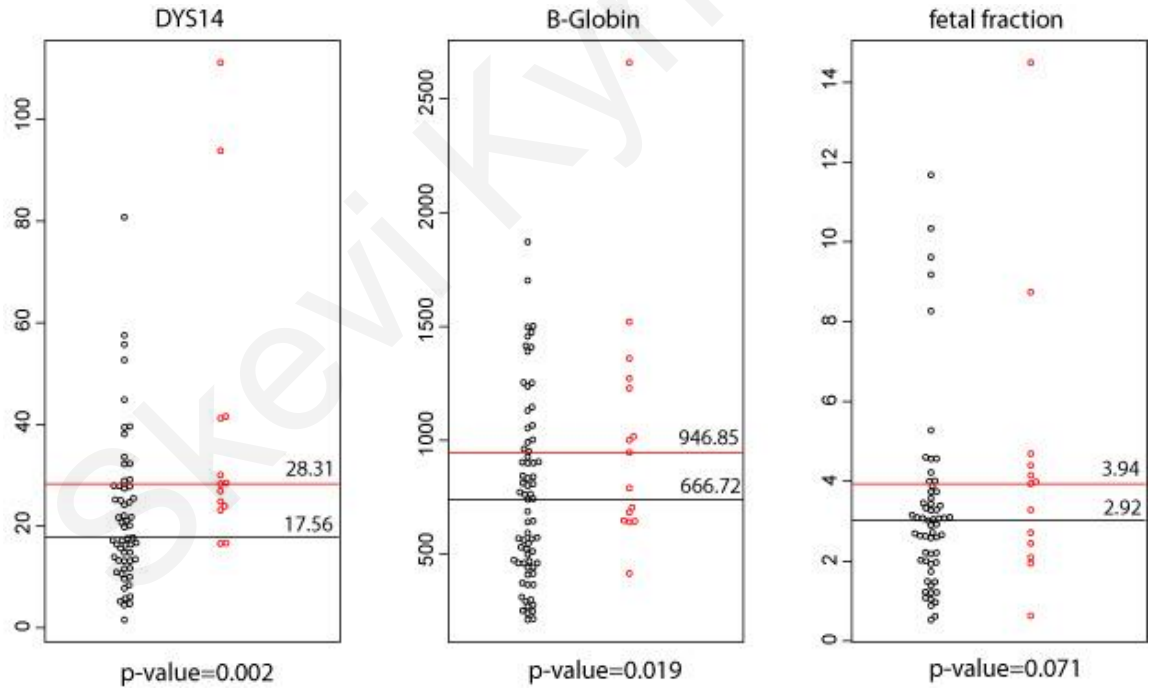


Figure 4-1: Association of normal and T21 pregnancies in terms of total and fetal amount and 'fetal fraction'. The samples are grouped in normal (black) and trisomy 21 (red) and are plotted with respect to their ffDNA (DYS14), total DNA (β-globin) and "fetal fraction" values. The horizontal lines represent the median values of each group (black for normal and red for trisomy 21) and the p-value shown below each plot corresponds to the comparison of the median values of the two groups. A p-value less than 0.05 indicates that the trisomy 21 samples have higher values than the normal samples.

4.2 Correlation of total and fetal amount in maternal plasma with MeDIP-qPCR methodology of T21 in maternal whole blood

This study further examined whether there is a correlation between the amounts of total DNA, cffDNA and the "fetal fraction" with the diagnosis of a trisomy 21 sample. These values were studied in correlation with the diagnostic D value and the "enrichment ratios" for each DMR. The enrichment ratios correspond to the amount of DNA that was enriched via the MeDIP process in comparison to known euploid samples used as controls and is calculated for each DMR as shown below.

Enrichment Ratio = Normalized Ct value of a Normal case or Trisomy 21 /Median of normalized Ct of normal controls

The diagnostic D-value combined these enrichment ratios for all the DMRs to give the diagnosis of trisomy 21 as previously described (Papageorgiou et al., 2010).

$$D = -6{,}331 + 0{,}959\ X_{EP4} + 1{,}188\ X_{EP5} + 0{,}424\ X_{EP6} + 0{,}621\ X_{EP7} + 0{,}028\ X_{EP8} + 0{,}387\ X_{EP10} - 0{,}683\ X_{EP11} + 0{,}897\ X_{EP12}$$

where XEPn = enrichment ratio Sample, EPn = DMR, and D is the diagnostic value.

The coefficient in front of each enrichment value showed the diagnostic power of each DMR as defined by Papageorgiou et al in 2010. A D-value greater than 0.798 classified a sample as trisomy 21 and as euploid otherwise.

All samples were examined for correlation with D-value and enrichment ratios. In addition normal samples and trisomy 21 samples were examined separately in order to identify specific trends. D-value and enrichment ratios of all samples were correlated with total DNA measured with β-globin in 83 cases. The same correlation studies were applied to the 70 pregnancies carrying male fetuses using ffDNA amount measured by DYS14 and the "fetal fraction". (Table 4-2 and Table 4-3, Figure 4-2 and Figure 4-3).

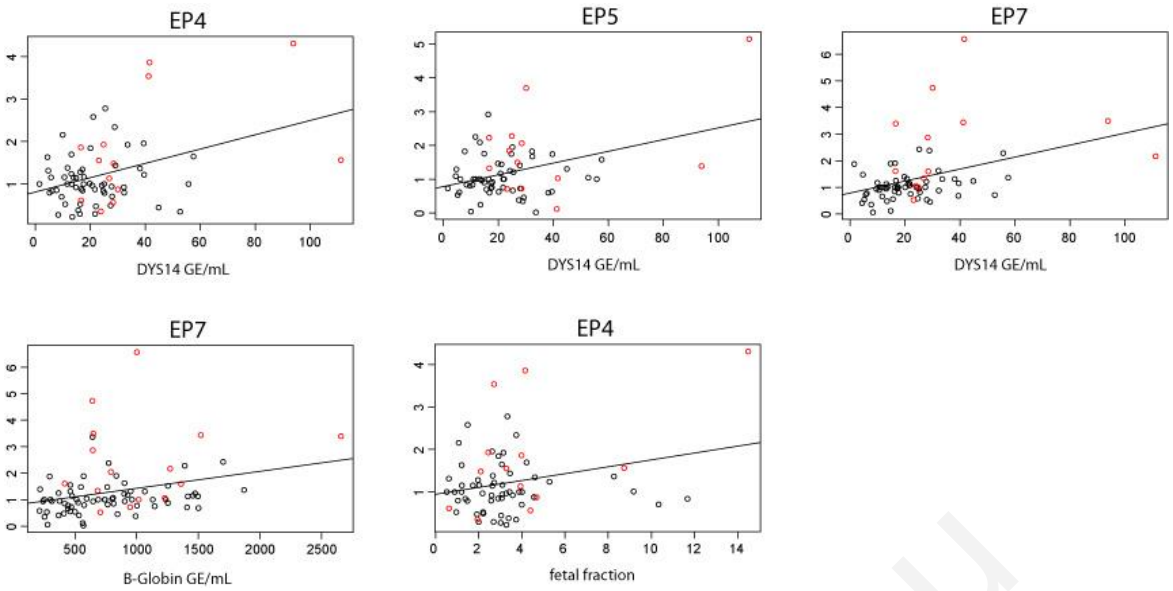
As indicated in table 4-3, the D-value showed no correlation with the amounts of ffDNA (DYS14) and the "fetal fraction" for any of the samples considered (p-value >0.05). The D-value showed low correlation (0.248, p-value=0.020, n=68 samples) with total DNA (β-globin) for the normal samples only (Table 4-3).More specifically, the enrichment ratios for the DMRs EP4 and EP5 showed moderate correlations

(0.385, p-value=0.0005 and 0.390, p-value=0.0004 respectively) with cffDNA (DYS14) for all pregnancies carrying male fetuses (n=70) (Table 4-2, Figure 4-2). Considering normal and trisomy 21 samples the first group showed no evidence of the presence of a correlation whereas the latter showed some evidence of moderate correlation (0.464, p-value=0.055 and 0.465, p-value=0.051 respectively for EP4 and EP5) (Table 4-2, Figure 4-2).The enrichment ratios for DMR EP4 also showed low correlation with the estimated "fetal fraction" for all 70 male samples (0.256, p-value=0.016) and moderate correlation (0.531, p-value=0.035) for the trisomy 21 samples only (Table 4-2, Figure 4-2).

For DMR EP7 the amount of cffDNA (DYS14) showed moderate correlation with the enrichment ratios with all samples (0.392, p-value=0.0004) and low correlation with the normal samples only (0.282, p-value=0.018) (Table 4-2, Figure 4-2). The same was observed for the total DNA (β-globin) with low correlations for all samples and normal samples only (0.282, p-value=0.005 and 0.263, p-value=0.015 respectively) but the "fetal fraction" showed no correlation with the enrichment ratios (Table 2, Figure 2).The rest of the markers displayed no association with the total DNA (β-globin), cffDNA (DYS14) and "fetal fraction" (DYS14/ β-globin) quantities (Table 4-2).

		EP1	EP4	EP5	EP6	EP7	EP10	EP12
cffDNA (DYS14) (GE/mL)	All	None	cor=0.385 p=0.0005	cor=0.390 p=0.0004	None	cor=0.392 p=0.0004	None	None
	Normal	None	None	None	None	cor=0.280 p=0.018	None	None
	Trisomy 21	None	cor=0.464 p=0.055	cor=0.465 p=0.051	None	None	None	None
Total DNA (β-globin) (GE/mL)	All	None	None	None	None	cor=0.282 p=0.005	None	None
	Normal	None	None	None	None	cor=0.263 p=0.015	None	None
	Trisomy 21	None	None	None	None	None	None	None
"fetal fraction" (%)	All	None	cor=0.256 p=0.016	None	None	None	None	None
	Normal	None	None	None	None	None	None	None
	Trisomy 21	none	cor=0.531 p=0.035	none	None	none	none	None

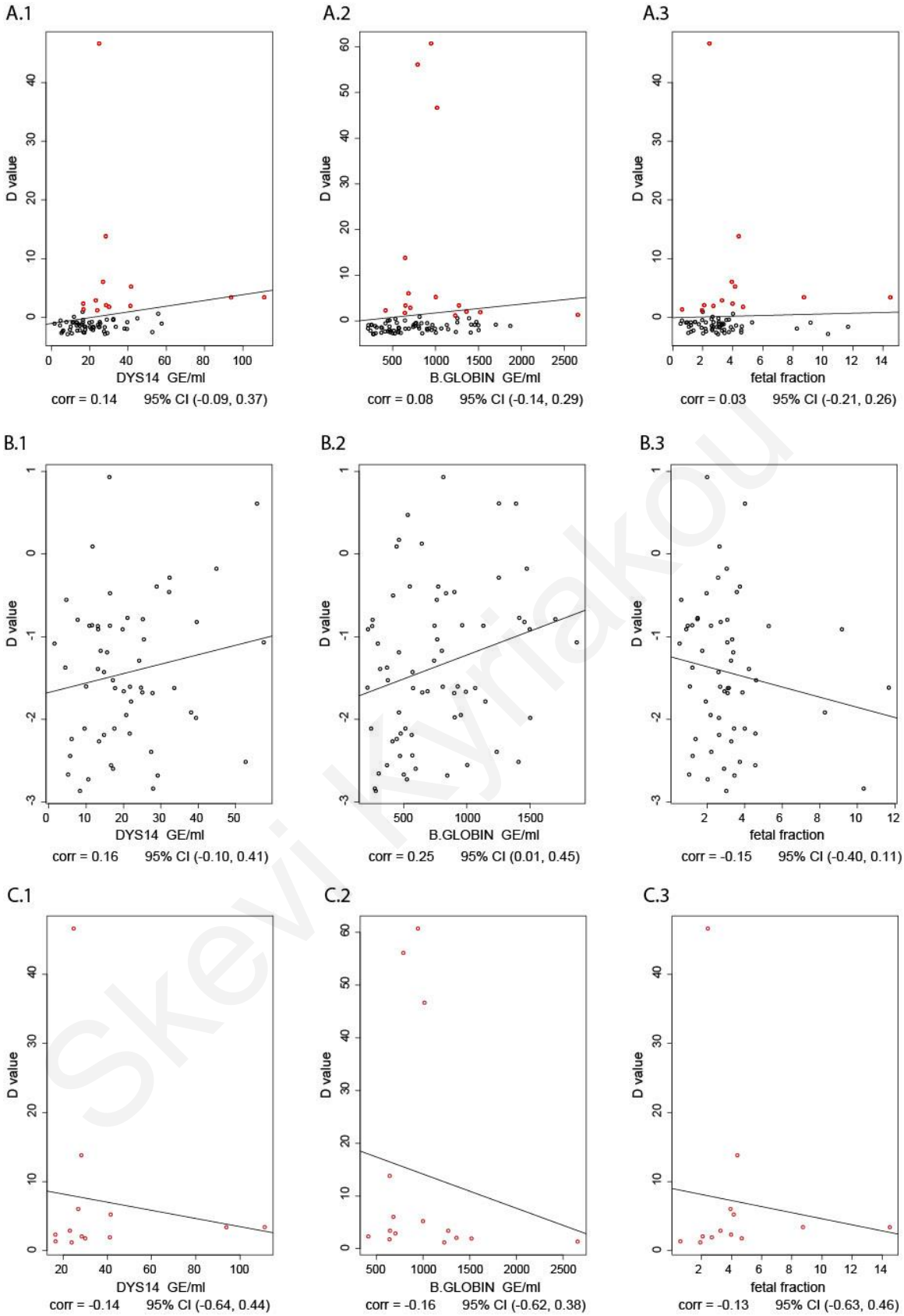
Table 4-2: Association of cffDNA(DYS14), total DNA(β-globin) and "fetal fraction" with the enrichment ratios of DMRs (EP1, EP4,EP5, EP6,EP7,EP10,EP12).



<u>Figure 4-2:</u> Association of enrichment ratios with cffDNA, total DNA and fetal fraction. The black circles correspond to normal samples and the red circles to trisomy 21 samples. The straight lines show the estimated linear relationship between the enrichment ratios and the corresponding quantities**.**

	D-VALUE		
	All samples	**Normal samples**	**Trisomy 21 samples**
ffDNA (DYS14) (GE/mL)	None	None	None
Total DNA (β-globin) (GE/mL)	None	correlation = 0.248 p-value=0.020	None
"fetal fraction" (%)	None	None	None

Table 4-3: Association of the D-value with the amounts of ffDNA (DYS14) and total DNA (β-globin) and the "fetal fraction" considering all samples and the normal and trisomy 21 samples separately. 'None' signifies that there was no significant evidence of the presence of an association (i.e. p-value>0.05)



<u>Figure 4-3:</u> Association of the D-value with the amounts of ffDNA (DYS14), total DNA (β-globin) and the "fetal fraction"

A.1, A.2, A.3. Plots of the association between the concentration of DYS14 (A.1), B.GLOBIN (A.2) and the estimated fetal fraction (A.3) with the D values for all the

samples studied. Normal samples are shown with black circles and Trisomy 21 samples are shown in red. The straight lines represent the estimated correlation between the measured quantities and the D values and these values are shown below each plot, along with the estimated 95% confidence intervals. If the confidence interval includes the value of 0 (i.e. "no association present") then there is no significant evidence to support that there is an association between the values at the 95% confidence level.

B.1, B.2, B.3. Plots of the association between the concentration of DYS14 (B.1), B.GLOBIN (B.2) and the estimated fetal fraction (B.3) with the D values for the Normal samples studied. Estimated correlations and their 95% confidence intervals are shown below each plot.

C.1, C.2, C.3. Plots of the association between the concentration of DYS14 (C.1), B.GLOBIN (C.2) and the estimated fetal fraction (C.3) with the D values for the Trisomy 21 samples studied. Estimated correlations and their 95% confidence intervals are shown below each plot.

4.3 Development of MeDIP methodology in maternal plasma

Fetal DNA is found to be more concentrated in maternal plasma compared to maternal whole blood. Having obtained this evidence, this study tried to develop the MeDIP-qPCR methodology of T21 (Papageorgiou et al.,2010; Tsaliki et al., 2012) using maternal plasma aiming to a more robust and sensitive methodology. The major limitation and challenge of this approach was the amount of DNA found in maternal plasma. In 1mL of maternal plasma there is only 1- 5ng of total DNA with a median of 3ng. All commercial MeDIP kits have a starting amount of 1μg which cannot be used in plasma DNA. In the current study, efforts were made for the downscaling of MeDIP starting quantity. First, 1000ng to 125ng (1:2 serial dilution) of total DNA of whole blood non pregnant and CVS underwent MeDIP-qPCR and tested on DMRs and control markers (Figure 4-4). These DMRs are hypomethylated in whole blood and hypermethylated in CVS. As a result the in house MeDIP protocol is working up to 125ng tested (Figure 4-4). Next, MeDIP performance was tested in starting quantities of 500ng to 1.95ng (1:2 dilution) using the same samples (Figure 4-5). As the starting

quantity decreases the difference between whole blood non pregnant and CVS decreases which means that performance of MeDIP is poor. Thus, the next step was to test the sensitivity of the assay starting from 250ng going down to 1.95ng total starting quantities. Spike in protocol was used where CVS was spiked in whole blood non pregnant to mimic pregnancy. Spikes of 2.5% fetal (CVS), 5%, 10%, 20%, 100% underwent MeDIP-qPCR (1:2 serial dilutions) (Figure 4-6). The sensitivity of the methodology was high only at 250ng and 125ng showing the expected trend which is as the fetal DNA increases the Ct value decreases. The remaining dilutions showed poor MeDIP performance and sensitivity wass lost. Due to the MeDIP poor performance at low DNA concentrations, some modifications on the protocol were applied. The antibody concentration and bead volume are critical steps in the protocol in order to avoid non-specific binding and therefore loose the specificity and sensitivity of the assay. First, titration of the antibody was performed at three different concentrations, 1ug, 0.5ug and 0.2ug, (Figure 4-7) showing good MedIP performance at 0.2ug of antibody only with a starting DNA quantity 5ng. Subsequent to this, bead volume titration was implemented (Figure 4-8) at three different volumes, 7ul, 5ul and 3ul, displaying precise results at 3ul bead volume only at 5ng starting DNA quantity. To further optimize the method and to keep reproducibility patterns, a more sensitive quantification technology was used replacing real time qPCR with digital PCR. Figure 4-9 shows 4 different DMRs tested with digital PCR indicating high sensitivity and specificity among the different spike in samples. After succeeding the desired sensitivity and specificity of a low DNA quantity MeDIP protocol, normal and trisomy 21 spikes in samples, 20%, 10% 5%, were tested for the correct classification of trisomy 21 samples with a starting quantity 5ng (Figures 4-10 – 4-12). Four different DMRs were tested using the newly developed MeDIP-dPCR protocol in a duplex form. Ratio values were calculated using the following equation:

Ratio = Unknown Sample/Average of Normals

As shown from the figures 4-10 – 4-12 the 20% and 10% spike in samples were successfully classified but in case of 5% spike in samples there was an indication that system fails to distinguish normal from trisomy 21 samples. Figure 4-10 show that DMRs 1 and 2 failed to classify 5% spike in samples correctly but DMR 3 and 4 successfully discriminate normal from trisomy 21 spike in samples at all fetal percentages (Figure 4-11). The sum of all DMRs (Figure 4-12) shows the ability to discriminate correctly at 20% and 10% spike in samples but 5%.

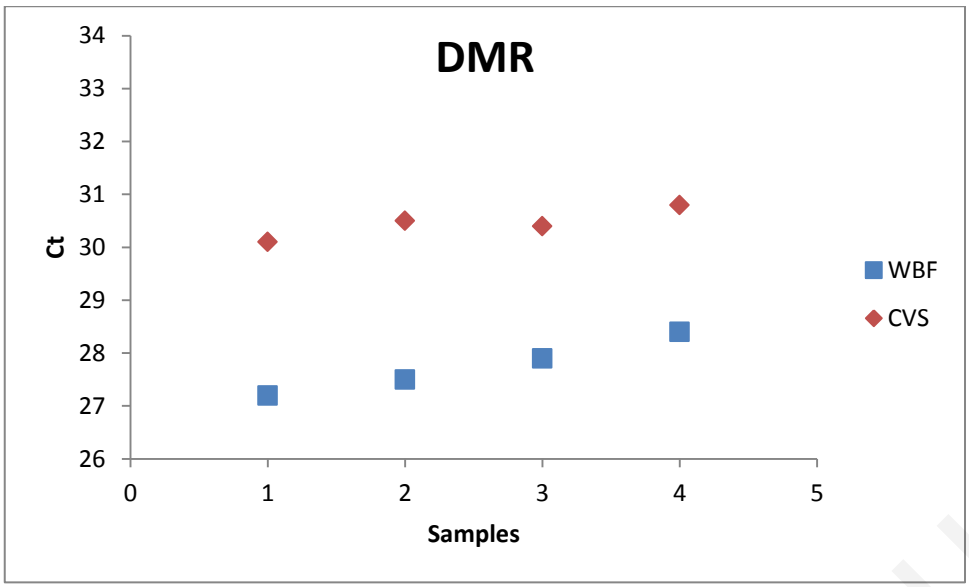


Figure 4-4: MeDIP-qPCR from different starting quantities. The red dots represent the Ct values obtained from whole blood non pregnant MeDIP samples (WBF). The green dots indicate the Ct values obtained from CVS MeDIP samples. The x-axis shows the different starting quantities (1= 1ug, 2= 500ng, 3= 250ng, 4=125ng). Experiment is performed in triplicates.

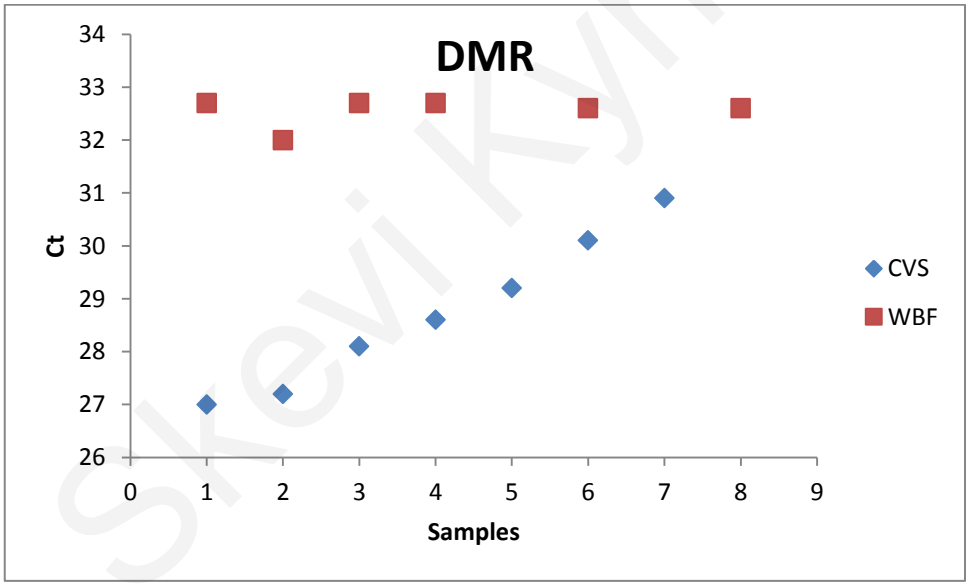


Figure 4-5: MeDIP-qPCR from different starting quantities. The red dots represent the Ct values obtained from whole blood non pregnant MeDIP samples (WBF). The blue dots indicate the Ct values obtained from CVS MeDIP samples. The x-axis shows the different starting quantities (1= 500ng, 2= 250ng, 3= 125ng, 4= 62.5ng, 5= 31.25, 6= 15.62, 7= 7.8, 8= 3.9, 9= 1.95). Experiment is performed in triplicates.

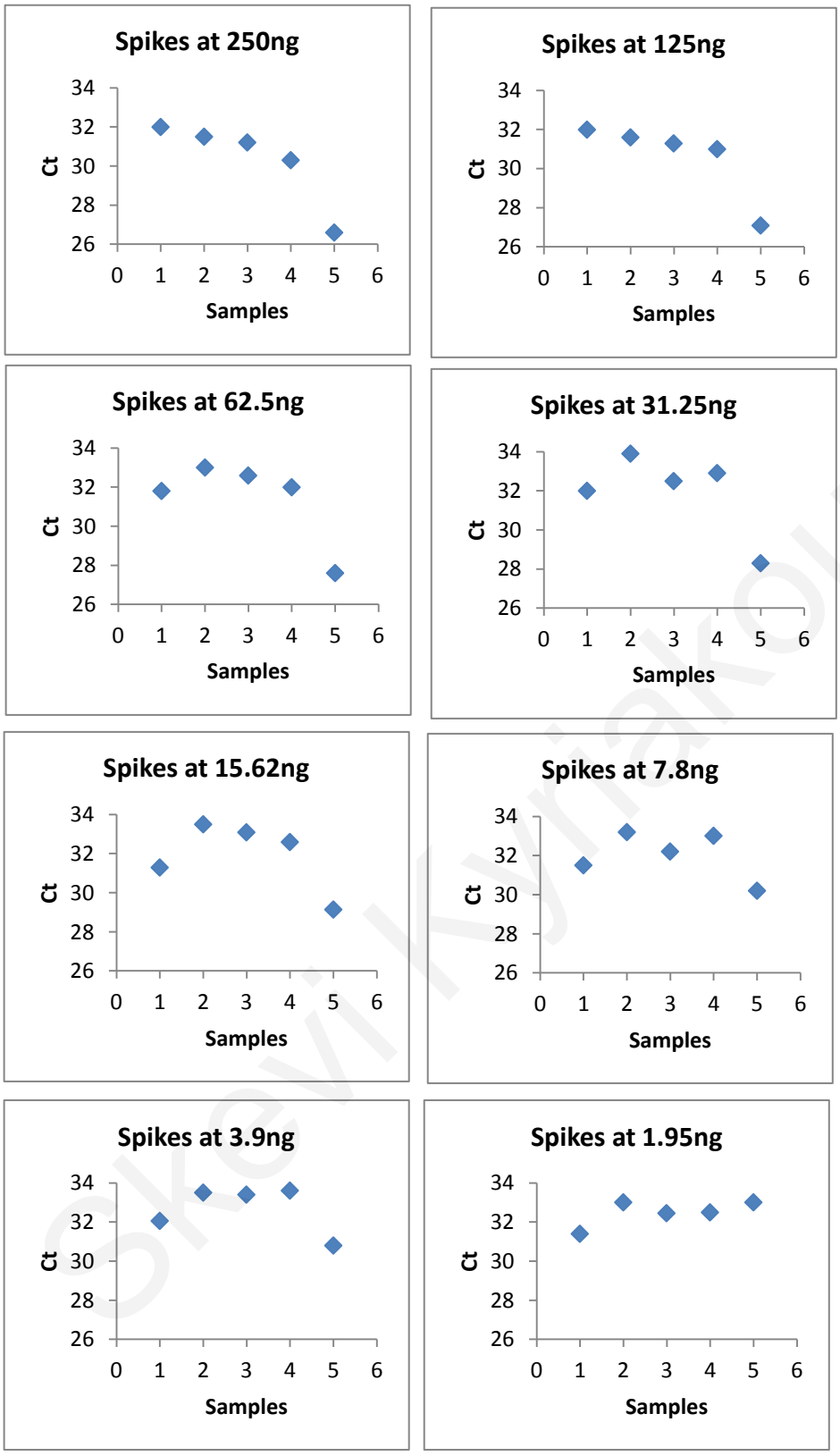
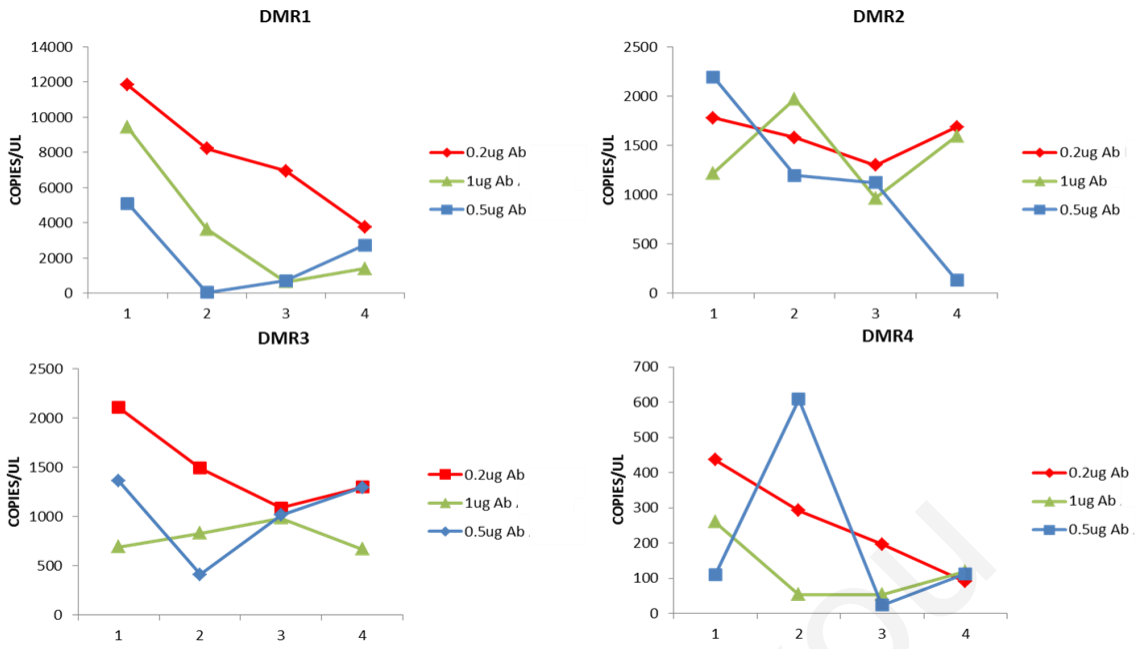
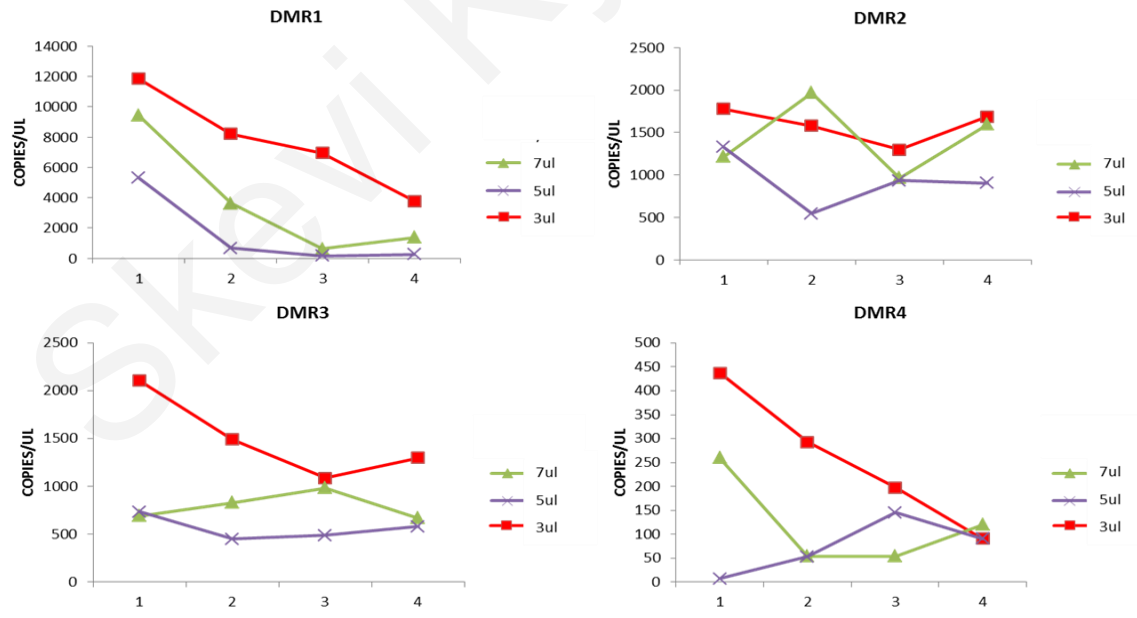


Figure 4-6: Sensitivity of MeDIP-qPCR methodology in low starting quantities. Each plot represents a different dilution and x-axis shows the different spikes (1= 2.5%, 2= 5%, 3= 10%, 4= 20%, 5= 100%). Experiment is performed in triplicates.



<u>Figure 4-7</u>: Titration of the antibody testing sensitivity and specificity of the assay using four different DMRs. X-axis shows points 1 to 4 which are spike in samples, 20%, 10%, 5% and 2.5% respectively. Experiment is performed in triplicates.



<u>Figure 4-8:</u> Titration of the bead volume testing sensitivity and specificity of the assay using four different DMRs. X-axis shows points 1 to 4 which are spike in samples, 20%, 10%, 5% and 2.5% respectively. Experiment is performed in triplicates.

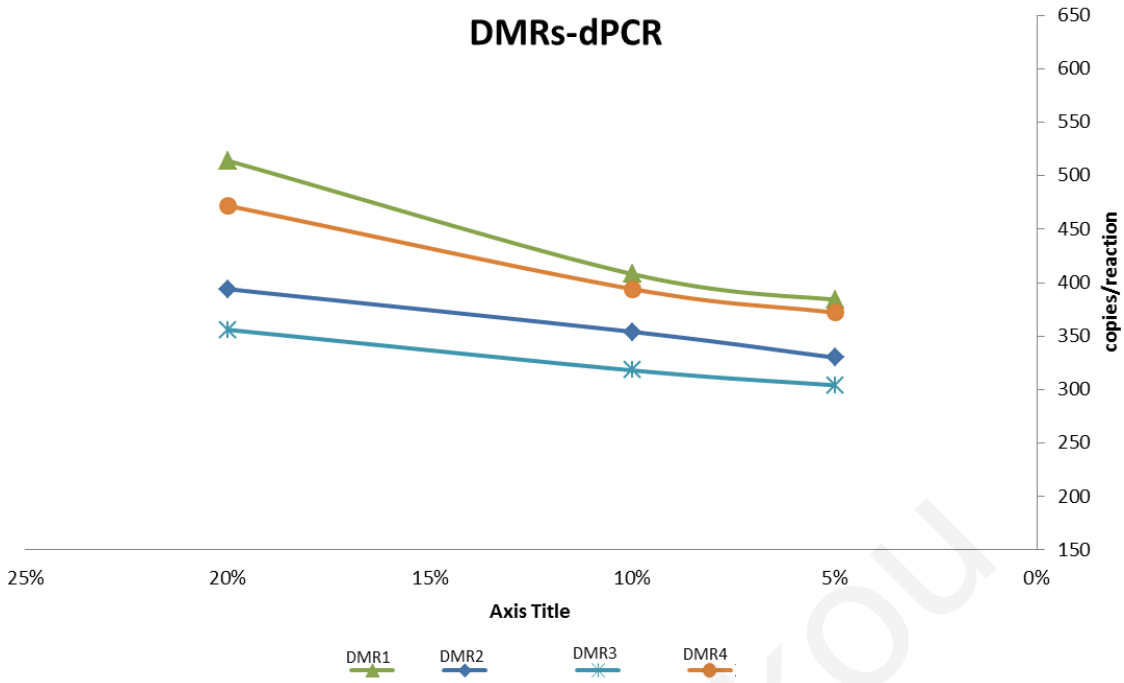


Figure 4-9: Sensitivity and Specificity of DMRs using digital PCR on 20%, 10% and 5% spike in samples at very low DNA quantities (5ng). Experiment is performed in triplicates.

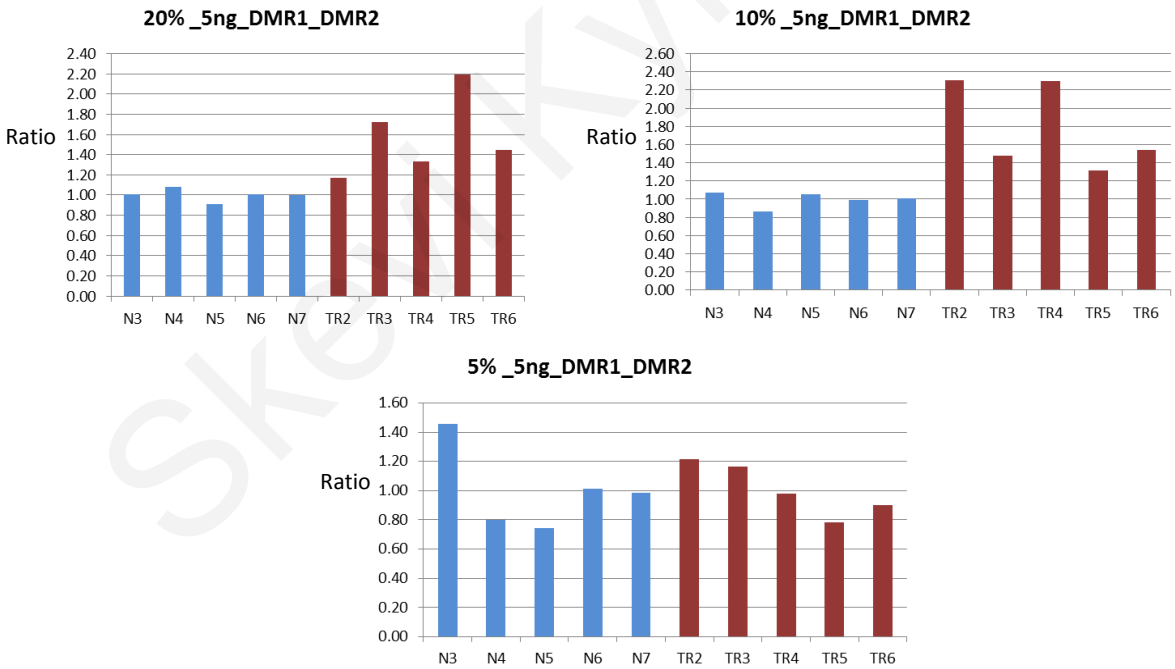


Figure 4-10: Discrimination of normal from trisomy 21 spike in samples using DMR1 and DMR2. Experiment is performed in triplicates.

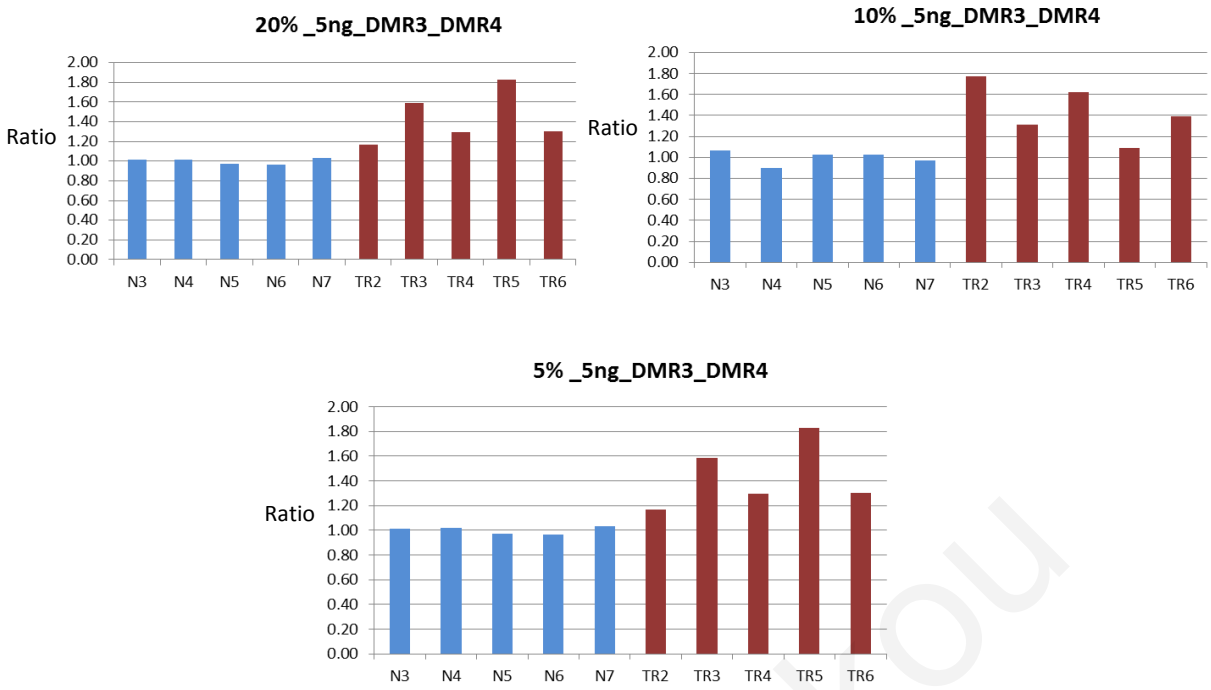


Figure 4-11: Discrimination of normal from trisomy 21 spike in samples using DMR3 and DMR4. Experiment is performed in triplicates.

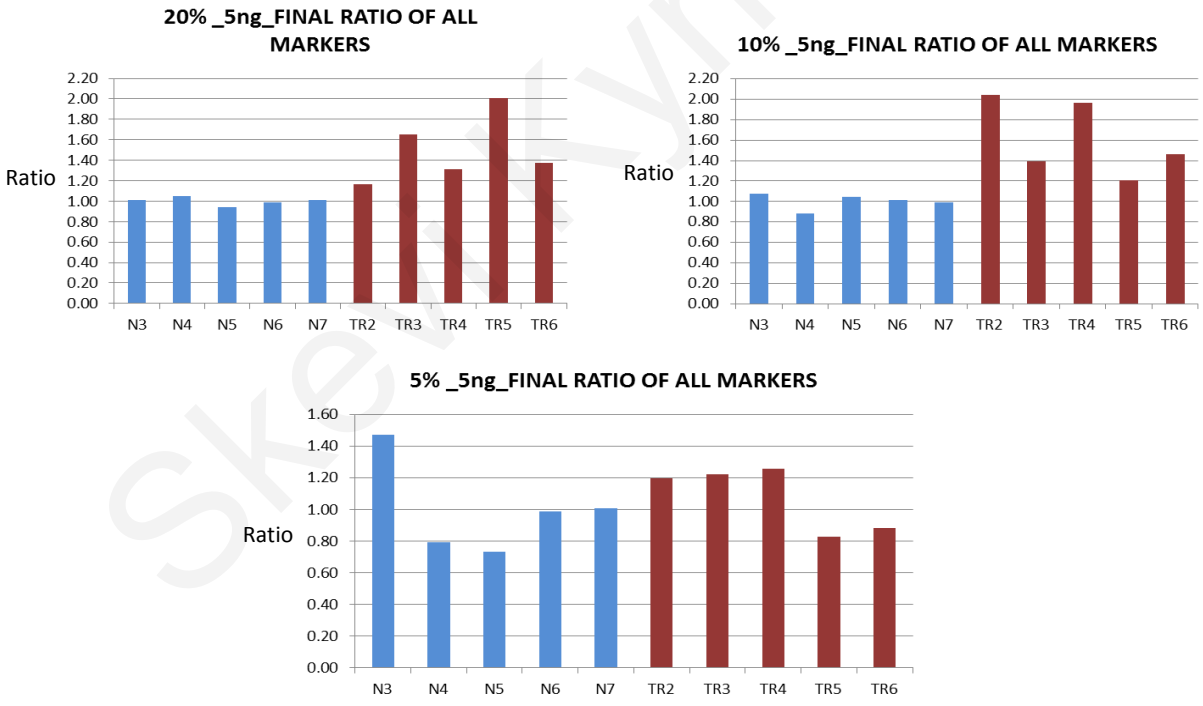


Figure 4-12: Discrimination of normal from trisomy 21 spike in samples using all DMRs. Experiment is performed in triplicates.

4.4 Identification of Differentially Expressed Genes and Association with Down syndrome Phenotype and Methylation Patterns

4.4.1 Analysis 1 and 2

Two types of analysis were performed to identify differentially expressed genes that can be used for the discrimination of T21 fetuses from normal fetuses. Analysis 1 compares trisomy 21 CVSs with normal CVSs. It identified 175 DEGs where 43 DEGs were up-regulated and 132 were down-regulated in trisomy 21 CVS (thus up-regulated in normal CVS). Figure 4-13 allocates each DEG to the corresponding chromosome. Table 4-4 shows the DEGs that are associated with Down syndrome according to the literature. Table 4-5 indicates the fold change of trisomy 21 CVSs cases ($T21_1$ and $T21_2$) against normal CVSs (N_1, N_2, N_3, N_4). Analysis 2 involved the identification of DEGs between mothers carrying trisomy 21 fetuses and mothers carrying normal foetuses. This type of analysis gave no results. No DEGs were identified when these two groups compared.

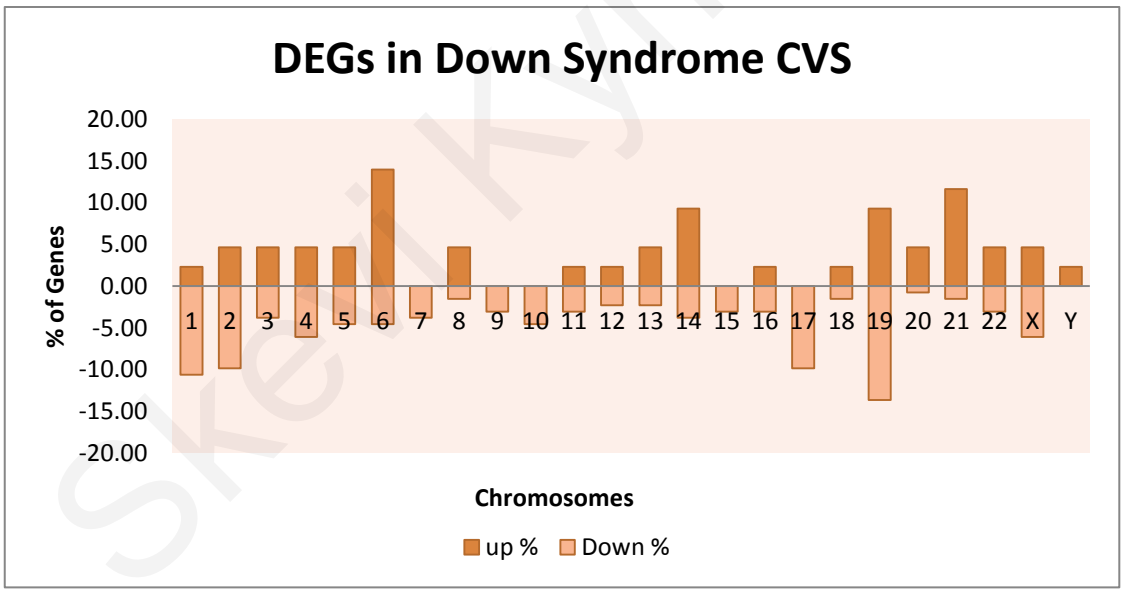


Figure 4-13 : Genes up- and down-regulated in trisomy 21 CVS aligned to each chromosome.

Gene	Gene Name	Chromosome	Regulation
PGF (Jeroen N.A. Pennings, et al., 2009)	**placental growth factor**	**chr14**	**down**
ADAM12 (Koster M.P.H, et al., 2011)	**ADAM metallopeptidase domain 12**	**chr10**	**down**
PSG1 (Bartels and Lindemman , 1988)	**pregnancy specific beta-1-glycoprotein 1**	**chr19**	**down**
CGA (Laurence A. Cole, et al., 1999)	**glycoprotein hormones, alpha polypeptide**	**chr6**	**down**
CGB (Laurence A. Cole, et al., 1999)	**chorionic gonadotropin, beta polypeptide**	**chr19**	**down**
INHA (N. J. Wald, et al., 1999)	**inhibin, alpha**	**chr2**	**down**
PAPPA (P.De Biasio, et al., 1999)	**pregnancy-associated plasma protein A, pappalysin 1**	**chr9**	**down**
STS (Kashork, et al., 2002)	**steroid sulfatase (microsomal), isozyme S**	**chrX**	**down**
COL6A1 (Mao R, et al., 2005)	**collagen, type VI, alpha 1**	**chr21**	**up**

Table 4-4 : Genes up- and down regulated in trisomy 21 compared to normal CVS associated with Down syndrome.

	Fold Change							
Gene	**$T21_1$/ N_1**	**$T21_1$/ N_2**	**$T21_1$/ N_3**	**$T21_1$/ N_4**	**$T21_2$/ N_1**	**$T21_2$/ N_2**	**$T21_2$/ N_3**	**$T21_2$/ N_4**
PGF	-4.42	-4.73	-5.37	-3.82	-3.80	-3.76	-4.27	-3.03
ADAM 12	-12.05	-7.26	-8.70	-2.82	-40.87	-24.63	-29.50	-9.58
PSG1	-7.85	-9.34	-4.68	-2.24	-60.10	-58.06	-35.96	-18.25
CGA	-4.15	-3.56	-2.85	-3.02	-5.88	-5.04	-4.03	-4.28
CGB	-22.51	-10.64	-9.83	-10.50	-90.92	-42.97	-39.69	-42.43
INHA	-8.09	-3.99	-6.00	-3.59	-7.06	-3.48	-5.24	-3.13
PAPPA	-4.93	-5.20	-6.46	-2.60	-58.47	-61.60	-76.61	-30.78
STS	-4.55	-3.20	-2.56	-2.65	-4.31	-3.04	-2.42	-2.51
COL6A 1	4.61	4.48	3.24	3.55	3.29	3.19	2.31	2.53

Table 4-5: Fold change difference of DEGs associated with Down syndrome.

4.4.2 Analysis 1A and 1B

Analysis 1A identified DEGs when comparing T21 CVS against normal CVS and the mothers, and analysis 1B the remaining DEGs.

Analysis 1A identified 12 DEGs up-regulated and 49 down regulated in trisomy 21 CVS against normal CVS and the mothers. Figure 4-14 aligns the DEGs into the corresponding chromosomes. Figure 4-15 shows the identified DEGs sharing similar phenotypes. These DEGs are categorized according to their associated disorders (figure 4-16). Analysis 1B consists of the remaining DEGs which are 31 up- and 83 down-regulated. These are DEGs between trisomy 21 CVS and normal CVSs but showed similar expression patterns with maternal cells. Figure 4-17 aligns the DEGs

to the corresponding chromosome and figure 4-18 shows the phenotypes that DEGs shared. Figure 4-19 associates the identified DEGs with disorders.

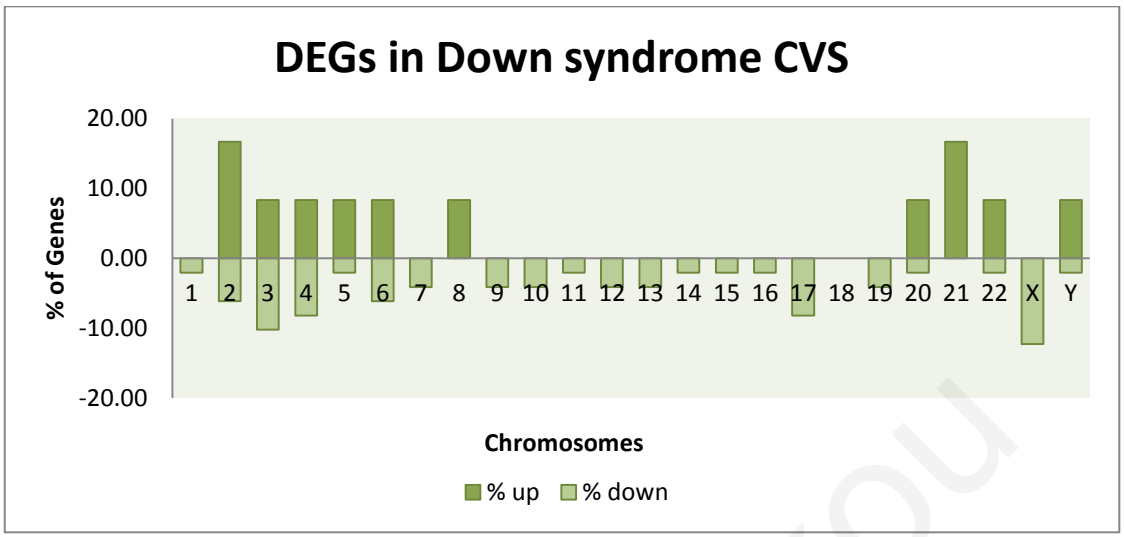


Figure 4-14: Genes up- and down-regulated in trisomy 21 CVS compared to normal CVS and maternal white blood cells aligned to each chromosome.

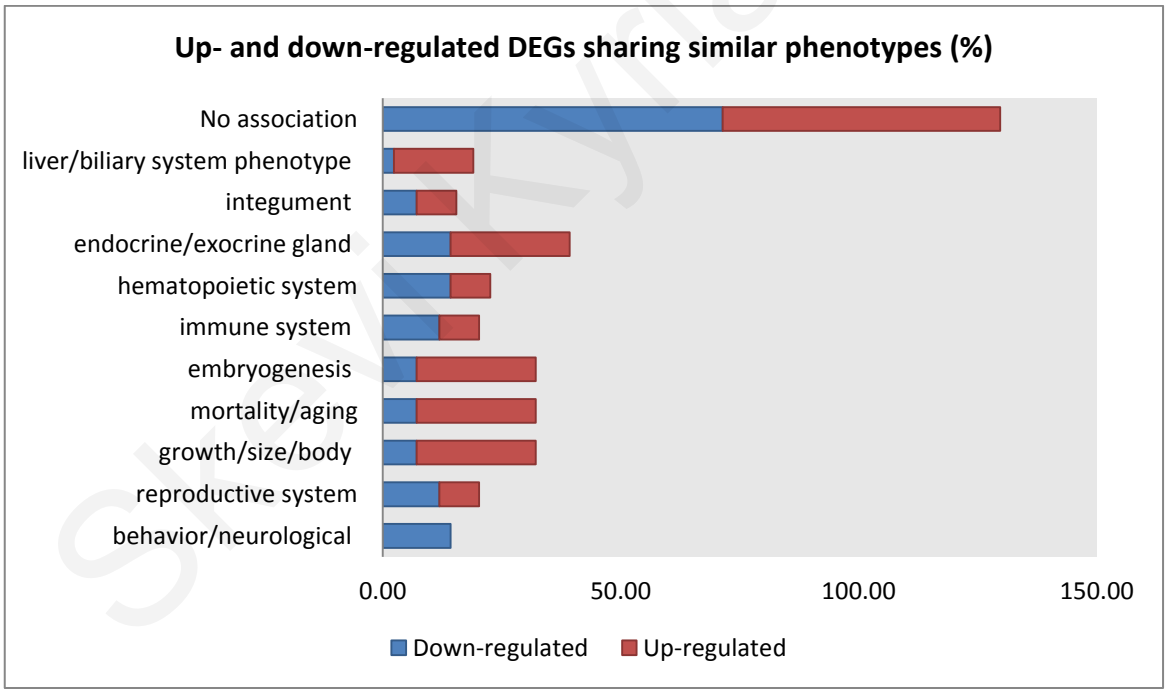


Figure 4-15: Genes up- and down-regulated in trisomy 21 CVS compared to normal CVS and maternal white blood cells sharing similar phenotypes.

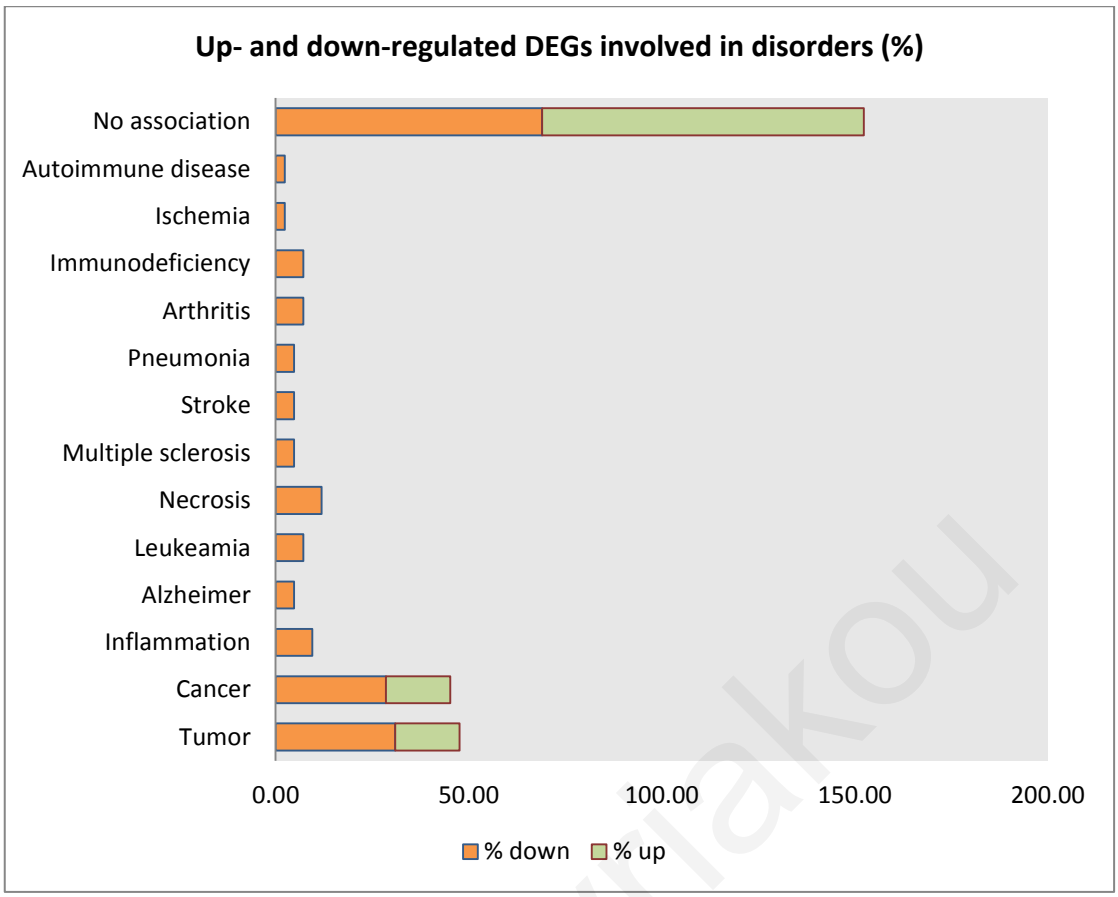


Figure 4-16: Genes up- and down-regulated in trisomy 21 CVS compared to normal CVS and maternal white blood cells associated with disorders.

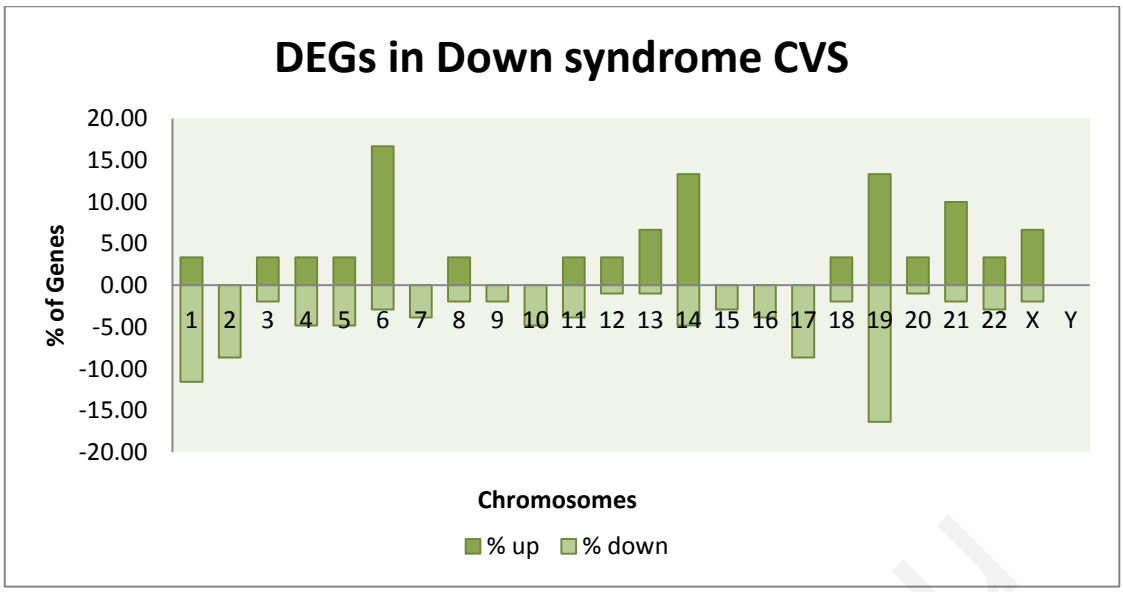


Figure 4-17: DEGs up- and down-regulated in trisomy 21 CVS against normal CVS and similar expressed against maternal white blood cells aligned to each chromosome.

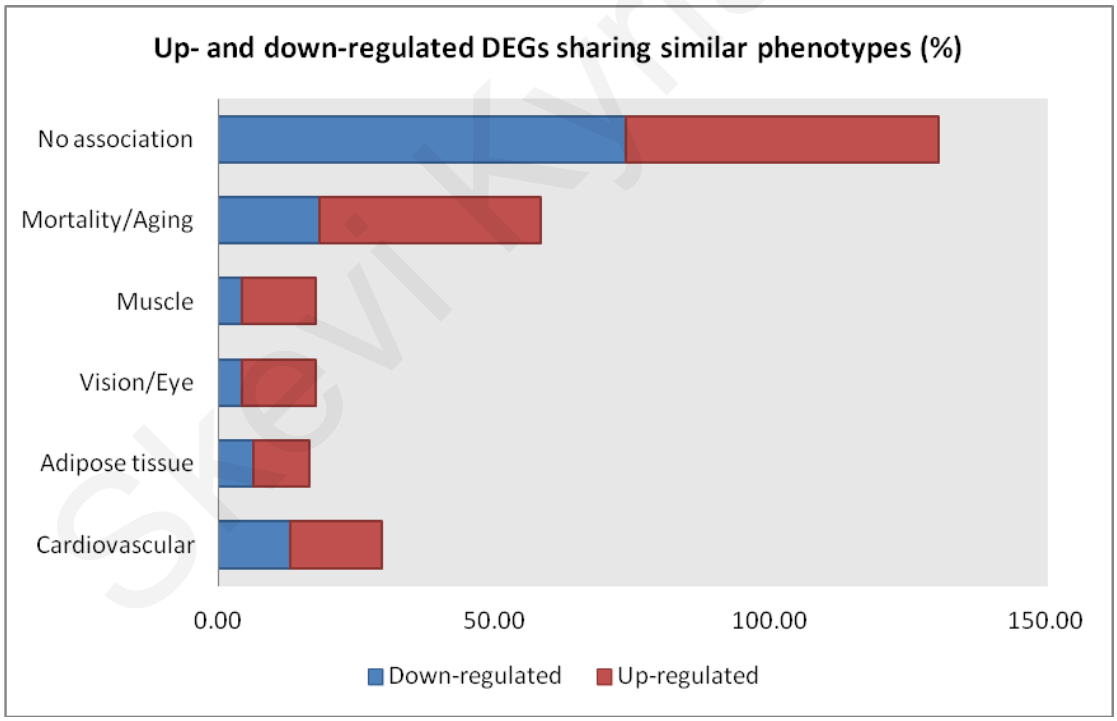


Figure 4-18: Genes up- and down-regulated in trisomy 21 CVS against normal CVS and similar expressed against maternal white blood cells sharing similar phenotypes.

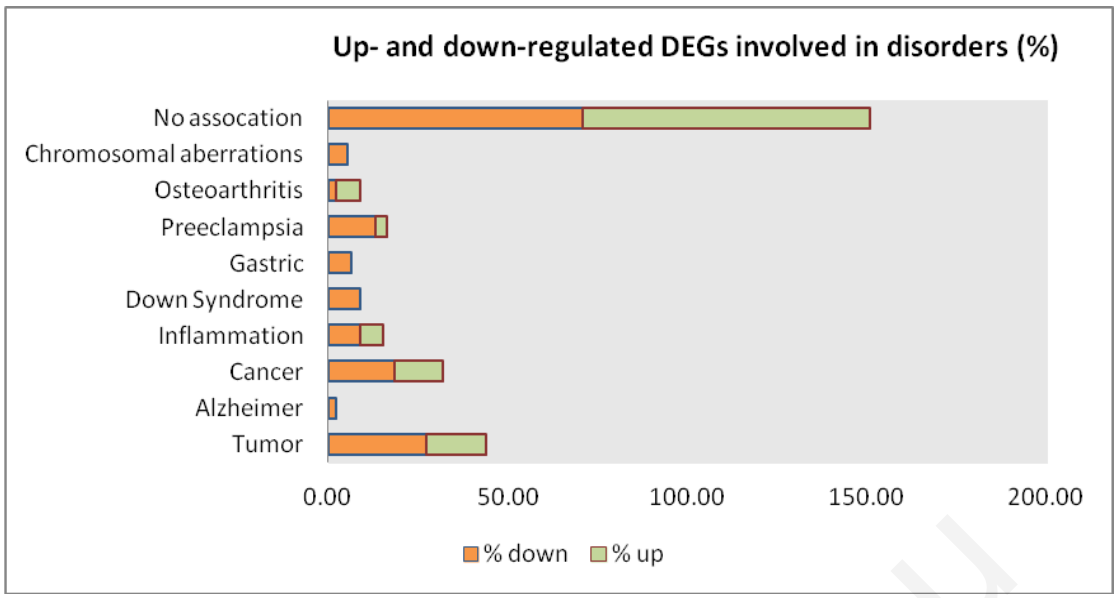


Figure 4-19: Genes up- and down-regulated in trisomy 21 CVS against normal CVS and similar expressed against maternal white blood cells associated with disorders.

4.4.3 Association of Differentially Expressed Genes with Methylation Patterns

No association of gene expression with methylation patterns was found. Similar methylation patterns among normal and T21 CVS and maternal tissue were observed.

5. Discussion

5.1 Influence of total and fetal amount in maternal plasma with the diagnosis of MeDIP-qPCR methodology of T21.

The variability in the amount of ffDNA, found in maternal plasma, between individuals has been a challenge in NIPD of trisomy 21. There are concerns whether or not the amount of ffDNA may influence non-invasive prenatal methodologies and, consequently, the correct classification of trisomy 21. Other techniques used in NIPD consider the fetal percentage and either perform a normalization step or/and set a cut off excluding a number of cases with low fetal percentage. In the current study, we present new data from a study of the absolute ffDNA, total DNA, and fetal fraction in maternal plasma in 83 samples and investigated whether these influence the enrichment ratios of DMRs and the correct classification of trisomy 21 using the MeDIP-qPCR based NIPD methodology applied in peripheral blood. To investigate the variability of ffDNA and total DNA among normal cases and trisomy 21 cases, we quantified 83 plasma samples of which 68 were normal and 15 were trisomy 21 cases. Collectively, our data exhibit a significant difference between normal and trisomy 21 cases in ffDNA and total DNA. These results confirm previously published data that describe abnormally high amounts of ffDNA in maternal plasma in trisomy 21 cases (Lo YM, et al., 1999). Together with the work of others, these results further extend our understanding of ffDNA release in maternal circulation. In contrast, our results indicate similar fetal fraction (ffDNA/total DNA) between normal and trisomy 21cases. As previously published, increase of ffDNA in trisomy 21is correlated with the increase in total DNA; therefore, the fetal fraction remains the same (Chiu RW, et al., 2011). Thus, the fetal fraction of normal and trisomy 21 samples shows no significant difference. On the basis of the abovementioned findings, we further investigated how total DNA and ffDNA affect the D-value and the enrichment ratios, which are the determining factors of correct classification of T21 cases. We performed correlation studies including all cases, normal only and trisomy 21 only cases. The D-value showed no correlation with the amounts of ffDNA and the fetal fraction for any of the samples considered. Although the variability of ffDNA amounts may pose a limitation in current methods of NIPD resulting in extensive sample analysis and sample exclusions,(Lo YM, et al., 2007; Chiu RW, et al., 2008, Ehrich M, et al., 2011) our results indicate that MeDIP-qPCR methodology from maternal whole blood is

simpler and can be applied safely for trisomy 21 classification without requiring ffDNA analysis between cases. The D-value combines the enrichment ratios for the seven DMRs (EP1, EP4, EP5, EP6, EP7, EP10, and EP12). To specifically investigate how the enrichment ratios are affected by total DNA, ffDNA, and fetal fraction, correlation studies were applied in all samples, normal only and trisomy 21 only. The enrichment ratios of a number of DMRs (EP4, EP5, and EP7) exhibited moderate correlation with ffDNA. The same analysis was applied using total DNA and fetal fraction. Only DMR EP7 was affected (weak correlation) by total DNA and EP4 by fetal fraction suggesting that D-value is not influenced significantly by the maternal background. This proposes that the diagnostic formula determining the D-value combines adequate number of DMRs to allow correct classification independently of the presence of moderate correlation of some markers with ffDNA. An additional explanation is the use of maternal peripheral blood that contains less than 0.5% ffDNA (appendix, table 2), compared with maternal plasma that contains approximately 3% quantified with qPCR. Consequently, the variability of ffDNA among maternal samples from different pregnancies is very small. Low variability may account for the low influence of ffDNA on our predicted model and ensures correct classification on the basis of methylation differences.

5.2 Towards the development of a Non-Invasive Prenatal Methodology using maternal plasma

This study turned its interest in maternal plasma where the fetal percentage is approximately ten folds higher than maternal peripheral blood. Our group has developed a MeDIP-qPCR methodology using peripheral blood (Papageorgiou et al., 2010) but this study aimed the development of a methodology, based on existing DMRs, using the challenging low DNA amounts of maternal plasma. Since fetal percentage is found to be much higher in maternal plasma than peripheral blood, indicates that a new methodology from maternal plasma will lead to a more robust and sensitive test for all aneuploidies. Enrichment of fetal DNA methylated sites can be achieved by three main approaches which are sodium bisulfite based, restriction enzymes based and methylated DNA immunoprecipitation based approaches. Sodium bisulfite treatment is based on the conversion of an epigenetic change into a genetic change by converting umethylated cytosines into uracils leaving unchanged the

methylated sites (Frommer M, et al., 1992).This genetic modification is then investigated by methylation specific PCR or by massive parallel sequencing (Herman J.G, et al., 1996; Gonzalgo M.L. and Jones P.A, 1997). Restriction enzyme approach is based on the recognition sites containing CG sequences which are methylated. Methylation sensitive restriction enzymes digest unmethylated sites leaving the methylated sites intact. The third approach is the methylated DNA immunoprecipitation method where methylated regions are captured by a monoclonal antibody and then magnetic beads bound to a secondary antibody. All three approaches have been used for the identification of DMRs between the fetus and the mother by different groups (Herman J.G, et al., 1996; Poon L.L, et al., 2002; Papageorgiou E.A, et al., 2009; Old R.W., et al., 2007). The latter approach was used by our team to identify around 2000 DMRs on chromosomes 13, 18, 21, X and Y (Papageorgiou E.A, et al., 2009). These DMRs are mostly located on non-genic regions with only around10% found in genic regions. Thus, our group developed a panel of DMRs which can be used for the identification of aneuploidies using methylated DNA immunoprecipitation approach. The efficiency of these methylation assays is assessed by many groups in the field and others. Sodium bisulfite approach is a chemical treatment which results in a high degree of DNA degradation, >90% of DNA (Grunau C, et al., 2001). Implementing of sodium bisulfite assay in maternal plasma sample where the total DNA is very low will lead to even lower fetal DNA copies thus the accuracy will be lost or reduced. Restriction enzyme approach requires high purity, high integrity and high quantities of samples which are not feasible using plasma DNA (Laird P.W., 2010). DNA immunoprecipitation assay can tolerate low purity and integrity samples, and is robust and cheaper compared to the other approaches. The only drawback is that it requires high DNA quantities which nit applicable using plasma DNA. Maternal Plasma DNA is of low purity, integrity and DNA quantity. This study is based on existing DMRs identified by DNA immunoprecipitation technique, thus it used the same technique to successfully develop a MeDIP-digital PCR methodology aiming the development of a non-invasive prenatal test. The first challenge was to downscale the immunoprecipitation assay from micrograms to less than ten nanograms of DNA starting material. This was not implemented using real time qPCR since from our findings the MeDIP-real time qPCR is achieved up to 1.95ng in terms of specificity but only up to 62.5ng in terms of sensitivity. The sensitivity is identified using hyper and hypo control markers for both maternal and placenta tissues and the sensitivity is measured using DMRs and spike-in samples at

different fetal percentages. The more fetal DNA was spike in non- pregnant DNA, the more copies of DNA should be enriched by MeDIP. This pattern was not achieved below 65.5ng of DNA using qPCR for quantification. Therefore the developed MeDIP-qPCR methodology is not performing well in low DNA amounts necessary for implemented in maternal plasma. A more sensitive technology was required to measure such low DNA amount. Digital PCR is a relatively new approach for the detection and absolute quantification of nucleic acids counting directly the target molecules. There are several advances of digital PCR compared to real time qPCR. The most useful advantage is the linear detection of very small differences. In a real scenario where fetal percentage of a trisomy 21 and a normal case is 5%, the expected difference between them is only 2.5% which is not detectable by real time qPCR. Digital PCR exhibits low quantitative bias and imprecision and high sensitivity compare to real time qPCR. Studies, on non-inasive prenatal field, reported fetal percentage two times higher, when measured with digital PCR, than real time qPCR (Lun M.F., et al., 2008). In addition, low abundance gene mutations, cancer related, were accurately detected with high sensitivity in plasma samples (Yung K.F., et al., 2009). A report by Sanders R. et al., in 2011, evaluated the quantitative abilities of digital PCR in low level detection targets. They demonstrated that it exhibits high precision, reproducibility and sensitivity at low copy measurments. Since digital PCR is reported to be more sensitive and is able to count number of copies present, immunoprecipitated products from 5ng of spike in samples at three different fetal concentrations were quantified using digital PCR. The evaluation of this assay was performed using four different DMRs on chromosome 21 which are hypermethylated in placenta and hypomethylated in maternal tissue. Number of copies per reaction should decrease as the fetal spike in concentration decreases. This is successfully achieved using the DNA immunoprecipitation assay followed by digital PCR. This proves our hypothesis that the DNA immunoprecipitation assay is robust enough to discriminate between small fetal concentrations differences among the huge maternal background and that a more sensitive quantification method was needed. This enrichment method is indeed working from very low DNA amounts and is specific and sensitive enough to enrich the few fetal methylated regions found in the maternal plasma. The only limitation was the technology initially used to quantify the immunoprecipitated product, real time qPCR, which was overcome when switching from qPCR to digital PCR. Since digital PCR was sensitive enough to correctly count and distinguish 20% from 10% and 5% of fetal percentages, one will expect that dPCR

is able to distinguish at least one normal and one T21 case with a 10% fetal amount. The difference between a normal and T21 with 10% fetal amount is only 5%, thus if digital PCR is able to distinguish 10% from 5%, it should be able to discriminate a T21 case from the normal one having 10% fetal DNA. But this is only a hypothesis. To prove this hypothesis, five different normal placentas and five different T21 placentas were spike-in non-pregnant plasma in order to mimic real cases. These placentas were spike-in at three different fetal quantities, 20%, 10% and 5% and evaluated with four different DMRs on chromosome 21. The results showed that MeDIP followed by digital PCR methodology is robust enough to discriminate T21 from normal spike in samples up to 10% fetal DNA. The hypothesis was proved and the results are very promising indicating that the methylation variability among foetuses does not interfere with the results. This is just an indication that real T21 cases can indeed discriminate from normal cases using the proposed non-invasive methodology. The next step is a pre-validation study using real cases and the development of an analytical pathway for the correct discrimination of normal from abnormal cases. Although the methodology seems to function very well using artificial samples, spike-in, there are many factors that may play a fundamental role in the correct diagnosis when using real cases. The non-pregnant plasma used for the experiments is a pool of many individual non pregnant women ranging from 20 to 40 years old. Thus, the maternal methylation variability was not taken into consideration. Each pregnant woman carries her own epigenetic characteristics which may influence the methodology. It is well known that methylation variability among individuals is high. Although, DMRs selected by our group are of minimum variability compared to others, still there is a degree of variability (Ioannides M. et al, 2014 submitted). In real pregnant cases, methylation variability exists among the fetal DNA and maternal DNA, thus none of the two is stable in terms of methylation. Although, this study tries to mimic a real case, there are limiting factors that may interfere with the proposed methodology when applied in real cases. Variability among individuals can be eliminated or reduced when using a large number of DMRs. Since all DMRs have been characterised in terms of their methylation variability (Ioannides M. et al, 2014, submitted), a diagnostic formula can be constructed using a specific coefficient for each DMR. This coefficient will represent the variability of each DMR and therefore a normalization step may eliminate or smooth down this effect. These are assumptions that need to be tested using real cases and advised by a biostatistician. In addition, this study proved that the fetal percentage does not interfere with the correct classification

of T21 when using the existing MeDIP-qPCR methodology. In the new developed methodology, proposed by this study, there is evidence that fetal fraction influences its performance and the diagnosis of T21. When spike-in samples at different fetal percentage were tested, a pattern was noticed from high percentage to low fetal percentage indicating that as the fetal fraction decreases the DNA copies decreases as well. No trend should have being appeared in case that fetal amount does not affect the methodology. This can be explained by the high fetal fraction found in maternal plasma compared to maternal peripheral blood. Subsequently, real cases with different fetal fraction cannot be compared within the same run. This is a challenging limitation of the method that needs to be considered in order to develop a robust diagnostic test. There are several ways to overcome this limitation. The most apparent but most challenging way is the use of fetal fraction in the normalization pipeline. In addition, grouping of samples in each run according to their fetal fraction and compared them with controls of similar fetal percentage could be a solution. This would be easy if there was a method to quantify both male and female fetal fractions. The only accurate method reported for measuring fetal fraction is by next generation sequencing targeting SNPs (Nicolaides et al., 2013). The proposed method avoids the use of sequencing since is more expensive, time consuming and needs a bioinformatician. Therefore, quantification and normalization or grouping according to fetal fraction in the proposed methodology is not a solution at this point. The only approach that could eventually allow to the proposed method to develop a robust diagnostic test is independent of fetal fraction. This can be achieved using horizontal analysis. Horizontal analysis is when normalization is performed within the same sample without the need of control samples. In the proposed methodology, DMRs on a reference euploid chromosome can be used. In a normal case, the reference and the testing chromosomes are euploid, thus calculating the ratio of the two should be close to 1. In a trisomy 21 case, testing chromosome has three copies where the reference chromosome has two copies, therefore the ratio should be above 1. This is an ideal scenario for the proposed method that needs to include DMRs with the minimum variability or a panel of multiple DMRs on reference and testing chromosomes. If this scenario works, the only way that fetal fraction may affect the methodology is in cases that fetal fraction is very low at a degree that the developed MeDIP method is not able to enrich the methylated fetal regions. These cases should show very low immunoprecipitated product after quantification and excluded from the analysis. Otherwise, non-specific enrichment (maternal instead of fetal) can be quantified and in

this case false positives and negatives may appear. All these are hypothesis that should be considered when applying the proposed methodology for validation studies.
Companies offering a non-invasive prenatal test use sequencing based methods. According to Jiang K., in 2013, Sequenom (MaternityT21 PLUS test) and Verinata (Verifi test) are the two companies that sequence the whole genome for the detection of aneuploidies on chromosomes 13, 18, 21 and sex chromosomes having similar sensitivity range, with the former being more expensive. Analysis for this whole genome sequencing assay includes horizontal analysis first and then compare it with a known euploid case within the same run. Ariosa (Harmony test) and Natera (Panorama test) companies use targeted sequencing and single nucleotide polymorphism respectively on chromosomes 13, 18, and 21 with the latter to be more expensive. Analysis, in case of Ariosa, is similar to others, Natera on the other hand has developed its own algorithm for interpretation of the results. All the above mentioned companies include a fetal percentage cut off and ignore any case below that threshold. The analysis part of a methodology from such low abundance targets its challenging and needs the appropriate expertise to accomplish it.

5.3 Discovery of Biomarkers for Non-Invasive Prenatal Diagnosis of Down syndrome.

The mechanism by which the extra chromosome 21 produces the phenotype of Down syndrome is still under discussion. Chromosome 21 carries around 350 genes according to NCBI database. In case of trisomy 21, there is an extra chromosome 21 which carries extra 350 genes. One can assume that these extra genes should be over-expressed in the trisomy 21 compared to euploid cases. This hypothesis has being studied by other groups using trisomy 21 tissues such as fetal cerebellum, adult cerebellum, adult brain, fetal heart and lymphoblastoid cells (Lockstone H.E., et al., 2007; Li, et al., 2006; Guedj F, et al., 2014; Mao R, et al., 2005). Findings from a number of these studies support this hypothesis but there are others that disagree. A study by Laffaire J, et al., in 2009 compared postnatal cerebellum, at three time points, of trisomy 21 and euploid tissues. The findings of this study are very interesting since they show the expression variability at different time points on postnatal cerebellum. They reported differentially expressed genes between normal and trisomy 21 at four postnatal time points. They share only 12 differentially expressed genes and only 1 is

on chromosome 21. Studies on cardiac tissues indicate over-expression of only 6 chromosome 21 genes, and reports on Down syndrome T-lymphocytes show over-expression of 172 genes from which only 5 are on chromosome 21 (Giannone S, et al., 2004). In contrast with these findings, studies on adult Down syndrome brains, lymphoblastoid cells and fetal brains and hearts reported the over-expression of genes on chromosome 21 compared to non-chromosome 21 genes (Lockstone H.E., et al., 2007; Li, et al., 2006; Guedj F, et al., 2014; Mao R, et al., 2005). The current study scanned the whole transcriptome of four normal CVSs and two trisomy 21 CVSs aiming the identification of differentially expressed genes that could later be used in non-invasive prenatal diagnosis. In total 43 genes are up-regulated in Down syndrome CVSs from which only 11.63% are located on chromosome 21 including COL6A1 which has already be reported in the literature. It is reported to be up-regulated in Down syndrome heart and brain tissues (Li, et al., 2006; Mao R, et al., 2005) but there are no data confirming the remaining identified chromosome 21 DEGs. The current study used more stringent criteria compared to other reported studies. The fold change used in this study is 2, compared to other studies, which is 1.5. Fold change of two is the default used by Agilent software to identify significant differences among genes. Lowering the fold change might increase the number of identified chromosome 21 DEGs. This is not ideal since this study aims the identification of biomarkers with significant difference between trisomy 21 and normal cases. The future use of the identified biomarkers is maternal plasma where only ~10% of fetal nucleic acids (placenta) are found. Thus there is a need for identification of high sensitivity and specificity biomarkers with high discrimination power. Studies that work on expression differences between Down syndrome and normal tissues report only the up-regulated identified DEGs. This study investigated both up- and down- regulated DEGs between trisomy 21 and normal CVSs, aiming their association with Down syndrome. In total 175 up- and down-regulated DEGs are identified. Only one up-regulated DEG located on chromosome 21 is confirmed by other studies but eight down-regulated DEGs are found to be associated with Down syndrome diagnosis. These DEGs are PGF, ADAM12, PSG1, CGA, CGB, INHA, PAPPA and STS. As I mentioned earlier in the introduction part, currently there are biochemical serum tests combined with ultrasound to diagnose chromosomal abnormalities with detection rate of 82-87% in the first trimester. Biochemical serum test in the first trimester consists of two pregnancy specific proteins which are PAPPA and hCG (CGA and CGB produces hCG). An abnormal level of PAPPA (lower levels than normal) or/and hCG

(higher levels than normal) together with the ultrasound findings indicate the need for an invasive prenatal test. Transcripts of these proteins are found as down-regulated DEGs in this study which agree with PAPPA protein behaviour but not with hCG (human chorionic gonadotropin). Biochemical test results are unknown to us thus it is not feasible to know the protein levels of the two trisomy 21 CVSs tested to be associated with the transcription levels. In addition, the observed variability in the fold change may explain the low detection rate of the current biochemical test. During the second trimester of gestation there are available the triple and the quadruple serum tests for the diagnosis of fetal chromosomal abnormalities. The triple test involves the AFP (alpha fetoprotein) (lower than normal), hCG and estriol (lower levels than normal). Estriol levels are controlled by STS protein (Kashork, et al., 2002). STS gene is found to be down-regulated in trisomy 21 cases in this study which can explain the low levels of estriol. Quadruple test consists of the triple test proteins and ads inhibin A (INHA) (higher levels in trisomy 21 than normal). In this study INHA is found to be down-regulated. All the proteins used in the biochemical tests are produced by the placenta during pregnancy except AFP which is produced by yolk sac and liver during fetal development. This justifies why AFP was not detected as DEG in trisomy 21 against normal CVSs. In 2003, a new potential screening protein was identified associated with the first trimester of gestation, ADAM12, which is produced by placenta (Koster M.P.H, et al., 2011). Low levels of this protein are found to be associated with Down syndrome and Edgwards syndrome (trisomy 18). In terms of transcription level, this gene is identified in this study as down-regulated DEG in trisomy 21 CVSs. Placenta growth factor, PGF (lower levels than normal), and pregnancy specific beta-1-glycoprotein 1, PSG1 (higher levels than normals), proteins are also associated with Down syndrome and are used by some groups for the identification of trisomy 21 during the second trimester. Both PGF and PSG1 transcription levels are shown to be less in trisomy 21 CVSs in this study. Protein levels are controlled by transcription and post-transcriptional mechanisms. It is reported that only 27% of protein levels are associated with transcription level of the genes (Straub, 2011). A more recent study reports a 40% association (Csardi G, et al., 2014). Thus the identified transcription level of DEGs does not necessarily follow their protein level.

Non-invasive prenatal diagnosis uses maternal plasma to discriminate normal from abnormal foetuses. Maternal plasma consists of approximately 90% maternal nucleic acids and 10% fetal nucleic acids. Thus maternal background is huge. Ideal

biomarkers for the diagnosis of fetal abnormalities would be the ones that can be detected along the maternal background. DEGs between trisomy 21 and normal CVS does not necessarily mean that are discriminative biomarkers. Genes that are differentially expressed from the normal CVS and the mother (Analysis 1A) could be used as discriminative biomarkers. This is because up-regulated DEGs would be easier to be detected in the maternal background making the method more robust. This group of DEGs can be detected using SNPs found only in placenta. Alternatively, discriminative biomarkers could be the DEGs that are up- or/and down-regulated between trisomy 21 and normal CVSs but stable transcription level in the mother (less than 2 fold change). This will allow us to target both maternal cells and placental RNA transcripts and compared normal versus trisomy 21 since only the placenta RNA transcripts change. This group of DEGs (Analysis 1B) is very promising since will be very easy to detect the biomarkers without the need of SNPs and without the need of high sensitivity and specificity technologies. All proteins already involved in the biochemical tests fall in the latter group of DEGs. Thus a panel of large number of biomarker has the potential to develop and high detection rate test.

There are studies reporting the significant deleterious impact of maternal immune system on fetus during development. Maternal antibodies reactivity against fetal brains proteins interacting fetal brain development may lead to multiple congenital and developmental disorder (Fox, et al., 2012). Studies on autism show evidence of maternal antibody reactivity against their fetuses (Braunschweig, et al., 2007; Singer, et al., 2007). In addition, reports on mouse showed fetal behavioural deficits after injection with maternal antibodies (Cerdeno, 2010; Smith S, et al., 2007).Taking in consideration these findings, this study tried to compare mothers carrying trisomy 21 fetuses and mothers carrying normal foetuses to identify DEGs. As a result, no DEGs were found to be differentially expressed between the two groups. This may empowered the findings of the first analysis and allow us to assume that the DEGs identified are due to the extra chromosome 21. This is just an assumption which needs to be confirmed with a larger scale study using a larger number of samples in both normal and abnormal cases. DEGs identified could also be due to the tissue used, placenta, which is involved in the developmental process. Developmental process undergoes various transcriptional modifications which may explain DEGs between tissues.

5.4 Association of Differentially Expressed Genes with Down syndrome phenotype and disorders

All DEGs were investigated in terms of common phenotypes and disorders. Analysis 1A up- and down-regulated DEGs reported ~60% and ~70% not to share any phenotypes respectively. The remaining ~40% and ~30% of DEGs share common phenotypes such immune system, integument (skin), exocrine/ endocrine glands, hematopoietic system, embryogenesis, mortality/aging, growth/size/body, reproductive system and behaviour/neurological phenotypes. Behaviour and neurological phenotypes are caused only be the down-regulated DEGs. The majority of these phenotypes are associated with Down syndrome such as the immune system, integument, aging, growth, reproductive system and behaviour/neurological (Down syndrome, 2012). We can only hypothesize that these DEGs may be responsible for the Down syndrome phenotype. A larger-scale study should be performed in order to confirm these findings and make the hypothesis stronger. The findings of this study suggest that both up- and down-regulated DEGs may lead to an abnormal phenotype.

DEGs from analysis 1A are investigated in terms of common disorders. Down-regulated DEGs share several disorders with a ~70% not to share any disorder. In contrast, the up-regulated DEGs only appear to be related with cancer and tumours and more than 80% do not share any disorder. This might be due to the low number of identified up-regulated DEGs compared to down-regulated. The down-regulated DEGs are related with autoimmune diseases, ischemia, immunodeficiency, arthritis, pneumonia, stroke, leukaemia, Alzheimer, inflammation, cancer and tumours. All these disorders are found to be associated with Down syndrome (Down syndrome, 2012).

DEGs from analysis 1B investigated for common phenotypes and disorders as well. More than 50% and 70% of the up-and down-regulated DEGs do not share any common phenotypes respectively. The remaining DEGs are related to mortality/aging, muscle, vision, adipose tissue and cardiovascular system. All these phenotypes are related to the Down syndrome phenotype and diseases.. Disorders related with analysis 1B are chromosomal aberrations, osteoarthritis, pre-eclampsia, gastric, Down syndrome, Inflammation, cancer, Alzheimer and tumours. The 8.7% of down-regulated genes are directly related with Down syndrome. The remaining DEGs are related to disorders that are found to be expressed in Down syndrome cases. These are very promising findings since identified DEGs are related to phenotypes and disorders that appeared in Down syndrome patients but a large scale study will confirm the

importance of these DEGs and their capability to be used as non-invasive prenatal biomarkers.

5.5 Association of Differentially Expressed Genes with Methylation Patterns

Gene bodies and 4000bp upstream and downstream of the gene bodies, where the promoter regions and transcription start sites are located, were investigated. No association of DEGs with methylation status was found. This suggests that genes are regulated by other mechanisms such as transcription factors (Straub, 2011; Csardi G, et al., 2014) or that methylation characteristics are an intra-organism effect. The methylation and expression patterns were not technical replicates but biological replicates thus further experimentation is needed to conclude whether methylation affects expression of genes in cases with Down syndrome. In addition DMRs are fetal specific not trisomy 21 specific. DMRs are differentially methylated in fetus compared to mothers, not trisomy 21 fetus compared to mothers and normal fetus. DEGs identified in this study are trisomy 21 specific. This may explain the absence of correlation between methylation and expression patterns of the regions identified.

Conclusions

In conclusion, the current study managed to develop a robust MeDIP-dPCR methodology for the identification of trisomy 21 from very low amounts of DNA found in maternal plasma. The MeDI-dPCR methodology is now ready for pre-validation in order to construct a diagnostic formula which will eventually discriminate Down syndrome cases from normal. The same approach could be used for the identification of additional chromosomal abnormalities based on methylated specific biomarkers. In addition, findings of this study led to the potential of identification of new biomarkers based on RNA transcription level of differentially expressed genes between trisomy 21 and normal CVS. Differentially expressed genes which are up-regulated in trisomy 21 CVS and are stable in maternal cells are ideal and eliminate the major issue of limited fetal nucleic acid targets. These biomarkers could eventually lead to an easy and fast non-invasive prenatal test without the need of a high sensitivity and pricey technique.

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APPENDIX

Karyotype	Sample Name	B-Globin (GE/mL)	DYS14 (GE/mL)
46,XY	S1	211.3870455	4.73524531
46,XY	S2	1130.461633	0.962486674
46,XY	S3	844.1750303	3.453821789
46,XY	S4	1415.279048	1.48891276
46,XY	S5	568.9043106	2.599915083
46,XY	S6	834.0337614	1.965154349
46,XY	S7	1236.617571	2.215417169
46,XY	S8	645.8779378	3.87685132
46,XY	S9	1252.722968	2.579852981
46,XY	S10	215.4201468	9.178453733
46,XY	S11	409.4740633	3.284015464
46,XY	S12	270.0932549	10.33932347
46,XY	S13	460.6586228	3.389369559
46,XY	S14	1390.024631	4.009483414
46,XY	S15	443.357987	1.379927516
46,XY	S16	761.3513366	0.617182039
46,XY	S17	311.6287771	4.220320303
46,XY	S18	1871.671212	3.073609702
46,XY	S19	573.0344075	3.097164773
46,XY	S20	372.9280682	4.602768729
46,XY	S21	523.6296753	2.023138595
46,XY	S22	365.9453734	1.210797709
46,XY	S23	764.4442641	3.328580272
46,XY	S24	468.7024675	1.22213471
46,XY	S25	800.893474	1.733282439
46,XY	S26	1502.004545	2.62463512
46,XY	S27	742.8297646	2.203853514
46,XY	S28	248.7332143	5.279493833
46,XY	S29	460.7833117	8.268602604
46,XY	S30	474.4905519	4.566423821

46,XY	S31	899.3585227	3.087618592
46,XY	S32	593.2081656	2.89883197
46,XY	S33	365.920763	4.560543189
46,XY	S34	962.3742776	1.205294686
46,XY	S35	732.9679345	1.144353629
46,XY	S36	1498.158022	0.882352108
46,XY	S37	1146.19043	1.921971881
46,XY	S38	1474.994878	3.041032412
46,XY	S39	1456.572943	2.717883736
46,XY	S40	1587.739052	1.828662182
46,XY	S41	808.2835752	2.689515058
46,XY	S42	687.5688604	2.919722333
46,XY	S43	717.4050915	3.053045946
46,XY	S44	1409.227913	3.7374836
46,XY	S45	812.7377354	1.996688134
46,XY	S46	926.1328734	4.058114245
46,XY	S47	1784.241981	1.691451688
46,XY	S48	711.266165	1.461219027
46,XY	S49	1121.603406	1.338304481
46,XY	S50	517.0735649	2.624123048
46,XY	S51	2211.939307	0.591249202
46,XY	S52	772.0658214	3.741303517
46,XY	S53	926.8081981	1.076748405
46,XY	S54	1065.964552	3.150321834
46,XY	S55	511.9091672	3.430920958
46,XY	S56	1050.601247	4.695996086
46,XY	S57	441.56877	2.654256447
46,XY	S58	951.2440617	2.179216262
46,XY	S59	1033.605041	1.496835049
46,XY	S60	781.3282652	1.581221045
46,XY	S61	700.6535574	4.000658058
46,XY	S62	730.6826288	3.502854923
46,XY	S63	1310.875569	0.796645551
46,XY	S64	330.5790893	9.006697559

46,XY	S65	1461.696603	2.465376892
46,XY	S66	390.8687771	5.434223906
46,XY	S67	1093.925801	4.903384576
46,XY	S68	718.0746407	2.104075218
46,XY	S69	680.7465758	5.096288916
46,XY	S70	1354.18751	1.311859756
46,XY	S71	326.7559708	3.156053189
46,XY	S72	927.9609821	1.529972877
46,XY	S73	1665.757357	2.744976012
46,XY	S74	1538.951696	1.915272865
46,XY	S75	839.8976337	9.616896746
46,XY	S76	1138.435219	2.530512006
46,XY	S77	1221.405081	3.32521506
46,XY	S78	906.0779464	3.076179326
46,XY	S79	1054.582244	4.263043516
46,XY	S80	900.72947	3.575741345
46,XY	S81	671.6990314	2.227072846
46,XY	S82	1676.235931	3.266781667
46,XY	S83	1702.332235	1.478640408
46,XY	S84	581.647381	3.239552113
46,XY	S85	569.5100541	9.228546946
46,XY	S86	601.5497835	5.899304601
46,XY	S87	1940.96664	0.790281206
46,XY	S88	386.329398	2.999587243
46,XY	S89	276.8946226	3.01701889
46,XY	S90	610.2306818	5.198046247
46,XY	S91	457.9832048	3.533532523
46,XY	S92	378.3554735	1.915377088
46,XY	S93	861.5551542	2.928450605
46,XY	S94	324.5587256	2.493561063
46,XY	S95	293.2859781	0.525899679
46,XY	S96	588.3660511	1.840983427
46,XY	S97	239.7489093	3.185241216
46,XY	S98	739.7379762	3.270173514

46,XY	S99	584.5480925	7.988226107
46,XY	S100	496.771043	5.179742539
46,XY	S101	301.69583	11.18196839
46,XY	S102	722.8612689	4.074378665
46,XY	S103	404.9324675	5.636958196
46,XY	S104	371.4936404	3.662236397
46,XY	S105	694.2138596	3.366863282
46,XY	S106	924.9782062	1.66598465
46,XY	S107	685.4734307	2.468042555
46,XY	S108	1376.346239	0.988044527
46,XY	S109	2664.42404	0.515153531
46,XY	S110	277.3400162	4.573049676
46,XX	S111	1053.219438	
46,XX	S112	1002.509745	
46,XX	S113	903.3008988	
46,XX	S114	1623.325909	
46,XX	S115	550.4913149	
46,XX	S116	909.2417045	
46,XX	S117	995.6398742	
46,XX	S118	755.038819	
46,XX	S119	363.7286661	
46,XX	S120	412.4778883	
46,XX	S121	742.9583333	
46,XX	S122	608.9872768	
46,XX	S123	327.4725528	
46,XX	S124	509.5831981	
46,XX	S125	688.1172348	
46,XX	S126	442.2099432	
46,XX	S127	743.3984848	
46,XX	S128	444.348796	
46,XX	S129	247.8855154	
46,XX	S130	273.5378044	
46,XX	S131	785.8402868	
46,XX	S132	715.6750406	

46,XX	S133	348.2574472	
46,XX	S134	592.1405303	
46,XX	S135	646.6311418	
46,XX	S136	486.1437635	
46,XX	S137	567.1136364	
46,XX	S138	230.3065544	
46,XX	S139	801.6212121	
46,XX	S140	799.8410714	
46,XX	S141	567.0822998	
46,XX	S142	1128.493141	
46,XX	S143	503.1606331	
46,XX	S144	361.017546	
46,XX	S145	133.7063596	
46,XX	S146	766.8932224	
46,XX	S147	356.222017	
46,XX	S148	741.8518466	
46,XX	S149	776.809145	
46,XX	S150	1239.732224	
46,XX	S151	1271.787679	
46,XY	S152	384.4593615	5.030145251
46,XY	S153	546.749398	4.405304974
46,XY	S154	613.603842	3.667228087
46,XY	S155	439.125974	4.6603509
46,XX	S156	608.9872768	
46,XY	S157	497.3282603	3.766324071
46,XX	S158	327.4725528	
46,XX	S159	969.5587662	
46,XY	S160	276.6863924	5.484623035
46,XY	S161	587.7576569	2.542797225
46,XY	S162	814.4416396	2.83046204
46,XY	S163	422.1719968	6.527750674
46,XY	S164	289.386411	6.251489644
46,XX	S165	506.6920996	0.108591953
46,XY	S166	231.1967735	6.645298112

46,XY	S167	568.8563447	4.880779273
46,XY	S168	69.09712284	5.278815424
46,XY	S169	136.738631	3.956373305
46,XY	S170	561.8407197	2.635364402
46,XY	S171	421.4971794	2.600176448
46,XY	S172	646.263961	3.367781405
46,XY	S173	731.1389069	3.39047263
46,XY	S174	344.0676732	4.594863502
46,XY	S175	249.4412514	4.317549741
46,XX	S176	353.1908888	
46,XX	S177	991.3333874	
46,XX	S178	206.8753653	
46,XX	S179	460.9584497	
46,XX	S180	321.8757779	
46,XX	S181	530.7389069	
46,XY	S182	453.4223485	1.548317513
46,XY	S183	956.6196429	2.188338191
46,XY	S184	583.4707792	2.527071265
46,XX	S185	641.8775568	
46,XX	S186	1253.969196	
46,XY	S187	785.3871483	2.564326972
46,XX	S188	300.0969372	
46,XX	S189	460.9173755	
46,XY	S190	251.8329004	3.072192096
46,XX	S191	414.1185823	
46,XX	S192	530.6366071	
46,XY	S193	499.2433442	1.037749822
46,XX	S194	546.8997511	
46,XY	S195	1228.7529	1.944590782
46,XY	S196	526.9857752	1.669347557
46,XX	S197	838.8310877	3.183576358
46,XX	S198	344.2877909	
46,XX	S199	310.985878	
46,XX	S200	311.5314786	

46,XY	S201	160.2584551	5.981309362
46,XX	S202	195.9092275	
46,XX	S203	372.3533009	
46,XX	S204	108.4720563	
46,XX	S205	379.891971	3.317366421
47,XY,+21	S206	1520.694912	41.20745989
47,XY,+21	S207	647.4843734	93.84219189
47,XY,+21	S208	1271.787679	111.1763887
47,XY,+21	S209	643.9067706	28.31063149
47,XY,+21	S210	2659.161924	16.63525679
47,XY,+21	S211	641.0833929	30.04853274
47,XY,+21	S212	415.3508019	16.55729119
47,XY,+21	S213	1001.062045	41.56570382
47,XY,+21	S214	704.8925974	23.13304951
47,XY,+21	S215	1016.72599	24.83995184
47,XY,+21	S216	683.1474594	26.8960276
47,XY,+21	S217	1359.751299	28.47460633
47,XY,+21	S218	3059.542045	100.3070414
47,XY,+21	S219	940.0474229	50.76827854
47,XX,+21	S220	790.1470671	
47,XX,+21	S221	766.9991613	
47,XX,+21	S222	748.5350379	
47,XX,+21	S223	946.8514448	
47,XX,+21	S224	969.5587662	

Table 1: Quantification and gender classification in maternal plasma.

Karyotype	Sample Name	B-Globin (GE/mL)	DYS14 (GE/mL)
46,XY	S1	5596798.312	9.819385714
46,XX	S2	5449175.022	
46,XY	S3	2802420	12.89807381
46,XX	S4	4022234.61	
46,XY	S5	4951624.242	4.005314935
46,XX	S6	4557235.065	

46,XY	S7	7464327.532	2.886097554
46,XY	S8	1690471.364	
46,XX	S9	5297089.394	
46,XY	S10	2221995.238	
46,XX	S11	4794433.593	
46,XX	S12	2947133.723	
46,XX	S13	2736793.506	
46,XY	S14	2154173.571	1.546364156
46,XY	S15	4358363.29	
46,XX	S16	3333183.42	
46,XY	S17	3196955.022	6.246415931
46,XY	S18	3729051.732	8.601781494
46,XX	S19	4216235.628	
46,XX	S20	6752767.532	
46,XX	S21	5137238.528	
46,XY	S22	5289600.433	4.744368019
46,XX	S23	4204492.597	
46,XX	S24	3430607.316	
46,XY	S25	4083315.758	2.264455216
46,XX	S26	3429685.065	0.444405455
46,XY	S27	4087896.234	1.348131461
46,XY	S28	2928327.229	1.090006558
46,XY	S29	3017399.307	5.443106851
46,XY	S30	7054004.329	2.340869524
46,XX	S31	3509552.641	5.752916899
46,XY	S32	3362411.385	1.437348788
46,XX	S33	7155783.81	
46,XX	S34	7596287.619	
46,XY	S35	7255781.991	21.18998182
46,XY	S36	2113195.801	0.555205823
46,XX	S37	2212133.766	
46,XX	S38	4210186.234	
46,XY	S39	6848158.442	19.60485974
46,XY	S40	7624280.519	7.511293182

47,XY,+21	S41	4943279.87	21.51815628
47,XX,+21	S42	9232391.472	0.909527221
47,XY,+21	S43	5275565.541	18.52938377
47,XY,+21	S44	3139013.68	4.958913506
47,XY,+21	S45	5439182.078	4.866201753
47,XY,+21	S46	3358279.524	
47,XY,+21	S47	5326588.268	
47,XY,+21	S48	3189938.442	
47,XY,+21	S49	2849497.403	
47,XX,+21	S50	5801630.736	
47,XX,+21	S51	4118242.641	
47,XX,+21	S52	4911057.662	
47,XX,+21	S53	2042125.022	
47,XX,+21	S54	3578731.645	
47,XX,+21	S55	3393621.775	
47,XX,+21	S56	4273615.195	
47,XY,+21	S57	6457923.16	7.048350433
47,XY,+21	S58	8199495.065	30.48030065
47,XX,+21	S59	12598222.73	
47,XX,+21	S60	6955423.896	
47,XY,+21	S61	10575988.66	25.22733983
47,XY,+21	S62	8490304.416	40.46015844
47,XY,+21	S63	4047990.433	25.07510216
46,XY	S64	2856768.139	
46,XY	S65	4402967.489	4.404304827
46,XY	S66	3784180.714	4.773474026
46,XX	S67	3176418.442	
46,XX	S68	1455441.45	
46,XX	S69	8181451.991	
46,XX	S70	5848200.087	
46,XX	S71	10180680.52	
46,XX	S72	11239185.11	
46,XX	S73	7415463.247	
46,XX	S74	7869890.519	

46,XX	S75	6344086.017	
46,XY	S76	7222261.039	
47,XY,+21	S77	5856090.519	30.57621212
47,XY,+21	S78	9731349.091	20.94629654
47,XY,+21	S79	7260664.242	45.87210281
47,XX,+21	S80	7421824.156	
69,XXY	S81	11685445.45	20.65833831
47,XX,+21	S82	7945184.416	
47,XX,+21	S83	6044207.013	
47,XY,+21	S84	6504299.957	67.67653571
46,XX	S85	7769369.481	
46,XX	S86	7045841.169	
46,XX	S87	6914279.61	
47,XX,+21	S88	6109378.485	
47,XY,+21	S89	11199171.43	40.61732121
47,XX,+21	S90	12172309.74	
46,XY	S91	8255673.377	8.66352316
46,XX	S92	7314442.792	
47,XX,+21	S93	8865416.45	
47,XY,+21	S94	7180616.234	13.52130693
46,XY	S95	5250337.706	12.84119329
46,XY	S96	9326572.9	4.646521558
46,XX	S97	7844929.394	
46,XY	S98	5550671.861	96.76551948
46,XY	S99	6174394.978	10.29608537
46,XX	S100	6787856.71	
46,XX	S101	6988223.377	
46,XX	S102	7025948.268	
46,XY	S103	6435607.013	39.25535866
46,XY	S104	8255964.762	
46,XY	S105	6022621.602	
46,XY	S106	8074044.719	
46,XX	S107	11860158.27	
46,XY	S108	18960406.06	

46,XY	S109	12992910.17	
47,XX,+21	S110	8571801.948	
47,XY,+21	S111	11444549.35	
46,XY	S112	10975599.35	
46,XY	S113	6502401.515	
46,XY	S114	12738806.93	
47,XY,+21	S115	11156304.63	
46,XX	S116	9475127.013	
46,XY	S117	11644762.77	
46,XX	S118	4610302.338	
46,XX	S119	11214848.83	
46,XY	S120	10447573.38	
46,XY	S121	6863448.052	
46,XX	S122	6723594.545	
46,XY	S123	6112849.567	
46,XY	S124	12973967.75	

Table 2: Quantification and gender classification in maternal whole blood

Sample Collection for DNA-assays

Maternal blood sample at the time of CVS or AF sampling (10-17 weeks of gestation)

- Take the sample preferably **before** CVS or AF collection.
- Take 15ml of peripheral blood in **EDTA tubes** and place them at 4-8^{o}C until processed. Important: the sample can be kept at +4^{o}C for a *maximum* of 4 hours before processing. Then process the sample as follows:
- Split each sample in 8 tubes A (8 x1ml) and 1 tube B (7ml). Please make sure to mix well the EDTA tube before aliquoding.

Processing of Tubes A (8 x1ml):

- Store in -80^{o}C.
- Label the tube with the patient's name along with the designation "**WBM**".

Processing of Tube B (7ml):

- Centrifuge maternal blood at 1600g for 10 min at 4^{o}C.
- Separate **Buffy Coat (I)** and **Plasma (II)**.
- Process Buffy Coat (I) as follows:

- Centrifuge at 2500g for 10 min at 4^{o}C.
- Remove any residual plasma.
- Store buffy coat at -80^{o}C.
- Label the tube with the patient's name and the designation "**BC**".

- Process Plasma (II) as follows:
  - Centrifuge at 16000g for 10 min at 4^{o}C.
  - Remove any residual cells.
  - Transfer plasma into new tube so that all blood cells are removed. Avoid transferring the last 5mm of plasma.
  - Store plasma in aliquots of 600ul at -80^{o}C.
  - Label the tube with the patient's name and the designation "**PL**".

Sample Collection for RNA-assays

Maternal blood sample at the time of CVS or AF sampling (10-17 weeks of gestation)

- Take the sample preferably **before** CVS or AF collection.
- Take 20ml of peripheral blood in **EDTA tubes** and place them at 4-8^{o}C until processed. Important: the sample can be kept at +4^{o}C for a *maximum* of 4 hours before processing. Then process the sample as follows:

A. Collection of White Blood Cells in Trizol

This procedure needs to be done as quickly as possible to avoid degradation of RNA

- Transfer 600ul of whole blood in two 1.5ml tubes (2x300ul). **Please keep the remaining blood for collection of plasma (refer to section B).**
- Add 900ul ice cold (4 ^{o}C) Erythrocyte Lysis Buffer (ELB) into each tube and leave at room temperature for 15 minutes
- Centrifuge at 3000rpm for 5 minutes
- Remove supernatant
- Add 1ml ice cold Erythrocytes Lysis buffer (ELB) into each tube and immediately centrifuge at 3000rpm for 5 minutes
- Remove supernatant
- Add 1ml Trizol into each tube
- Store at -80 ^{o}C
- Label the tube with the patient's name, a patient's unique code and the designation "**WCR**".

Note:

- You will be provided with Trizol which must be stored at 4 ^{o}C
- You will be provided with Erythrocyte Lysis Buffer which must be stored at room temperature

B. Collection of Plasma

- Centrifuge maternal blood at 1600g for 10 min at 4^{o}C.
- Separate **Buffy Coat (I)** and **Plasma (II)**.

a) Process Buffy Coat (I) as follows:
   - Centrifuge at 2500g for 10 min at 4^{o}C.
   - Remove any residual plasma.
   - Store buffy coat at -80^{o}C.
   - Label the tube with the patient's name, a patient's unique code and the designation "**BCR**".

b) Process Plasma (II) as follows:
   - Centrifuge at 16000g for 10 min at 4^{o}C.
   - Remove any residual cells.
   - Transfer plasma into new tube so that all blood cells are removed. Avoid transferring the last 5mm of plasma.
   - Store plasma in aliquots of 600ul at -80^{o}C.
   - Label the tube with the patient's name, a patient's unique code and the designation "**PLR**".

Note:

- Plasma should be collected within 6 hours after blood drawn

C. Collection of CVS in Trizol

- Add CVS tissue in 1ml Trizol
- Make sure that the tissue is fully submerged in Trizol reagent by making sure that no remaining tissue is left on the walls of the tubes.
- Store at -80^{o}C.
- Label the tube with the patient's name, a patient's unique code and the designation "**CVSR**".

Note:

- Tissue must be submerged in Trizol **within 4 hours** after harvest

Fetal Quantification in plasma using Taqman Real-Time PCR

Materials Required:

- ✓ iPrep PureLink Virus Kit (Invitrogen Cat. No: IS1008)
- ✓ 430-500μL of Plasma Sample
- ✓ Human Genomic DNA: Male (Promega Cat. No: G1471)- for standard curve use
- ✓ Human Genomic DNA: Male (verified male sample of unknown identity)
- ✓ Human Genomic DNA: Female (verified female sample of unknown identity)
- ✓ Primers: GTGCACCTGACTCCTGAGGAGA (b-globin-345F), CCTTGATACCAACCTGCCCAG (b-globin-455R), GGGCCAATGTTGTATCCTTCTC (DYS14F), GCCCATCGGTCACTTACACTTC (DYS14R)
- ✓ Probes: AAGGTGAACGTGGATGAAGTTGGTGG (b-globin 402T), TCTAGTGGAGAGGTGCTC (DYS)
- ✓ TaQman Universal Master Mix (Applied Biosystems Cat. No: 4304477)
- ✓ HPLC-dH20
- ✓ 384-Optical Reaction Plate (Applied Biosystems Cat. No: 4309849)
- ✓ MicroAmp Optical Adhesive Films (Applied Biosystems Cat. No: 4311971)

Plasma DNA Extraction using iPrep PureLink Virus Kit (IS10008)

Prior to starting, load **430-500μL** for each sample in tubes (use elution tubes found in the iPrep kit).

Insert the iPrep™ Card: Viral DNA/RNA prior to turning on the instrument.

1. Ensure the power switch on the iPrep™ Instrument is on the OFF position.

2. Open the iPrep™ Card Slot and insert the iPrep™ Card into the slot in the correct orientation (arrow on the card is at the top and card label is facing your left side).

3. Using the power switch located on the left side of the instrument, turn the instrument ON.

If the card is fully inserted in the correct orientation, all axes return to their original positions automatically. The digital display shows the version for the iPrep™ which changes in a few seconds to display the Main menu.

4. Press Start to run a protocol.

5. Open the iPrep™ instrument door. Remove the iPrep™ Cartridge Rack, and iPrep™ Tip and Tube Rack to set up the platform.

6. Remove the desired number of iPrep™ PureLink™ Virus Cartridges from the box. To collect any solution from the foil, tap the cartridge to deposit the solution at the bottom of the tube.

Note: You can load 1–13 cartridges on the rack depending on the number of samples that you wish to process.

7. Insert the iPrep™ Sample Processing Tube in the heated tube position of the cartridge (position 11) for each of the iPrep™ Virus Cartridge that is used.

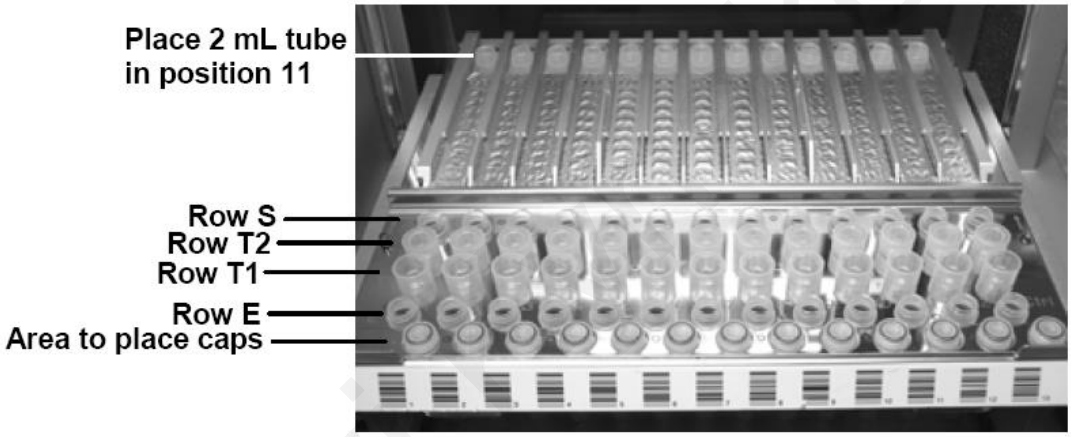
8. Load the cartridges on the iPrep™ Cartridge Rack and insert the loaded rack on the iPrep™ platform.

9. Load the iPrep™ Tip and Tube Rack as follows:

• Load the first row (labeled as E) with 1–13 elution tubes without caps (you may place the caps on the rack as shown in the figure below). **Elution tubes must be labeled on the cap and side.**

• Load the third row (labeled as T2) with iPrep™ Tips iniPrep™ Tip Holders.

• Load the fourth row (labeled as S) with iPrep™ Sample and Elution Tubes containing samples.



10. Read the sample and elution tube barcode, if needed (otherwise press Enter).

11. Insert the iPrep Tip and Tube rack on the iPrep™ platform.

12. Close the iPrep™ instrument door.

13. Press Enter to continue.

14. When prompted, select the appropriate lysis mode (**inline**), sample volume (**400µL**), and elution volume (**50µL**).

15. Ensure that you have loaded the cartridges, tubes, and tips in the appropriate positions and that elution tubes do not have any caps. Make sure that you have loaded a 2mL tube in the heated tube position of the cartridge (position 11).

16. Press Start. The automated purification protocol begins and various steps of the protocol including the approximate time remaining are displayed on the digital display (**approximately 40 minutes**).

Important: Do not open the door once the protocol has begun.

17. At the end of the run, the instrument beeps briefly and the digital display shows "Protocol Finished" for 10 seconds. The Main menu appears after 10 seconds.

18. Open the instrument door. Remove and cap the elution tubes containing the purified DNA (**approximately 50μL per sample**). Use the DNA for the desired downstream application or store the purified DNA at –20 C°.

19. Discard the used cartridges, tips, and sample tubes into biohazard waste. Do not reuse the cartridges.

Preparation of Primers and Probes

List of Primers and Probes (ordered by Applied Biosystems)

Name	Sequence	Concentration
b-globin-345F Primer **1**	GTGCACCTGACTCCTGAGGAGA (Sedrak *et al.*)	10nmol
b-globin-455R Primer **2**	CCTTGATACCAACCTGCCCAG (Sedrak *et al.*)	10nmol
DYS14forward Primer **1**	GGGCCAATGTTGTATCCTTCTC (Zimmermann *et al.)*	10nmol
DYS14reverse Primer **2**	GCCCATCGGTCACTTACACTTC (Zimmermann *et al.)*	10nmol

Upon arrival, each Primer is diluted to 100μM stock by the addition of HPLC-dH20.

Corresponding Probes for each Primer set

Name	Sequence	Reporter	Quencher	Concentration
b-globin 402T **Set 1**	AAGGTGAACGTGGATGAAGTTGGTGG (Sedrak *et al.*)	FAM	TAMRA	100μM
DYS **Set 2**	TCTAGTGGAGAGGTGCTC (Zimmermann *et al.)*	VIC	MGBNFQ	100μM

Note: Probes are already in solution so there is no need for addition of water.

Preparation of working solutions of Primers and Probes

Prepare a working stock solution for each Primer/Probe set for b-globin (Set 1) and DYS14 (Set 2).

Each Primer/Probe mix is prepared using the Stock concentrations of 100μM prepared above.

Working stock solutions:

Primer 1 4.5μL
Primer 2 4.5μL
Probe 3μL
dH20 288μL
Total **300μL**

This mix can be aliquoted and stored in -20 C° for future use.

Set up the standards (D1-D5)

Note: The standards must be prepared fresh every time prior to plate preparation.

Use Human Genomic DNA: Male Product code: G1471 Promega (concentration varies)

- Measure the concentration each time prior to use
- A standard curve is included for each Primer/Probe set and each standard is run in triplicate
- Prepare serial dilutions

1:10 **(D1)**
1:100 **(D2)**
1:1000 **(D3)**
1: 10000 **(D4)**
1:100000 **(D5)**

Preparation of serial dilutions: Add 36μL of dH20 in 5 Eppendorf tubes. Add 4μL of male control DNA in the first tube containing 36μL of dH20. Mix well by vortexing (this is D1). Remove 4μL of D1 and mix in second tube (this is D2). Mix well by vortexing. Continue serial dilutions until D5.

Preparation of control DNA (positive control, negative control)

Prepare dilutions of male control DNA at 4ng/μL and female control DNA at 4ng/μL. These samples are used in every plate as controls.

Preparation of TaqMan Reactions

Each Taqman reaction contains the following:

5μL DNA (directly added to the plate)

10μL Taqman Mix

4μL Primer/Probe Mix (prepared above)

1μL dH20

Total 20μL

Prepare a mix of

1. Taqman reagent (10μL per sample)

2. Primer/Probe Mix (working stock solution) (4μL per sample)

3. dH20 (1μL per sample)

Note: Calculate the required volume of the mix for all reactions in triplicate but estimate at least 5 additional reactions.

384-well plate preparation (A simple schematic of the plate is shown below.)

- Add DNA (5μL) for standards (D1-D5), samples and controls (positive: male, negative: female)
- Add dH20 water in 2 wells that are used as blanks for each Primer set (b)
- Add Taqman/Primer/Probe/dH20 mix to each well (15μL)

Real-time PCR step

Set up the following program on the ABI 7900HT Fast Real-time PCR System:

- 2 minutes incubation at 50 C°
- 10 minutes denaturation 95 C°
- 45 cycles of 95 C° for 15 seconds and 60 C° for 1 minute

Pre-analytical Quality Control checklist

Both data sets (b-globin and DYS14) are evaluated for the following parameters:

- **Standard curve slope check** (from the exported files)

The slope values of the standard curves should be within the acceptable range of -3.32 ± 0.25. Outliers can be removed in order to improve slope values that do not lie in this range. Following this process if any slope values are still not within the acceptable range then the experiment should be repeated.

- **Ct values variability check**

The variation of the Ct values between the three replicate reactions of each sample should not exceed 0.4 Ct. If 0.4 Ct is exceeded then the most distant replicate from the median Ct value should be excluded and the remaining replicates should be considered for the analysis.

If one of the three replicates gives "Undetermined" value and the remaining two have values > 0.4 Ct apart then the experiment for that sample should be repeated.

- **Presence of contamination check**

The Blank wells used as controls should not show presence of any product. "Undetermined" or a Ct value > 35 is expected. If the Ct value for a Blank well is < 35 then there is indication for contaminants and the experiment should be repeated.

Quality assessment of the DYS14 data set

Evaluate the male and female controls of the DYS14 data set as follows:

- The percentage of (DYS14/b-globin) of the male control should be in the range of 50% ± 10%.
- The female control should be "Undetermined" or have a Ct value > 35 that is associated with non-specific amplification.

If any of the above conditions fails then the experiment is repeated.

MeDIP Protocol

- ✓ **Use 10X IP Buffer**
- ✓ **Prepare 1X IP Buffer**

For 50 ml solution add:

- ✓ 45ml HPLC water
- ✓ 5ml 10X IP buffer
- ✓ Mix well and store at room temperature

Reagent	Full name of reagent	Company	Catalogue Number
Antibody (for MeDIP)	5-mC 33D3 Monoclonal Antibody (Premium) – 2ug/ul, 500ug	Diagenode	Previous Cat no: MAb-081-500 New Cat no: C15200081-500
Beads (for MeDIP)	Dynabeads M-280 Sheep anti-Mouse IgG – 10ml (~10mg/mL)	Life Technologies	11202D
pH shift buffer (for pH shift)	TF2.7 Elution buffer – Ethanolamine pH11 – 30mL	Ademtech	
Lysis binding buffer (for clean-up)	Lysis binding buffer – 25mL	Ademtech	
Beads (for clean-up)	Silica-Masterbeads – 3.5mL	Ademtech	
qPCR MasterMix Plus for SYBR Green I (for qPCR)	qPCR MasterMix Plus for SYBR® Assay ROX– 50 mL, 4000 RXN (25 µL)	Eurogentec	RT-SN2X-20+

Methodology

DAY 1 - Samples and spikes preparation:

1. DNA extraction of non-pregnant whole blood sample and CVS.
2. Measure the concentration of the samples using qPCR.
3. Preparation of the spikes. For 1 series of experiment: 3 spikes (20%, 10%, 5%) – 3 IPs per spike.
4. Shear the spike in samples (sonication) using the bioruptor from Diagenode. The sonicator settings are 7 cycles of 30s ON/OFF**.**

5. Store the spikes at -80^{o}C if not use immediately.

DAY 2- IP protocol:

6. Place the spikes on ice to thaw.
7. **Proceed with the washes of beads (washed beads needed at step 13). For a series of 20%, 10% and 5% - 3IPs per spike, total 9 IPs, prepare the following master mix (prepare a separate tube for each series of spikes):**

MM in a standard 2ml tube:

- 3ul dynabeads/reaction (+2extra reactions) => 3ul/reaction x 11 IP reactions = 33ul beads
- 1700ul PBS-BSA 0.05%

IMPORTANT! When adding the dynabeads mix with your tip 3 times (to clean your tip). The container of the dynabeads should be placed always in an upright position in the fridge and when used so that the dynabeads are at all times in an aqueous solution. Mix the dynabeads well before using them. Check if there are any remaining dynabeads at the bottom of the container and continue the gentle mix ***(NO VORTEX)*** *until all the dynabeads are removed from the bottom to end up to a homogeneous solution.*

8. Incubate 5’ at room temperature (RT) with vortex.
9. Capture for 2’ and pipette off the supernatant.

IMPORTANT! Remove the buffer by using a 1000p pipette as follows: Place the tip to the bottom of the tube while being on the magnetic rack with slow movements so that you remove any bubbles that may exist without disturbing the column of the dynabeads. Slowly remove the buffer.

10. Add 1700ul PBS-BSA 0.05%.

IMPORTANT! Make sure that you do not leave the dynabeads without solution for more than 10 sec. Dryness of the dynabeads may reduce their activity. The washes of the dynabeads before use are essential for the removal of preservatives.

11. Incubate 5' at room temperature (RT) with vortex.
12. Capture for 2' and pipette off the supernatant.
13. Add 1700ul 1xIP buffer (quick wash).
14. Capture for 2' and pipette off the supernatant.
15. Add 33ul 1xIP buffer (enough for 9IPs & 2 extra – 3ul beads per IP).
16. Keep the washed beads on ice until needed.
17. Transfer 44ul of sample per IP reaction into a 96 well LowBind plate (total wells needed = 3 IP reactions x 3 spikes = 9 wells).
18. Denature for 10'@95^{o}C in a PCR machine.
19. Transfer immediately on ice and keep on ice until it cools down (5 minutes).
20. Spin down the plate and add separately to each IP reaction:
    - **1ul** of Ab Diagenode@0.2uG (diluted 1:10 in 1xIP buffer).
      Note: stock Ab is @2uG and needs 1:10 dilution.
    - **5ul** of 10xIP buffer.
    - **3ul** of washed beads (Dynabeads).
21. Incubate for 3 hours @ 4 ^{o}C with rotation (20rpm).
22. Spin down the plate, capture for 2', pipette off the supernatant and add 150ul 1xIP buffer.
23. Incubate for 10' at room temperature on a rotator (40rpm).
24. Repeat for 2 times (total 3 washes).

pH shift:

25. After the last washing step, place the 96 well LowBind plate on the magnet for 2 min.
26. Pipette off the supernatant - **remove the entire remaining buffer**.
27. Remove the plate from the magnet and resuspend the beads with **25µl of TF2.7 Elution Buffer** by pipetting. Mix by vortexing.
28. Incubate 5 min at RT under agitation (1000 rpm).
29. Spin down the plate and place it on the magnet for 2 min.
30. Slowly transfer the supernatant into a new clean 96 well LowBind plate.

DNA Purification post-MeDIP (clean-up)

For Nucleic acid Binding:

31. Add **25µL of Lysis Binding Buffer** to 25µL of TF2.7 eluate in the 96 well LowBind plate.
32. Add **25µL of Isopropanol** and **3.5µL of Silica-Masterbeads** (105µg) to the reaction mixture. Mix by pipetting.
33. Incubate 5 min at room temperature under agitation (1000 rpm).

Washing

34. Spin down the plate and place it on the plate on the magnet for 2 min.
35. Pipette off the supernatant.
36. Remove the plate from the magnet and resuspend the beads with **75µl of Washing Buffer** (Ethanol 70%) by pipetting. Mix by vortexing.
37. Spin down the plate and place it on the plate on the magnet for 2 min.
38. Pipette off the supernatant.
39. Remove the plate from the magnet and resuspend the beads with **75µl of Washing Buffer** (Ethanol 70%) by pipetting. Mix by vortexing.
40. Spin down the plate and place it on the plate on the magnet for 2 min.
41. Pipette off the supernatant.

Elution

42. Remove the plate from the magnet and resuspend the beads with **25µl of DNase RNase free water** by pipetting. Mix by vortexing.
43. Incubate 5 min at 50°C under agitation (1000 rpm).
44. Spin down the plate and place it on the plate on the magnet for 2 min.
45. Slowly transfer the supernatant into a new clean microtube.

46. After clean-up, pool the 3IPs of the same spike.

47. Store the pool IP sample at -20^{o}C for further use.

DAY 3 – qPCR/dPCR

qPCR Protocol

1. Proceed with real time qPCR (relative quantification) – triplicate reactions per sample.
   Note:

   - Total reaction volume 20ul:
     - ⇨ 4ul sample
     - ⇨ 6ul primer (1uM) –
     - ⇨ 10ul SYBR green

BIO-RAD Real-Time thermocycler (CXF384 Real-Time System) conditions:

STAGE		**TIME (min)**	**TEMPERATURE (˚C)**
Stage 1 Initial denaturation and enzyme activation	Step 1	10:00	95
Stage 2 Amplification-Extension	Step1 Step 2 (39 repeats)	0:15 1:00	95 60
Stage 3 Melting curve*	Step 1 Step 2 Step 3	0:15 0:15 0:15	95 60 95

* The melting curve is necessary to evaluate the specificity of the amplification reaction.

ddPCR Protocol (Duplex Reaction)

1. Prepare 20X working stock of Probe/Primer Mix

2. Prepare PCR Reaction Master Mix

Reagent	Amount/rxn (ul)	X ……. Samples
2X ddPCR Mix	10 ul	
20X Probe/Primer Mix 1	1 ul	
20X Probe/Primer Mix 2	1 ul	
Total	12 ul	

Note : Keep mix at RT

3. Add 12ul of reaction mix in 200ul PCR tube

4. Add 8ul of DNA in each tube to a total of 20ul. Mix by pipetting up and down twice *slowly*.
5. Transfer ALL 20ul of PCR reaction in the cartridge/holder apparatus in the appropriate well

6. Add 70ul of oil in the appropriate well

7. Ensure that no bubbles are present in the sample wells

8. Cover wells with a new membrane and place in the droplet generator

9. After droplets are generated: Remove cartridge/holder from the Generator and *slowly* add the sample in the appropriate well of a 96-well plate

   Note: Cover each column with a scotch tape, if more than one column is used on the plate.

10. Place aluminium film (red line facing upwards) on the plate and heat seal.

11. Place plate in a thermal cycler for PCR and start the appropriate program

12. Once PCR is finished: Turn on PC and Reader and lunch the QuantaSoft software

13. Place plate in the adaptor and secure black cover. Place adaptor with plate in its position in the reader and close door

14. To Set up a new Plate and Begin reading

15. Analyze the results using the QuantaSoft program