CHAPTER I

INTRODUCTION

1. Statement and significance of the problem

Down syndrome is the most common cause of mental retardation, occurring in about one out of every 800 newborns, with the incidence increasing markedly in the offspring of women over 35 years old. This condition derives its name from John Langdon Down who first described the syndrome in 1866 (Muller and Young, 2001a).

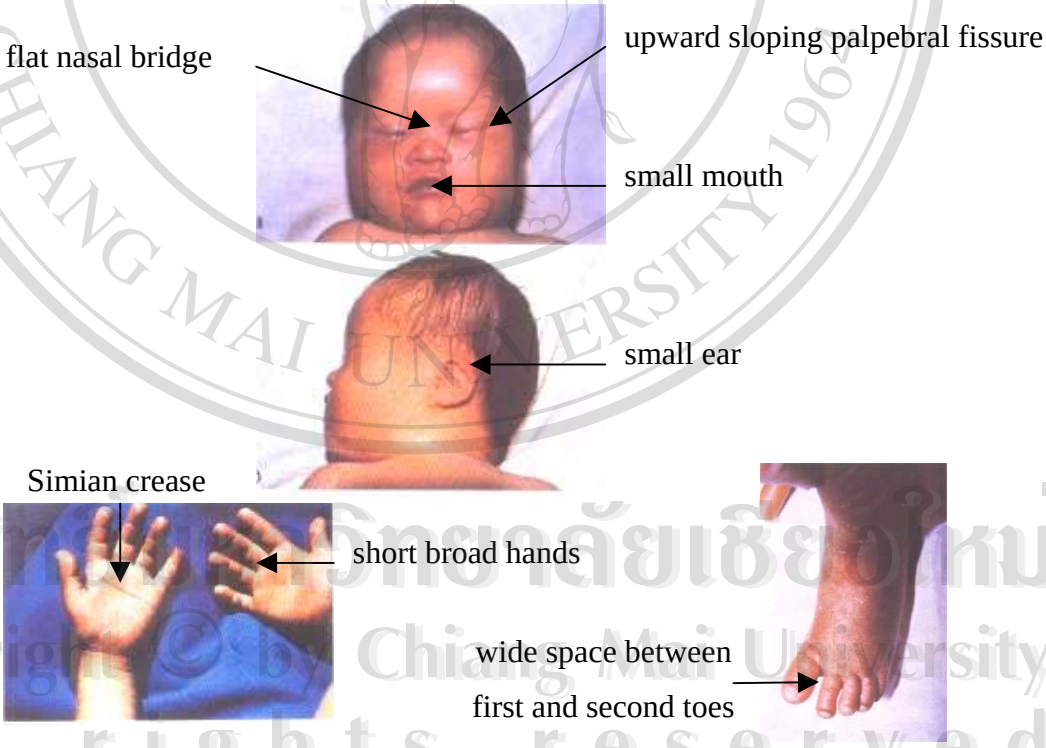

Figure 1 Phenotype of a Down syndrome patient.

(Modified from http://www.ram-hosp.co.th/books/17down.htm)

This phenotype is usually the facial characteristics of an upward sloping palpebral fissure, flat nasal bridge, epicanthus, small mouth, short broad hands and wide space between first and second toes, hypotonia, small ears, Simian crease, congenital cardiac anomalies and mental retardation.

The chromosomal basis of Down syndrome was first established in 1959 by Lejeune *et al*. This chromosomal disorder is the result of having an extra chromosome 21 (Trisomy 21). In most people with Down syndrome, an error occurs during the meiotic division and they end up with 47 chromosomes instead of 46. The detection rate rises with increasing maternal age due to the increased risk for nondisjunction of the smallest chromosome.

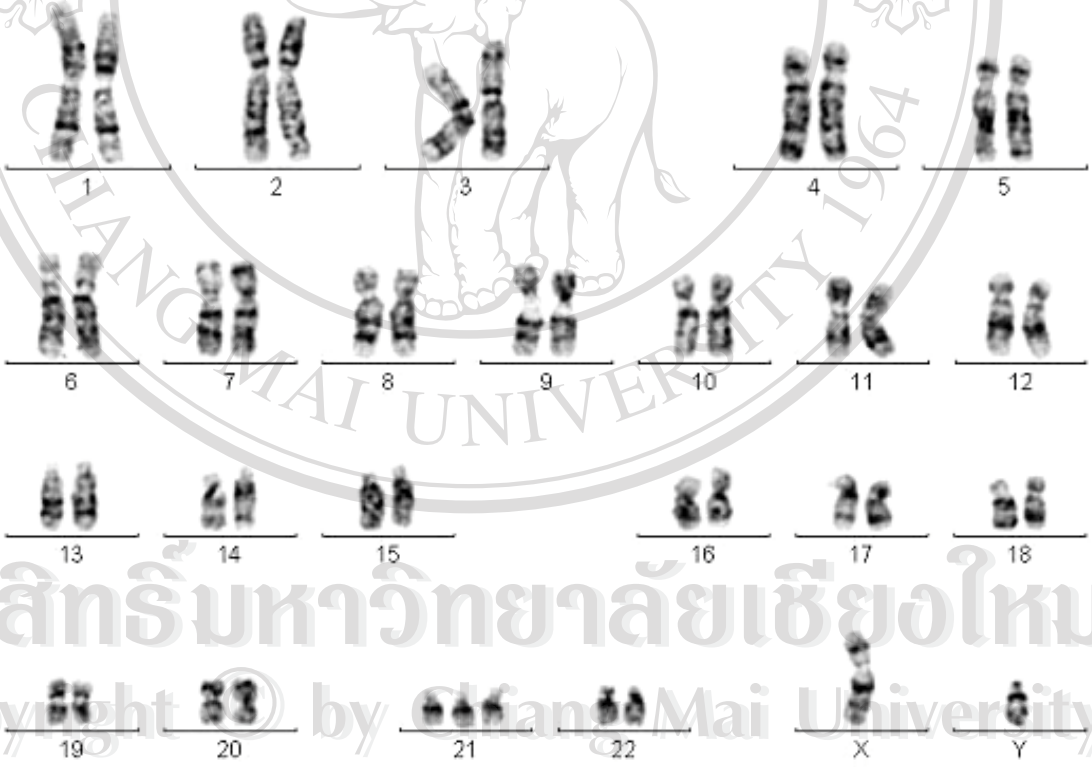

Figure 2 Karyotype of a Down syndrome male patient (47,XY,+21).

Prenatal diagnosis with a view to identify fetal genetic disorders started in the early 1970s. Since its inception, the most common reason for prenatal diagnosis is increased risk of having a child with trisomy 21. This risk depends on maternal age, and it can be assessed by maternal serum marker screening and fetal ultrasonography (nuchal translucency). Other indications for prenatal diagnosis of chromosome disorders include additional structural fetal abnormalities detected by ultrasonography, a previous child with a chromosome disorder or either parent being a carrier of chromosome rearrangement (Hultén *et al*., 2003).

Prenatal chromosome diagnosis is accomplished by conventional cytogenetic banding of metaphase chromosomes, obtained from fetal trophoblasts (from chorionic villus biopsy), amniocytes (from amniotic fluid) or fetal lymphocytes (from cord blood). This technique is accurate and reliable allowing the detection of variety of numerical and structural aberrations. The primary disadvantage of the conventional cytogenetics is that the fetal tissue must be cultivated for several days prior to analysis. It takes 10 days to 3 weeks to obtain results and has a culture failure rate of about 1% (Thein *et al*., 2000; Jobanputra *et al*., 2002).

Fluorescence *in situ* hybridization (FISH) was first introduced in 1986 as a potential powerful tool in clinical cytogenetics (Cremer *et al*., 1986; Julien *et al*., 1986). The use of interphase FISH for rapid prenatal diagnosis of numerical chromosome abnormalities from direct preparation of amniocytes is now widespread. FISH technique allows identification of specific nucleic acid sequences from chromosomes, even in non-dividing interphase stage. Advances in molecular techniques, including chromosome specific probes and *in situ* hybridization technique have generated considerable demand for extremely rapid results, particularly as they can be applied to uncultured cells (Martin *et al*., 1996; Jobanputra *et al*., 2002). During the late 1980s and early 1990s, technical issues were the focus of research. Specific probes, determination of cell types suitable for use with FISH, and more effective techniques for cell preparation and signal detection were intensively studies (Philip *et al*., 1994; Feldman *et al*., 2000). FISH analysis for detection of aneuploidies of chromosome 13, 18, 21, X and Y has been successfully performed with a high degree of concordance with cytogenetic results. Since Thailand is a

developing country, most prenatal chromosome analysis is performed with conventional cytogenetic techniques. To use DNA probes with the FISH technique is relatively expensive, and many families cannot afford it. This study attempted to produce chromosome 21 derived-probe by micro-FISH technique. The sensitivity, specificity and accuracy of the probe were evaluated for detection of chromosome 21 in uncultured amniocytes.

2. Literature review

2.1 Down syndrome (Trisomy 21)

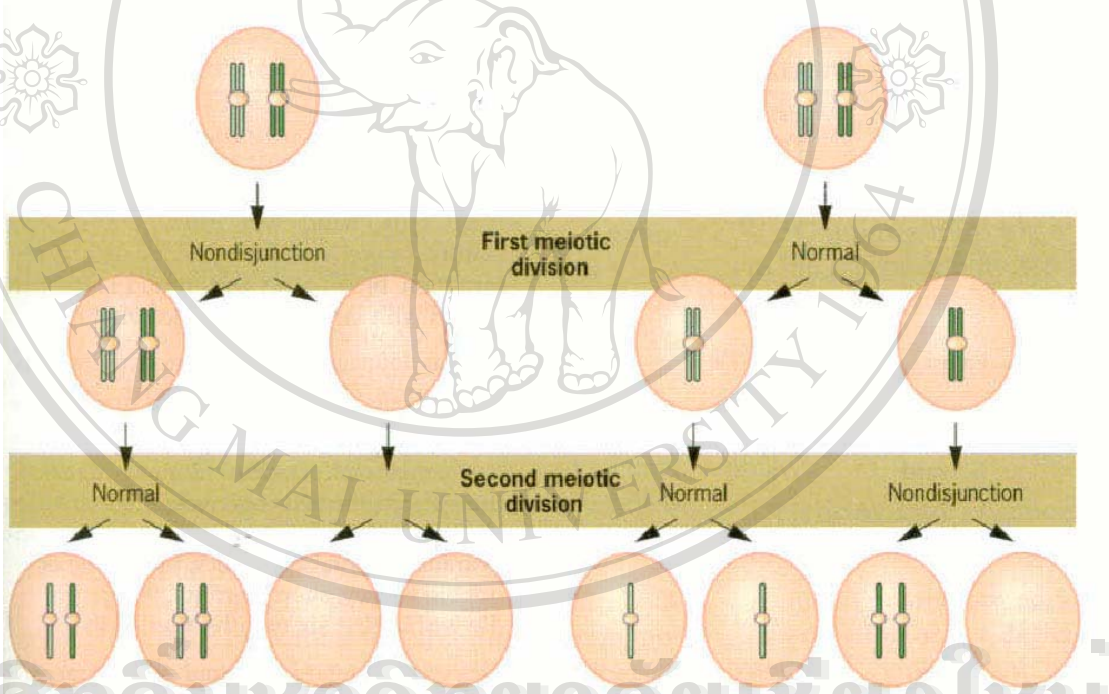

Figure 3 Schematic representation of the nondisjunction mechanism. (Modified from Snustad DP and Simmons MJ, 1997).

Down syndrome is one of the most common chromosome abnormalities detected in livebirth. There are three copies of chromosome 21 found in patients with Down syndrome rather than the normal two copies. Pathogenesis and associated

factors of trisomy is mainly nondisjunction that is a failure of segregation of chromosomes or chromatids in mieotic division (Friedman *et al*., 1996). If it occurs during the first division of meiosis (meiosis I) it results from the failure of homologous chromosome to segregate. If it occurs in meiosis II division, the sister chromatids are failures to separate. Both events produce gametes that are disomic and nullisomic for specific chromosomes, and fertilization produces aneuploid zygotes, either trisomic or monosomic.

Nondisjunction is a common event that appears to occur at a higher frequency in oogenesis than in spermatogenesis. Studies using cytogenetic and DNA polymorphism have shown that in approximately 90% of the cases, the extra chromosome in Down syndrome patients comes from the mother. The majority of the extra chromosomes were derived from meiosis I errors and only about 5% occur during spermatogenesis. Thus, meiosis I errors account for 76 to 80% of maternal meiotic errors. Maternal meiosis II errors constitute 20 to 24% of maternal meiotic errors. In rare families in which there is paternal nondisjunction, most of the errors occur in meiosis II. The mean maternal and paternal ages are similar to the mean reproductive age in western societies (Bianca, 2002). Mitotic nondisjunction in somatic cells, like a meiosis II error, is the failure of sister chromatids to segregate at anaphase. This result of a trisomic and a monosomic daughter cell. In 5% of trisomic individuals, the supernumerary chromosome 21 appears to result from an error in mitosis. In these cases, no advanced maternal age and no preference for which chromosome 21 is duplicated in the mitotic error

The only clear influence on the etiology of nondisjunction is the age of the mother. The occurrence of trisomy in livebirths and spontaneous abortions is increased with the age of the mother. The incidence of Down syndrome in different maternal ages is summarized in table 1.

Table1 Incidence of Down syndrome in relation to maternal ages (Muller and Young, 2001a).

Maternal age at delivery (years)	Incidence of Down syndrome
20	1 in 1500
25	1 in 1350
30	1 in 900
35	1 in 400
36	1 in 300
37	1 in 250
38	1 in 200
39	1 in 150
40	1 in 100
41	1 in 85
42	1 in 65
43	1 in 50
44	1 in 40
45	1 in 30

The genetic abnormalities causing Down syndrome are free trisomy 21 (95%), unbalanced translocation (4%) between chromosome 21 and other acrocentric chromosomes, most often chromosome 14 or 21 and mosaicism with two cell lines, one normal and one trisomy 21 (1%)(Bianca, 2002). It is well known that de novo t (14;21) trisomies have originated in maternal germ cells. In most cases, the t(21;21) is an isochromosome (dup21q) rather than the result of a Robertsonian translocation caused by a fusion between 2 heterologous chromatids. About half of the cases were of paternal origin and half of maternal origin.

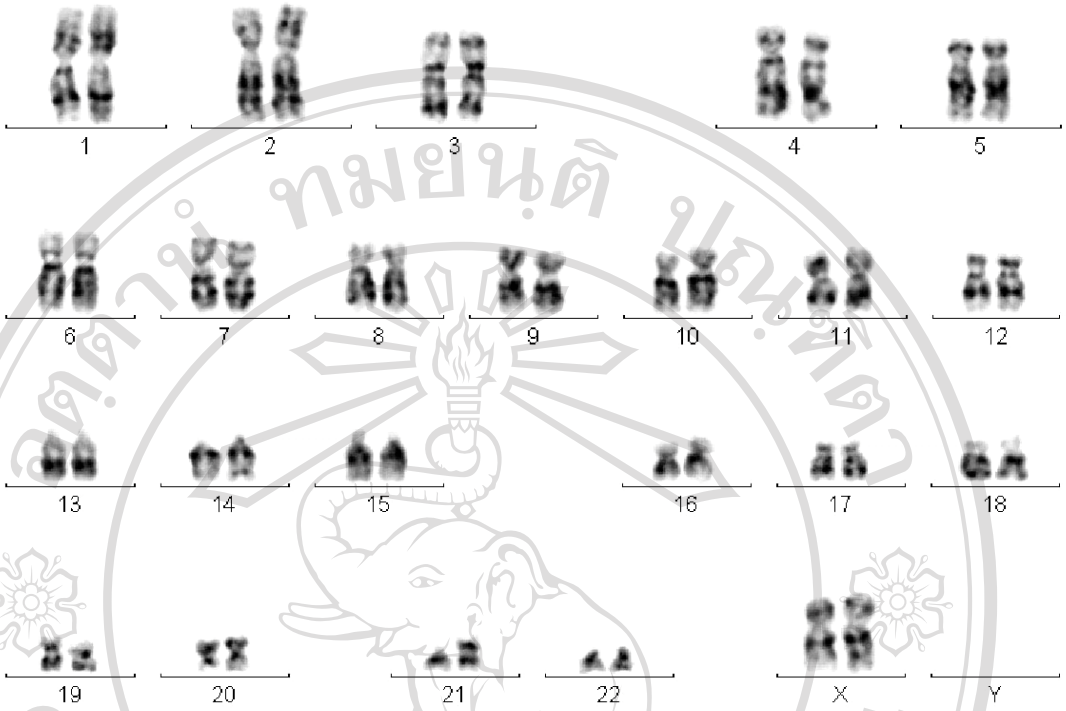

Figure 4 Karyotype of a female patient with translocation between chromosome 21 and 21 or 46,XX,+21,der(21;21)(q10;q10) from Rajanagarindra Institute of Child Development, Chiang Mai.

Efforts have been made to correlate the various clinical features in Down syndrome with trisomy for specific region of chromosome 21, by studying children with partial trisomy for different regions. Very rarely, Down syndrome is diagnosed in a patient in whom only a part of the long arm of chromosome 21 is present in triplicate, and a Down syndrome patient with no cytogenetically visible chromosome abnormality is even more rarely identified. These patients are of particular interest because they can show what region of chromosome 21 is likely to be responsible for the Down syndrome phenotype (the "critical region") and what regions can be triplicated without causing the phenotype (Thompson *et al*., 1991a). There is some support for a Down syndrome "critical region" at the distal end of the long arm (21q22) as children with trisomy for this region usually have typical Down syndrome

facial features. Chromosome 21 is a "gene-poor" chromosome with a high ratio of AT to GT sequences. At present the only reasonably well-established genotype-phenotype correlation in trisomy 21 is the high incidence of Alzheimer's disease which is attributed to an amyloid precursor protein gene dosage effect (Muller and Young, 2001a).

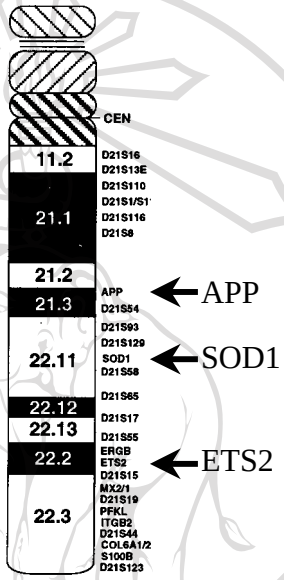

Figure 5 Schematic of chromosome 21, showing the location of selected genes. (Modified from Korenberg *et al*., 1994)

The chromosome 21 contains 225 genes and 59 pseudogenes. About 41% of the genes that were identified on chromosome 21 have no functional attributes (Hattori *et al*., 2000). The critical segment to chromosome band 21q22 is expected to contain at least 50 to 100 genes. The "Down syndrome critical region" 21q22.2-22.3, when present in three copies seems to be responsible for at least some of the major phenotypic features in Down syndrome such as the characteristic faces, lowered IQ, short stature, and heart defects. The genes that may be within the critical region include those for superoxide dismutase (SOD1); cystathionine β-synthase (CBS), the enzyme that is deficient in homocystinuria; α crystallin lens protein (CRYA1) and the est-2 oncogene (ETS2) (Thompson *et al*., 1991b). The genes for superoxide

dismutase (SOD1) and amyloid precursor protein (APP) located proximal to band 21q22.1 may be excluded from a significant contribution to the Down syndrome phenotype, while parts of band 21q22.2 and 21q22.3 including locus D21S55 may be the minimal region necessary for the generation of many Down syndrome features. Studies by Korenberg *et al*., 1994 suggested that instead of a single critical region, many chromosome 21 regions are responsible for various Down syndrome features (Blancato, 1999). Additional phenotypic characteristics may map outside the D21S55 region. A "phenotypic map" was constructed that included 25 features and assigned regions of 2 to 20 Mb as likely to contain the gene responsible. So, Down syndrome is a contiguous gene syndrome and makes it unlikely that a single Down syndrome chromosomal region is responsible for most of the Down syndrome phenotypic features (Bianca, 2002).

With regard to natural history, children with Down syndrome show a broad range of intellectual ability with IQ scores ranging from 25 to 75. The average IQ of young adults with Down syndrome is around 40 to 45 (Muller and Young, 2001a). About half of children with Down syndrome are born with congenital heart disease. The most frequent lesion are atriventricular septal defect (45% of newborns with Down syndrome) and ventricular septal defects (35%), isolated secundum atrial septal defects (8%), isolated persistent patent ductus arteriosus (7%), isolated tetralogy of Fallot (4%), and other lesions (1%) can also arise (Roizen and Patterson, 2003). In the absence of a severe cardiac anomaly, which leads to early death in 15-20% of cases, the average life expectancy is 50-60 years (Muller and Young, 2001a). In a survey of 17,897 individuals with Down syndrome complied by US Centers of Disease Control and Prevention National Center for Health Statistics for 1983-1997, the mean age of death increased from 25 years in 1983 to 49 years in 1997 ($p < 0.0001$) (Roizen and Patterson, 2003; Yang *et al*., 2002). Moreover, individual with Down syndrome also exhibit a risk of leukemia, immunological deficiencies, reproductive problems, thyroid diseases, diabetes mellitus, celiac disease, atlantoaxial instability, epilepsy and other health problem such as, eye, ear, nose, throat, oral, dental and orthopedics problems.

2.2 Prenatal cytogenetic diagnosis

The study of chromosomes, their structure, and their inheritance is called cytogenetics. The science of modern human cytogenetics date from 1956, when Tjio and Levan developed effective techniques for chromosome analysis and established that the normal human chromosome number is 46. Since that time, much has been learned about human chromosomes, their normal structure, their molecular composition, the locations of the genes that they contain, and their numerous and varied abnormalities (Thompson *et al.*, 1991b). Prenatal diagnosis for chromosome abnormalities has been available for over 30 years. The most common referral indication is a raised risk of Down syndrome. There are a variety of non-invasive and invasive techniques available for prenatal diagnosis. Each of them can be applied only during specific time periods during the pregnancy for greatest utility. Ultrasound, which is more commonly used to screen pregnancies, may also be used as a diagnostic tool for structural anomalies which are known to be familial but where no chromosomal or molecular defect is known (Stranc *et al.*, 1997). The fetal-derived tissues must first be obtained to perform prenatal diagnosis. All of the commonly used methods that yield fetal tissues that fetal karyotype can be obtained such at amniocentesis, chorionic villus sampling (CVS) and cordocentesis (percutaneous umbilical blood sampling, PUBS) are invasive. Because of the gestation at which it is performed and the attendant risk, cordocentesis is not commonly used, except in those women who come to attention late in pregnancy which is usually done after 18 weeks of gestation and are at increased risk of a chromosome anomaly in the fetus. Both midtrimester amniocentesis and chorionic villus sampling are now well-established techniques for obtaining genetic information about the fetus. Other less commonly used method is early amniocentesis. Recent evidence from the large Canadian Trial Group, significant differences for early amniocentesis (11^{th} to 12^{th} gestational weeks) compared with mid-trimester amniocentesis (15^{th} to 16^{th} weeks) were found. Total fetal losses including pre-procedure, post-procedure, stillbirth and neonatal death in the early amniocentesis group (7.6%) was higher than in the late amniocentesis group (5.9%). Furthermore, early amniocentesis was associated with a higher incidence of talipes or clubfoot (1.3% compared to 0.1%) and postprocedural amniotic fluid leakage (3.5% compared to 1.7%) (The Canadian Early and Mid-Trimester

Amniocentesis Trial (CMET) Group, 1998; Canadian Colledge of Medical Geneticist and the Genetics Committee of the Society of Obstetricians and Gynaecologists of Canada, 2001).

Amniocentesis

Amniocentesis is the withdrawal of amniotic fluid from the amniotic sac surrounding the fetus. For over two decades this has been the primary technique utilized for the diagnosis of fetal genetic disorders. Amniocentesis is usually performed on an outpatient basis at about the 16th week of gestation after the last menstrual period (Thompson *et al*., 1999c). At this time the uterus is easily accessible to a transabdominal approach, and a sufficient volume of amniotic fluid (approximately 200 ml) exists to permit 15-20 ml to be withdrawn safely. This technique is performed under ultrasound guidance, which at this gestation age also affords the opportunity for evaluation of fetal anatomy, outlining the position of the fetus and placenta. The amniotic fluid contains cells of fetal origin that can be cultured for diagnostic tests. The sample is spun down to yield a pellet of cells and supernatant fluid. The cell pellet is resuspended in a culture medium with fetal calf serum which stimulates cell growth. While most of these cells in the amniotic fluid which have been shed from the amnion, fetal skin, gastrointestinal and urinary tracts epithelium will be non-viable, a small proportion will grow (Muller and Young, 2001b). It takes 10-14 days for reliable karyotyping on amniotic fluid cells and up to 3 weeks in total turnaround time; thus where pregnancy termination is necessary after an amniocentesis at 16 weeks it can usually be done at 18-19 week (Stranc *et al*., 1997). In addition, supernatant amniotic fluid can be used for measurement of substances such as amniotic fluid α-fetoprotein (AFAFP), hormones, enzymes, etc. The results of laboratory studies on amniotic cell cultures are highly accurate (more than 99% for most biochemical and cytogenetic studies). Significant maternal injury from amniocentesis is rare. There is a small risk of inducing miscarriage, estimated to be approximately 0.5 percent. Maternal infection is a rare complication. To prevent Rh immunization of the mother, administration of Rh immune globulin is routine for Rh-negative women (Thompson *et al*., 1991c). The amniotic fluid is generally similar

in appearance to white wine. Occasionally blood-tinged amniotic fluid may be obtained, generally due to maternal bleeding into the amniotic cavity at the time of the procedure. If the patient has previously had a history of antepartum bleeding, the amniotic fluid may be brown or dark red in colour due to blood pigments being absorbed across the chorio-amnion membranes. The presence of discolored fluid on amniocentesis is associated with an increased risk of pregnancy loss (Canadian Colledge of Medical Geneticist; the Genetics Committee of the Society of Obstetricians and Gynaecologists of Canada, 2001).

With the development of higher resolution ultrasound equipment, some centers have begun offering amniocentesis before 15 weeks gestation, usually between 10 and 14 weeks. The majority of the procedures have been performed during the 13^{th} and 14^{th} weeks of gestation. There is evidence that early amniocentesis is associated with a higher fetal loss rate and a more frequent occurrence of certain congenital abnormalities (Department of Chemical Pathology, The Chinese University of Hong Kong, 1999).

Chorionic villus sampling (CVS)

This procedure is usually carried out at 10-12 weeks gestation under ultrasound guidance by either transcervical or transabdominal aspiration of chorionic villi (Muller and Young, 2001b). The transcervical technique is used for the majority of the posterior placental locations, while the transabdominal technique is better suited for the fundal and anterior placental location. The upper gestational age of 12 weeks is generally considered for the transcervical technique. Both the trancervical and tranabdominal technique usually obtain 5 to 25 mg of chorionic tissue which is aspirated into the catheter of the needle. In contrast to amniocentesis, which obtains amniotic fluid, the CVS obtains chorionic tissue from the developing placenta. These are fetal in origin being derived from the outer cell layer of the blastocyst, i.e. the trophoblast. These cells can then be analyzed by a variety of techniques. The most common test employed on cells obtained by CVS is chromosome analysis to determine the karyotype of the fetus. The cells can also be grown in culture for biochemical or molecular biologic analysis. CVS is usually done earlier than

amniocentesis, and culturing differences make the turnaround time for karyotyping shorter than it is with amniocentesis (7-14 vs 21 days) (Stranc *et al*., 1997). CVS may be associated with a higher rate of procedure-related fetal loss (an additional 0.5% to 1.0%) when compared to standard amniocentesis, although this did not reach statistical significance in comparative trials. Recently, a possible association with fetal limb reduction anomalies, particularly with CVS performed before the ninth week of pregnancy has been reported. As with amniocentesis, the risk of causing Rhesus sensitization may be avoided by administration of Rh immune globulin with the procedure (American Academy of Pediatrics, Committee on Genetics, 1994). There is also the possibility that maternal bloods cells in the developing placental will be sampled instead of fetal cells and confound chromosome analysis.

Cordocentesis

Cordocentesis or percutaneous umbilical blood sampling (PUBS) or fetal blood sampling (FBS) can be used to obtain fetal blood from as early as 12 weeks gestation age until full term with acceptable results (Randolph, 1999), but is usually used after 16 weeks. The procedure-related loss rate at a mean gestation of 29.1±5 weeks at the time sampling was 0.9%. Indications for cordocentesis include: fetal karyotyping when congenital malformations of intrauterine growth retardation are identified by ultrasound, viral infections, hematological abnormalities including Rh or the immune hemolytic disease, maternal or fetal platelet disorders, and inborn errors of metabolism. This procedure is an ultrasound-guided freehand or needle guide technique which allows insertion of a 22 gauge spinal needle into the umbilical cord vessel at either the placental insertion of the umbilical cord or into a free loop of umbilical cord. Depending on the indications for the test and the gestational age of the fetus, one to three ml of blood is removed for analysis. Fetal chromosomes can usually be obtained within 48 to 72 hours by culturing fetal white blood cells (Canadian Colledge of Medical Geneticist and the Genetics Committee of the Society of Obstetricians and Gynaecologists of Canada, 2001). The major advantage of cordocentesis is that it allows direct access to the fetus, not only for diagnostic but also for therapeutic management. Maternal complications from FBS are uncommon

but include amnionitis, infection, rhesus sensitization and transplacental haemorrhage. Fetal loss rates following FBS have been reported to be approximately 1% in several large series. The presence of structural abnormalities or severe growth retardation of the fetus is associated with a much increased fetal loss rate. Other fetal complications include infection, premature rupture of membranes, haemorrhage, severe bradycardia and umbilical cord thrombosis (Department of Chemical Pathology, The Chinese University of Hong Kong, 1999).

Traditionally, chromosome diagnosis is accomplished by karyotyping, that is, analysis of chromosomes by microscopy followed by the lining up of each chromosome pair.

Karyotyping

Each species has a characteristic chromosome complement (karyotype) in terms of the number and the morphology of its chromosomes. The genes are in linear order along the chromosomes, each gene having a precise position or locus. The gene map, the map of the chromosomal location of the genes, is also characteristic of each species and is, as far as we known, the same in all individuals within a species. The 46 chromosomes of human somatic cells constitute 23 pairs. Of those 23 pairs, 22 are alike in males and females and are called autosomes. The remaining pair comprises the sex chromosomes XX in females and XY in males. Members of pair (described as homologous chromosomes or homologs) carry matching genetic information; that is, they have the same gene loci in the same sequence, though at any specific locus they may have either identical or slightly different forms, which are called alleles (Thompson *et al*., 1997a).

The advent of certain specialized staining techniques that include Giemsa or G-banding, quinacrine mustard or Q-banding, and reverse or R-banding, arbitary identification of individual chromosome pairs was based on the size and position of the centromere. A chromosome with its centromere in the middle is metacentric, one with centromere closer to one end is submetacentric, and one with the centromere almost at one end is acrocentric. The alternating dark and light stained demarcations called bands appear along the length of each chromosome. The banding patterns produced are specific for each chromosome pair, thus enabling the identification not

only of individual chromosomes, but of regions within each chromosome as well (Tharapel, 1999).

When designating a karyotype, the first item specified is the total number of chromosomes including the sex chromosomes present in that cell, followed by a comma, and the sex chromosomes, in that order. Thus a normal female karyotype is written as 46,XX and a normal male karyotype as 46,XY. The characters are contiguous, without spaces between items. Most karyotypes can be described using the "short form" of the nomenclature. For a "long form" in which abnormal chromosomes can be described by using the ISCN (International System for Human Cytogenetic Nomenclature) 1995 (Tharapel, 1999).

However, the conventional cytogenetic have the culture step for preparing metaphase chromosomes and take around 2-3 weeks for karyotyping. It is recognized that long waiting times for results may cause much psychological suffering and this has been one of the main reasons for the introduction of molecular methods for prenatal diagnosis of common chromosome disorders. This type of approach does not require cell culture and reports can routinely be issued within 1-2 days (Hultén *et al*., 2003).

2.3 Fluorescence *in situ* hybridization (FISH)

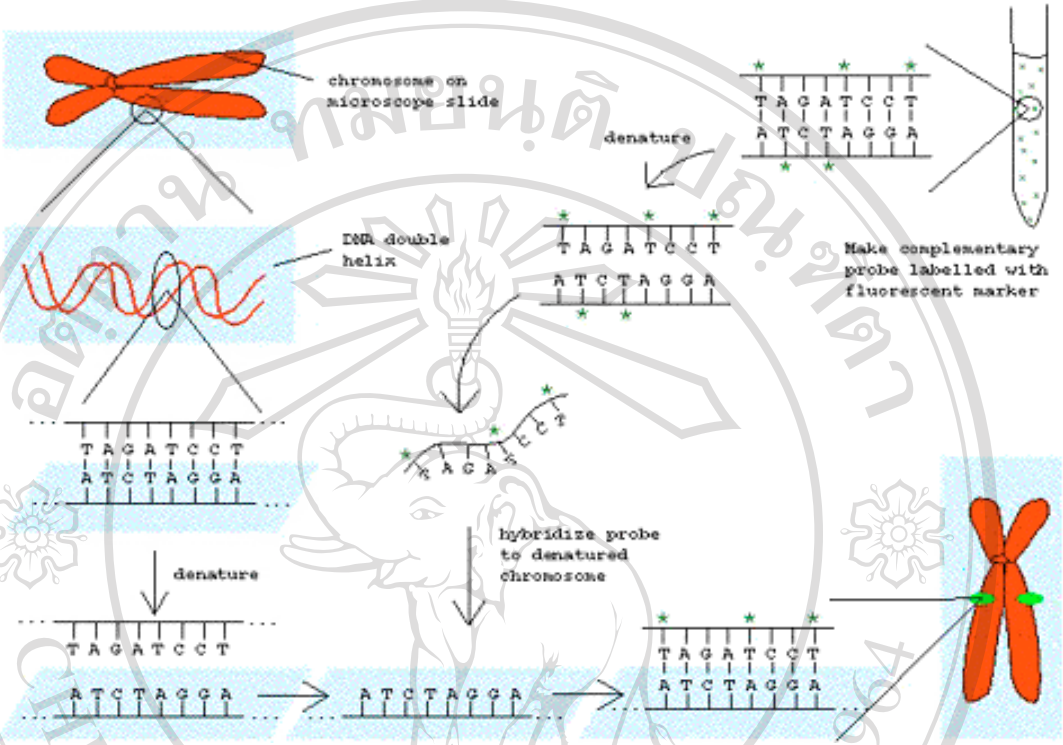

Figure 6 Principle of the Fluorescence *in situ* hybridization.

(Modified from www.udl.es/.../imatges_ metodes/in_situFISH.gif)

The technique of fluorescence *in situ* hybridization relies on the unique ability of a portion of single-stranded DNA, known as a probe, to anneal or hybridize with its complementary target DNA sequence, wherever it is located in the genome. Since the probe and target sequences are initially double-stranded DNA, the initial step in any FISH analysis is to denature both probe and target sequences with heat and/or chemicals such as formamide or alkali, to break the chemical bonds holding the two strands together in order to form single-strand DNA. In a subsequent step, the labeled probe is the annealed to its complementary DNA sequences on target nuclei or metaphase chromosomes that have been fixed on a microscopic slide. The probe is visualized either directly by the incorporation of a fluorochrome-conjugated

nucleotide labeling (e.g. fluorescein-dUTP) or indirectly with the incorporation of a reporter molecule (e.g. biotin-dUTP or digoxigenin-dUTP), which is released in a follow-up step. After hybridization and washing, the signal is visualized directly by fluorescent microscope (Pergament, 2000b). The methods of detection and visualization of the signal depend on the type of reporter molecule which was incorporated into the probes. The indirect methods use digoxigenin (detected by specific antibodies such as antidigoxigenin) or biotin (detected by streptavidin), whereas direct methods using fluorescein or other fluorochromes directly coupled to the nucleotide can be detected directly. Two commonly used fluorescent labels are fluorescein isothiocyanate (FITC) and Texas red (TR). A fluorescence microscope equipped with filters appropriate to the specific excitation and emission properties of the fluorochrome is used in the detection system. Thus, the number of copies of a specific chromosome is determined by *in situ* hybridization with a chromosome-specific probe by simply counting the number of signals obtained in the hybridized nuclei (Phillips and Reed, 1996; Pergament, 2000b).

There are three categories of chromosome specific probes, each offering different applications.

Repetitive probes

The repeated sequences most commonly used are the α-satellite DNA which is a 171 bp DNA monomer that is tandemly repeated n times. This entire block of tandemly repeated DNA is the copies n number of times in a higher order repeat at the centromere of each chromosome. The centromeres of most individual chromosomes can be distinguished and the probes unique to those chromosomes can be produced. The exception is the shared homology that exists between the centromeres of chromosome 13 and 21 and between those of chromosome 14 and 22. Other satellite DNAs include β-satellite which is a 63-bp unit that repeats in the same fashion as α-satellite DNA and is found at the tip of the acrocentric chromosomes (pairs 13, 14, 15, 21, and 22) , classic satellite I DNA which is an AATGG repeat found on chromosome 1, 9, 16, and Y ; and the telemeric repeat found at the ends of both arms of all chromosomes which is conserved over species and is composed of a TTAGGG that repeats n number of times.

Repeat sequence probes are useful for determining the number of specific chromosomes present (ploidy determination) and can be used on both interphase and metaphase cells. These probes are robust because the targets are large and repeated many times which allows the hybridization to take place rapidly and produce very large signals (Blancato, 1999).

Whole Chromosome Probes

Whole chromosome probes that known as whole chromosome "paint" (WCPs) are composed of numerous unique and repetitive sequences each derived from one entire chromosome. These can be produced using somatic cell hybrids via flow sorting of the specific chromosome, or by microdissection of specific chromosomes with subsequent PCR amplification of the DNA. Whole chromosomes probe are also called painting probes because of the painted appearance of the metaphase chromosomes after hybridization. These probes are designed for use on metaphase chromosome preparation only. Their use in interphase results in splotchy, undefined signals because the interphase chromatin to which they hybridize is decondensed as opposed to the compact condensed state of metaphase chromosome. Whole chromosome paints for all human chromosomes are used to determine the composition of marker chromosome; to detect subtle or cryptic translocations; to confirm the presence of deletions, duplications and insertions; and to analyse the complex rearrangements involving multiple chromosomes.

Chromosome arm-specific probes represent a subset of WCPs, made from the individual short and long arms of each chromosome. There are used for similar purposes, but allow the focus to be narrowed to one chromosome arm. As the facility of probe development increases, laboratories are devising probe systems that allow investigation of specific abnormalities. These probes may be composed of yeast artificial chromosomes (YACS); contiguous cosmids; or chromosome arms, bands, or sub-bands that have been isolated through microdissection. Depending on the size of the probe and the specific application, these can be used on interphase and/or metaphase preparations (Blancato, 1999).

Unique Sequence Probes

Unique sequence probes have target regions that are not repeated in the genome and may code for genes. The various sorts of FISH unique sequence probes currently used in clinical cytogenetics laboratories are aimed at identification of microdeletion syndromes; oncogenes such as m-myc, c-myc, and her-2-neu; and unique sequences in subtelomeric regions. Subtelomeric probes can be produced from the chromosomal regions proximal to the telomere and contain unique sequences that are specific for each chromosome. Unique sequences in close proximity to the end of chromosomes are used for studies of cryptic translocations and for gene mapping. In a standard amnio-FISH screening, α-satellite probes for chromosomes 18, X, and Y and unique sequence probes for the long arms of chromosome 13 and 21 are used. The centromeres of chromosome 13 and 21 cannot be distinguished with α-satellite probes, and hence sequence, nonoverlapping cosmid contigs derived from these chromosomes are used for prenatal studies. This creates some difficulty because such unique sequence probes produce smaller hybridization signals. And longer hybridization times are required (Blancato, 1999).

The first developed probes were derived from DNA of flow-sorted whole chromosomes and used for prenatal diagnosis of trisomy 21, 13 or 18 by Kue *et al.* (1999). For aneuploidy diagnosis, FISH with smaller probes is advantageous as signals appear as more distinct dots. Normal samples are expected to show two dots per nucleus, whereas those that are trisomic will show three dots.

There is now a large number of reports in the literature highlighting the efficacy of rapid prenatal aneuploidy diagnosis, using FISH probes on interphase nuclei. The first prospective FISH study on interphase amniocytes, using probes for single copy-like signals of chromosomes 21, 18, 13, X and Y appeared more than a decade ago (Klinger *et al*., 1992).

Klinger *et al*. (1992) compared aneuploidy detection by fluorescence *in situ* hybridization of interphase nuclei with the results obtained by cytogenetic analysis. They constructed probes derived from specific subregions of human chromosomes 21, 18, 13, X, and Y that give a single copy-like signal when used in conjunction with suppression hybridization. The locus-specific probe sets that they constructed

generated bright, easily detected hybridization signals that were spatially resolved in interphase nuclei. The combination of chromosome-specific probe sets composed primarily of cosmid contigs and optimized hybridization/detection allowed accurate chromosome enumeration in uncultured human amniotic fluid cells, consistent with the results obtained by traditional cytogenetic analysis. In their study the hybridization pattern of all trisomic sample (n=21) was clearly distinct from that seen in normal cells demonstrating the sensitivity and specificity of interphase FISH analysis.

Ward *et al*. (1993) described the results of the first clinical program which utilized FISH for the rapid detection of chromosome aneuploidies in uncultured amniocytes. Region-specific DNA probes to chromosomes 13, 18, 21, X, and Y were used to determine ploidy by analysis of the signal number in hybridized nuclei. For the current study, the prenatal diagnostic application of the FISH assay was possible by the development of DNA probes with high signal-to-noise ratio, good spatial resolution of the fluorescent signals, and high hybridization/detection efficiencies in association with the development of novel cell-handling techniques (Klinger *et al*., 1992). The overall detection rate for aneuploidies was 73.3%, with an accuracy of informative FISH results for aneuploidies of 93.9%. Compared to cytogenetics, the accuracy of informative results for euploid and aneuploid was 99.8%, and the specificity was 99.9%.

Rapid detection of prenatal aneuploidy using interphase FISH on a large scale was successfully initiated (Klinger *et al*., 1992; Ward *et al*., 1993). The results of the FISH analyses were routinely reported to the referring physician within 2 days of receipt of the specimen. However, FISH should not be used as an independent, stand-alone technology for prenatal diagnosis. The existence of chromosome abnormalities which are not detected by current FISH protocols and the lack of widespread experience with this new technology require that the FISH protocols should be utilized as an adjunctive test to traditional cytogenetic analysis (Ward *et al*., 1993). Although these two studies formed the basis of the clinical protocols for the application of FISH to prenatal diagnosis, they had several obstacles that delayed wide acceptance of FISH as a highly reliable method for routine prenatal diagnosis

(Jobanputra *et al*., 2002). Their probes were prepared by their own laboratories and indirectly labeled and "home brewed" and are not commercially available (Jalal *et al*., 1998). Most cytogenetic laboratories are not qualified and equipped to synthesize DNA probes and to perform necessary quality-control studies. Furthermore, the assay conditions should be modified for each set of probes because the quality and characteristics of the probes are the key factors for successful FISH analysis (Feldman *et al*., 2000). Nowadays, several studies have used commercially available FISH probes with a standardized technique (Jobanputra *et al*., 2002; Luquet *et al*., 2002; Witter *et al*., 2002 etc.).

Eiben *et al*. (1999) performed FISH on uncultured amniocytes from 12 weeks of gestation to the third trimester using commercially available specific DNA probes for chromosome 13, 18, 21, X and Y. For all analyzable disorders the FISH results were in complete agreement with standard cytogenetics. Neither false-positive nor false negative results were obtained using FISH. In 57 cases the FISH results were trisomy 21 for most of the aneuploidies. FISH was performed successfully in 3,150 prenatal cases. Very high rates of chromosomal aberration (28.6%) were found in very early gestational ages (week 11-13) because of ultrasonographic aberrations. This rate is declining substantially in the later gestational weeks. In their study, they suggested the following guidelines for further clinical management. In cases with pathological FISH data without ultrasonographically visible changes, they waited for standard karyotype analysis before irreversible consequences were discussed with the pregnant woman. Whenever the FISH result is aberrant and corresponding aberrations have been detected by ultrasound, rapid decisions may be possible and necessary. In their hospital legal abortions have been performed in cases of trisomy 21 and 18, in triploidies and in monosomy X cases with multiple visible abnormalities. In most cases of the normal FISH result renders sudden relief to the pregnant women, especially, the FISH results could be obtained within 24 hour.

Feldman *et al*. (2000) found that the previously published studies had a high percentage of samples not suitable for FISH and many uninformative and problematic results were the other major limitations. Thus, the purpose of their study was to determine the accuracy of FISH in detection of aneuploidies in real clinical practice

with very high-risk pregnancies, i.e., those with fetal anomalies detected by ultrasound. FISH studies with multicolor, commercially available, specific probes for chromosomes 13, 18, 21, X, and Y were routinely performed and the results were also compared with standard karyotyping. The referring physicians on 301 cases (7.2%) ordered rountine FISH studies. Aneuploidies were detected in 32 samples (10.6%) (14 cases of trisomy 21, 10 of trisomy 18, 3 of trisomy 13, 4 of monosomy X, and 1 of triploidy). The sensitivity, specificity, and predictive values for the rountine FISH analysis as a method to detect aneuploidies of the chromosome 13, 18, 21, X, and Y in their study group were all 100%. They believed that the major problems of most studies were the result of unsatisfactory criteria for interpretation of results. At least 50 interphase amniocytes or lymphocytes or 100 trophoblasts from CVS, were examined for each probe and the criteria were chosen for diagnosis if at least 85% of the cells were euploidy or aneuploidy. These modifications were the basis for the zero uninformative FISH results in their group.

Weremowicz *et al*. (2001) performed FISH with probes specific for chromosome 13, 18, 21, X, and Y on 911 of amniotic fluid samples (8.2%) submitted over an 8-year period**.** There were 5 cases (6%) which produced a false-negative FISH result. A majority of the false-negative or uninformative results among aneuploid samples involved chromosome 21. Two different chromosome 21-specific DNA probes were used in this series; it was found that the cosmid probe (Oncor) used initially produced a relatively small signal with a lower efficiency of hybridization compared to the LSI21 (Vysis) probe that is currently used. However, the LSI21 probe tends to produce an indistinct, granular hybridization signal in some cases that compromises scoring of hybridization signals. Accuracy and reproducibility of FISH analyses is critically dependent upon the specificity and sensitivity of the probes. These results indicate good performance of the commercially available probe set, with a 94% detection rate of all aneuplodies and, at most, a 0.1% false-positive rate, in informative samples.

Jobanputra *et al*. (2002) evaluated FISH in prenatal diagnosis of aneuploidies in high-risk pregnancies in Indian people. Prenatal diagnosis was performed in 88 high-risk pregnancies using FISH and cytogenetic analysis. Multicolour commercially available FISH probes specific for chromosome 13, 18, 21, X, and Y

were used. A minimum of 50 nuclei of CVS and AF samples or 20 metaphases of cord blood sample were scored for each probe. The hybridization efficiency of the 5 probes used for the detection of aneuploidies was 100%. The overall mean interphase trisomic signal pattern of chromosome 21 was 97.3%. The criteria for interpretation of the results was defined as normal samples in which ≥ 80% and defined as abnormal specimen in which ≥70% of the nuclei/metaphase spread. However they suggested that the cut-off point could be optimized to as high as 90% for both disomic as well as trisomic prenatal samples.

Luquet *et al* (2002) determined the accuracy of the technique when FISH is preformed routinely and suggested protocols for the use of FISH results in the clinical management of pregnancies. The commercial probe (AneuVysion EC assay kit) for the 13, 18, 21, X, and Y chromosomes were used according to the manufacturer's instructions. For each probe, 50 nuclei, whenever possible, were examined by two trained investigators. FISH was successful in 1,968 cases (98.40%), and 210 aneuploidies (10.7%) were detected. FISH was entirely successful in 1,882 cases (94.1%). In 86 cases (4.3%), FISH was partially successful. In 32 cases (1.6%), FISH was not successful. Most of the cases where FISH failed or was only partially successful were observed when the amniocentesis was performed before the 15th weeks of gestation, because there were not enough nuclei. In cases of trisomy, the mean frequency of nuclei with three signals was 85%, 70% and 86% for chromosomes 13, 18, and 21, respectively. However, they thought that the cost of the FISH test was borne by the laboratories so interphase FISH is performed only in very high-risk pregnancies and in late gestational ages.

Witters *et al*. (2002) performed direct FISH for chromosome 21 in all 5,049 samples. They found that FISH is a reliable technique for the rapid prenatal diagnosis of trisomy 21 as all 70 cases of Down syndrome were identified by interphase FISH and confirmed by conventional cytogenetics (sensitivity = 100%) without false-positive result (specificity = 100%). Direct FISH for aneuploidies for four other chromosomes (13, 18, X and Y) was not rountinely performed in all samples, but following ultrasonographic indications. All numerical chromosomal anomalies detected by FISH (5 of trisomy 13, 15 of trisomy 18, 14 of sex chromosomal

aneuploidies, 3 of triploidy) were also confirmed by conventional cytogenetics and there were no false-positive results.

FISH has been found to be highly effective for rapidly determining the number of specified chromosomes in interphase cells (Cremer *et al*., 1988; Lichter *et al*., 1988). The results of these efforts were technical advances such as commercially available highly specific and reliable probes, direct labeling, and multicolour computerized signal detection systems (Divane *et al*., 1994; Jalal *et al*., 1998). Advances in molecular techniques, including chromosome-specific probes and *in situ* hybridization techniques have generated considerable demand for extremely rapid results particularly as they can be applied to uncultured cells (Martin *et al*., 1996). Despite the fact that this commercial probe is specific, the cost of the test must be considered. Moreover, there are still chromosome aberrations (like marker chromosomes and de novo unbalanced translocations) that cannot easily be identified by FISH technique. For the characterization of these aberrations micro-FISH has been developed (Engelen *et al*., 1998a). Some cytogenetics laboratories are qualified and equipped to synthesize DNA probes and to perform quality-control studies, so that the FISH-probe could be constructed by using micro-FISH technique.

2.4 Micro-FISH

Micro-FISH comprises the physical dissections of GTG-band chromosomes using a dissection needle or a laser beam. This is followed by a degenerate oligonucleotide primed-polymerase chain reaction (DOP-PCR) to amplify the dissected DNA and by labeling of the PCR product obtained with a reporter molecule (biotin or digoxigenin). Subsequently, the DOP-PCR product is annealed to metaphase spreads from normal subjects and this hybridization is detected using FISH (Engelen *et al*., 1998a; 2002). This method was introduced as a novel procedure and termed micro-FISH by Meltzer *et al*. in 1992. In the same year, Telenius *et al*. developed DOP-PCR that consists of a PCR using a 22 bp oligonucleotide with 6 degenerate bases close to the 3'end while the 5'end contains a rare restriction-endonuclease recognition site (5'-CCGACTCGAGNNNNNNATGTGG-3').

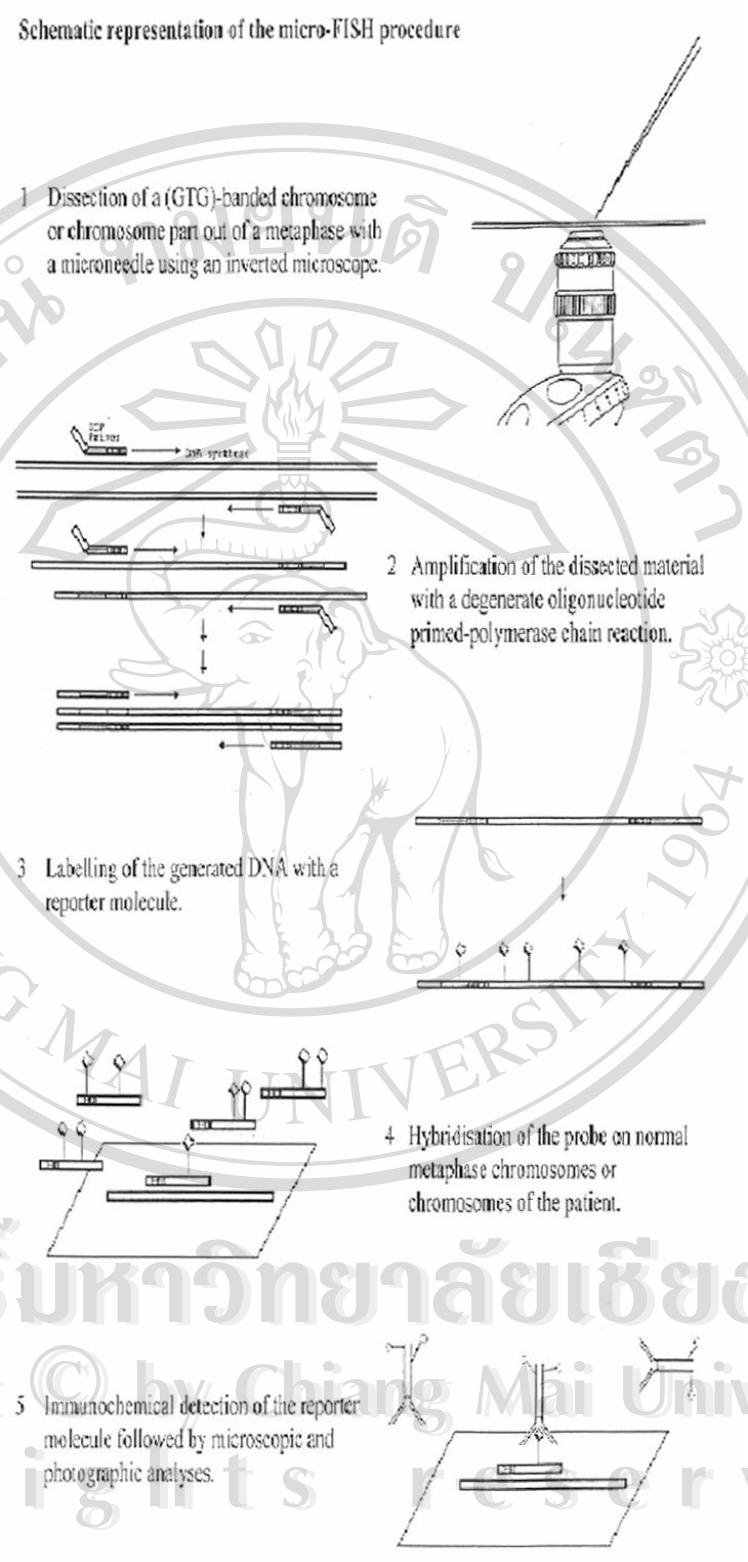

Figure 7 Schematic representation of the micro-FISH technique. (Modified from Engelen, 2002)

The PCR process can be achieved by cyclical alterations of temperature facilitating the DNA-strand separation, hybridization of primers and polymerization as follows. First, the target DNA is separated into two strands by heating to 92-98°C. The temperature is then reduced to between 37 and 55°C to allow the primers to anneal (the actual temperature depends on the primer lengths and sequences). Following annealing, the temperature is then increased to 60-72°C for optimal polymerization. If the PCR was 100% efficient, one target molecule will become 2^n after n cycles. In practice, 20-40 cycles are commonly used (Bermingham and Luettich, 2003). Efficient amplification of DNA by DOP-PCR relies on two fundamental requirements: (1) initial low annealing temperature cycles, which allow the primer to initiate PCR from short target sequences and (2) primer degeneracy. The six degenerate positions create a pool of 4^6 primers of different sequences as opposed to the single sequence of a nondegenerate primer (Telenius *et al*., 1992). Examining the PCR products after gel electrophoresis and ethidium bromide staining can monitor the quality of the DOP-PCR product. If the DOP-PCR product is used for microcloning, part of the product should be hybridize *in situ* to metaphase chromosomes to make sure that only the dissected region displays a FISH signal. The most important factor for the quality of the DOP-PCR product after microdissection of an eventually constructed library is the effective prevention of contaminant DNA during the microdissection and microcloning steps.

Microdissection, the technique to pick up chromosomal regions with a glass needle was first introduced by Scalenge *et al*. in 1981. It was used for the cloning in bacteria of genomic sequences from *Drosophila* salivary gland polytene chromosome bands. Microdissection and microcloning of mammalian chromosomes was successfully applied using mouse chromosome in 1984 by Rohme *et al*. and human chromosomes in 1986 by Bates *et al*. Since no PCR technology was available at that time, a large number of chromosome fragments (100-200 copies) had to be dessected prior to microcloning. The number of clones obtained in these libraries was far from being sufficient to represent the complete dissected region. The introduction of PCR technique and sequence independent DNA amplification enabled Lüdecke *et al*., (1989) to amplify dissected chromosomal material in vitro. These improvements

allowed them to reduce the number of dissected fragments from more than 100 to approximately 25 and to use GTG-banded chromosomes for microdissection (Senger *et al*., 1990). DOP-PCR improvements enabled synthesis of sufficient PCR product for chromosome painting from only a single dissected chromosome (Guan *et al*., 1993). Since the introduction of micro-FISH in 1992 the application of this methods have been developed to generate different DOP-PCR probes.

Meltzer *et al*. (1992b) generated a micro-FISH probe by microdissection of the material distal to 6q14 and hybridized it back to tumour metaphase chromosomes and to normal lymphocyte chromosomes. The results indicate that the dissected material was amplified and that it was derived from the terminal long arm of chromosome 21 (q21-qter). The micro-FISH method described in this report eliminated the microchemical techniques by using DNA a oligonucleotide primer to directly prime DNA synthesis at intervals along the microdissected DNA template. The method presented here is sufficiently rapid that a skilled worker could prepare one or more probes a day, which would allow the preparation of hundreds of probes specific for different regions of the human genome. Furthermore, prior to this study, microdissection was restricted to normal metaphase prepared from peripheral blood lymphocytes and had not been applied to abnormal human chromosomes. This method permits application of this technology to any source of metaphase chromosomes.

Viersbach *et al*. (1994) used micro-FISH to characterize a marker in a boy with a 46,X,+mar karyotype and demonstrated that the marker was a t(X;Y) chromosome. Furthermore, they dissected two marker chromosomes in a child with a 48,XY,+mar1,+mar2 karyotypes. Reverse chromosome painting revealed that the centromeres of the chromosome 13 and 21 displayed fluorescent signals; that these repetitive DNA probes were specific for the centromere of chromosome 13 and 21, which gave positive signals on both marker chromosomes. A phenomenon always seen when micro-FISH includes the short arm of an acrocentric chromosome is that the short arms of (most of) the acrocentric chromosomes display fluorescent signals after reverse painting with the DOP-PCR product. This cross-hybridization was first

reported and later confirmed by Sun *et al*. (1995) who characterized a marker derived from chromosome 21 (Engelen *et al*. 1998a).

Guan *et al*. (1994) generated WCPs for 15 normal human chromosomes (including 1, 3, 6, 7, 9, 12, 13, 14, 15, 17, 19, 20, 21, 22 and X) and their specificity demonstrated by FISH. All 15 WCPs were characterized by FISH to normal human peripheral lymphocyte metaphase chromosomes and to interphase nuclei. All centromere regions of the target chromosomes were hybridized and displayed fluorescent signal intensities similar to those on arm regions except chromosome 1 and 9, which displayed stronger signals. The short arms of the acrocentric chromosomes (13-15, 21 and 22) were not dissected to avoid cross hybridization during FISH. Therefore, none of the p arms of all acrocentric chromosomes were hybridized by their corresponding WCPs. However, WCP-X specifically hybridized to the short arm (Ypter-p11.1) and proximal long arm (Yq11) of chromosome Y, provided cytologic evidence for the pseudoautosomal region of the X. The complex structural chromosome rearrangements, including a nonreciprocal translocation, t(1;7)(q21;p11); a reciprocal translocation, t(3;13)(p11;q11); and two rearrangements involving these different chromosomes, t(1;7;13) and t(1;3;13) could be determined. The three-color FISH was performed using a mix of three D-group WCPs which were labeled with biotin (green), spectrum orange (red), and both (yellow) respectively, by PCR. By this three-color FISH it is readily possible to distinguish D-group chromosomes without banding and to detect numerical and structural aberrations involving these chromosomes. Finally, the WCPs prepared by microdissection can also effectively hybridize to their corresponding chromatin domains in interphase nuclei.

Yokoyama and Sukuragawa (1995) modified proteinase K treatment and DOP-shuttle-PCR method to improve the simple generation of chromosome region-specific painting probes. Five segments each were dissected from the region 21q22-qter and 11q23→qter, respectively. This method achieved relaxation of highly condensed chromosomal DNA, reduction of endogenous and extraneous contamination, and efficient and highly sensitive amplification of dissected chromosomal DNA.

Engelen *et al*. (1998a) developed a simple and efficient method for the dissection of marker chromosomes, micronuclei and chromosome regions. Before microdissection, metaphases were overlaid with milli-Q water to rehydrate the chromosomes which made them soft and sticky. The dissected chromosome fragments were dissolved without proteinase-K or topoisomerase treatment and directly amplified using DOP-PCR. The modified microdissection method described here enabled fast and reliable dissection of chromosomes and regions of chromosomes. Furthermore, relaxation of the dissected material was achieved without extra additives, an efficient DOP-PCR reaction mix was composed and reduction of the risk of extraneous contamination was reached by omitting sequenase addition.

Liehr *et al*. (2001) developed 24 multicolor-banding (MCB) probe sets, a specific one for each human chromosome, by the creation of microdissection libraries are detailed for the first time. Glass-needle based microdissection was performed to create 138 region-specific partial chromosome painting probes covering the whole human genome. More than $3x10^6$ FISH-experiments can be done from one microdissection derived probe by application of skillfully chosen DOP-PCR amplification and re-amplification steps without loss of hybridization quality. Thus, in practical use the microdissection derived probes do not have any disadvantages concerning the availability of the probe, compared to cloned probes.

Nantakarn *et al*. (2002) modified micro-FISH technique from Engelen *et al*. 1998a to characterize the two de novo rearrangments in peripheral blood samples. One marker of a small ring chromosome appeared to be derived from the pericentromeric region on the short arm and long arm (Xp11.1-q12) of the X chromosome and the second aberrant was identified as an isodicentric X chromosome or idic(X)(q28).

Pangjaidee *et al*. (2003) determined the origin of all six marker chromosomes by using the micro-FISH technique. In this investigation, 6 structural aberrant chromosomes were characterized. There were dup(9)(p21), idic(X)(q28), der(17)t (17;4)(q25;q28), del(X)(q23), r(18)(p11.3q23), and r(X)(p11q12) chromosomes.

Horpauphan *et al*. (2003) applied micro-FISH techniques to produce whole chromosome 9 and 22 probes for detection of the Ph^1 chromosome in metaphase chromosomes and interphase nuclei of CML patients. The chromosome 9 derived probes could not detect the Ph^1 chromosome in interphase nuclei. The signals on the interphase nuclei were not informative because of the cross-hybridization of the probe.

The whole process of microdissection, DOP-PCR, probe lebeling and reverse painting can be completed within three days which makes this method perfectly suitable for prenatal diagnosis (Muller-Navia *et al*., 1995; Engelen *et al*., 1998a). However, the accuracy and limitations of interphase FISH have to be determined because of the undefined signal of WCPs in interphase cells. Moreover, cross-hybridization between short arms of the acrocentric chromosomes (13-15, 21 and 22) has been an unavoidable problem for most currently used WCPs and appears to be a consequence of common DNA sequences (Guan *et al*., 1994). Nowadays, this problem has been solved and the advances of micro-FISH techniques allow us to generate painting probes, in addition to chromosome 21 specific probe for Down syndrome detection.

2. Objectives

In this study

1. To generate chromosome 21 specific probe by the development of micro-FISH technique
2. To determine the t(21;21) and 21q derived-probes which hybridized on normal metaphase chromosome
3. To apply the chromosome 21 specific probe on uncultured amniocytes for prenatal diagnosis in Down syndrome high-risk pregnancy