CHAPTER II

MATERIALS AND METHODS

The peripheral blood and amniotic fluid samples for the production of chromosome 21 specific probes and evaluation of the derived-probes were collected from the Departments of Pediatrics and the Departments of Obstetrics and Gynecology. The samples were prepared for obtaining the karyotyping and the micro-FISH results at the Human Genetics Laboratory, Department of Anatomy, Faculty of Medicine, Chiang Mai University.

1. Preparation of DNA probes

1.1 Metaphase chromosome preparation

Chromosomes were prepared from peripheral blood lymphocytes using the synchronization method of Dutrillaux and Viegas-Pequignut (1981) with only minor modifications. Blood samples were cultivated in RPMI 1640 medium containing fetal bovine serum, antibiotics and phytohemagglutinin (PHA-stimulated peripheral blood lymphocytes). The cultures were incubated at 37ºC for 72 hours and were exposed to colcemid (0.1 μg/ml) for 30 minutes prior to harvesting. Suspension was centrifuged for 5 minutes at 1200 rpm, and washed in phosphate-buffered saline (PBS). Following centrifugation, the cells were processed by the addition of 5 ml of 0.075 M KCl, hypotonic solution for twice. Hypotonic treatment increases cell volume and disrupts the cell membrane of the red blood cells (allowing their removal). After that, lymphocyte was pelleted by centrifugating and the pellet was resuspended by adding 5 ml of fixative (3:1 methanol and acetic acid) for 3 times. For preparing microdissected slides, 1-2 drops of the cell suspension was dropped on 22 x 50 mm cover slips and dried on a 37ºC hotplate, and then rinsed in distilled water an incubated 37ºC overnight. All of the above steps were performed under sterile conditions.

The chromosome preparation on cover slips for microdissection in this studies were prepared from normal cases and the patients who had translocation between chromosome 21 and 21.

1.2 Microdissection of the chromosome

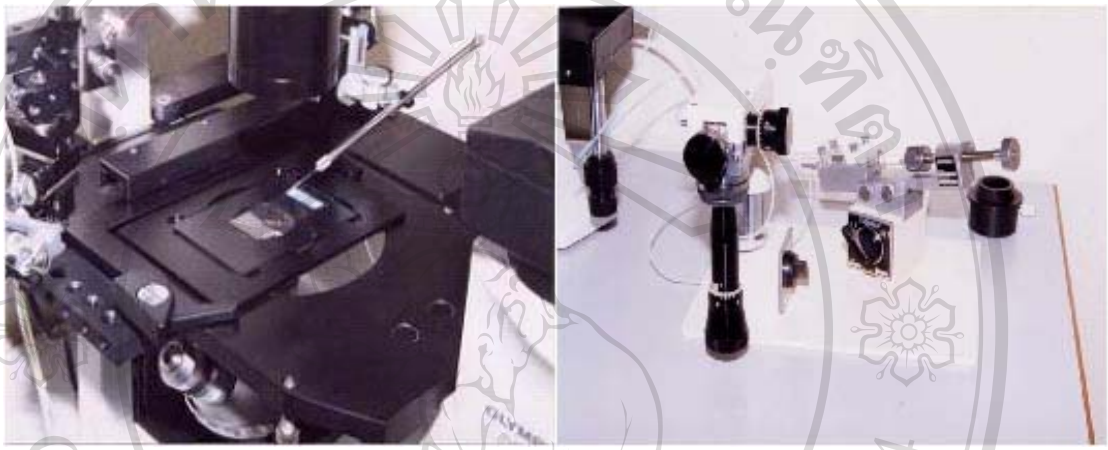
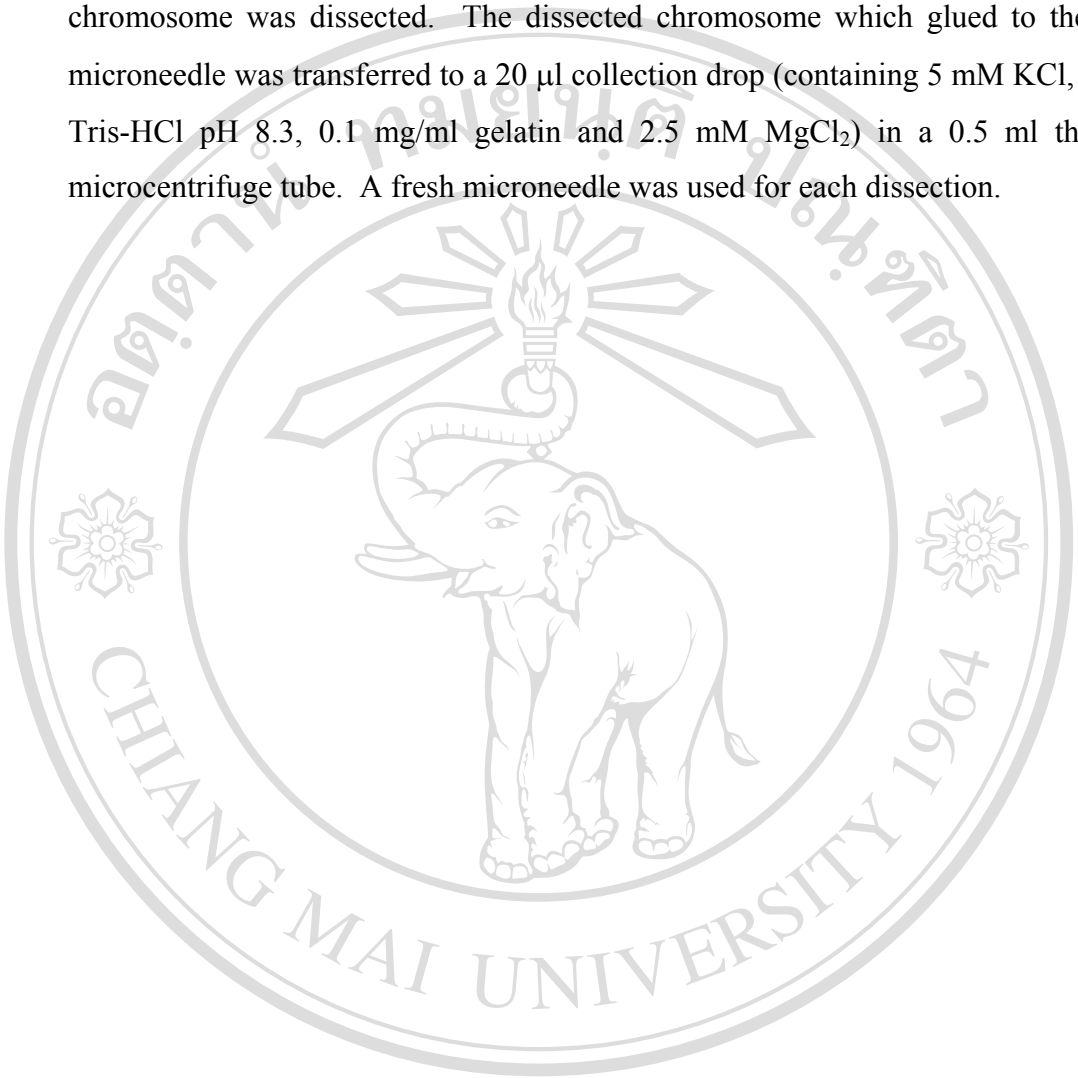


Figure 8 An inverted microscope (Olympus; CK40) equipped with micromanipulator (Narishige MMO-220-D). (Modified from Pangjaidee, 2003).

Microdissection was performed with siliconized glass needles (a diameter of about 0.3-0.5 μm) which had been equipped with a pipette puller (Narishige Model PC-10) and controlled by a micromanipulator (Narishige MMO-220-D). To avoid contamination with extraneous DNA, the microcentrifuge tubes including the collection drop and pipettips were treated with UV light for 30 minutes. After high resolution G-banding of microdissected coverslip was performed by the chromosome digestion with 2.5% trypsin solution followed by staining with 10% Giemsa stain in Sorensen's buffer for 3 minutes, 5-10 copies of the long arm of chromosome 21 or t (21;21) chromosome were dissected. A metaphase with well spread chromosomes was selected to cover with 1 μl milli-Q water in order to rehydrate the chromosomes. The wet and sticky t(21;21) chromosome could be collected as a whole by using only one microneedle. To collect the 21q chromosome region, a slightly different procedure was followed. The short arm and centromere of chromosome 21 were

dissected when the chromosome was still dry; subsequently a new microneedle was installed, the metaphase was covered with 1 µl milli-Q water and the 21q chromosome was dissected. The dissected chromosome which glued to the tip of microneedle was transferred to a 20 µl collection drop (containing 5 mM KCl, 10 mM Tris-HCl pH 8.3, 0.1 mg/ml gelatin and 2.5 mM $MgCl_2$) in a 0.5 ml thin wall microcentrifuge tube. A fresh microneedle was used for each dissection.



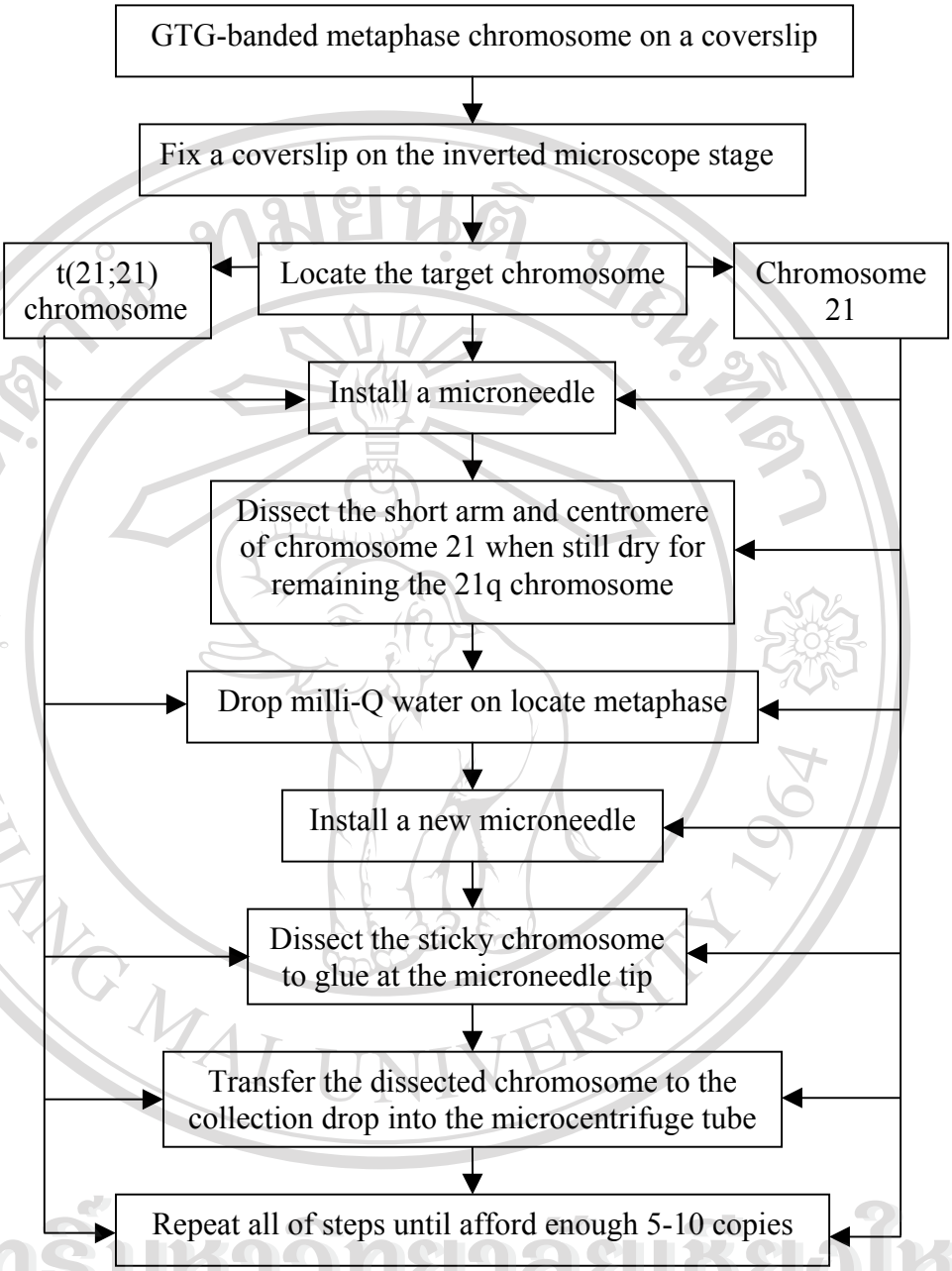


Figure 9 Summary of the dissection of a GTG-banded chromosome or chromosome region out of a metaphase with a microneedle using an inverted microscope.

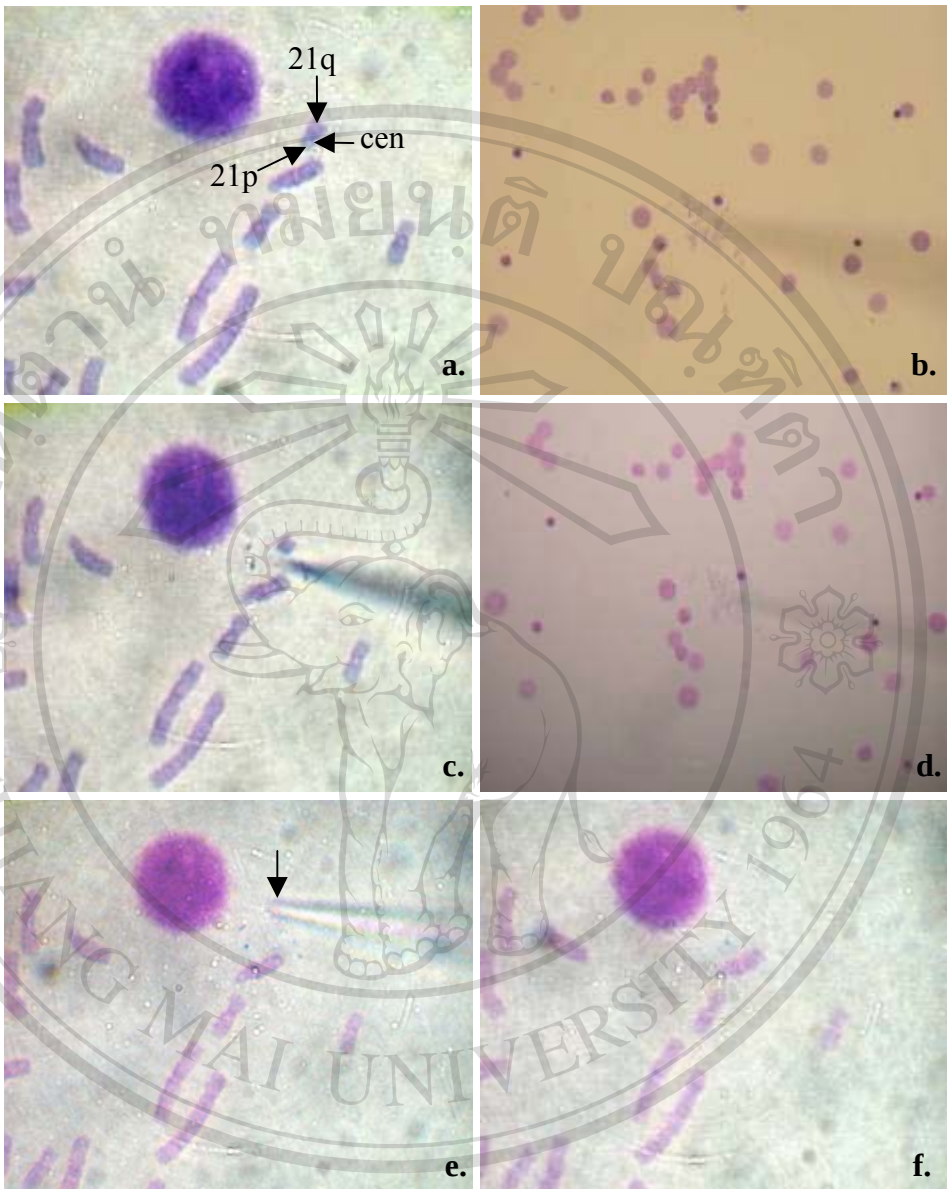


Figure 10 The procedure of chromosome microdissection.

(a) Location of a metaphase with chromosome 21 (at arrow)

(b) Installation of microneedle over the located metaphase

(c) Dissect short arm and centromere of chromosome 21

(d) Metaphase covered with milli-Q water

(e) Dissect long arm of chromosome 21 (arrow pointed at tip of the needle)

(f) The target chromosome 21 was dissected away and absence from metaphase

1.3 Amplification of Chromosomal DNA

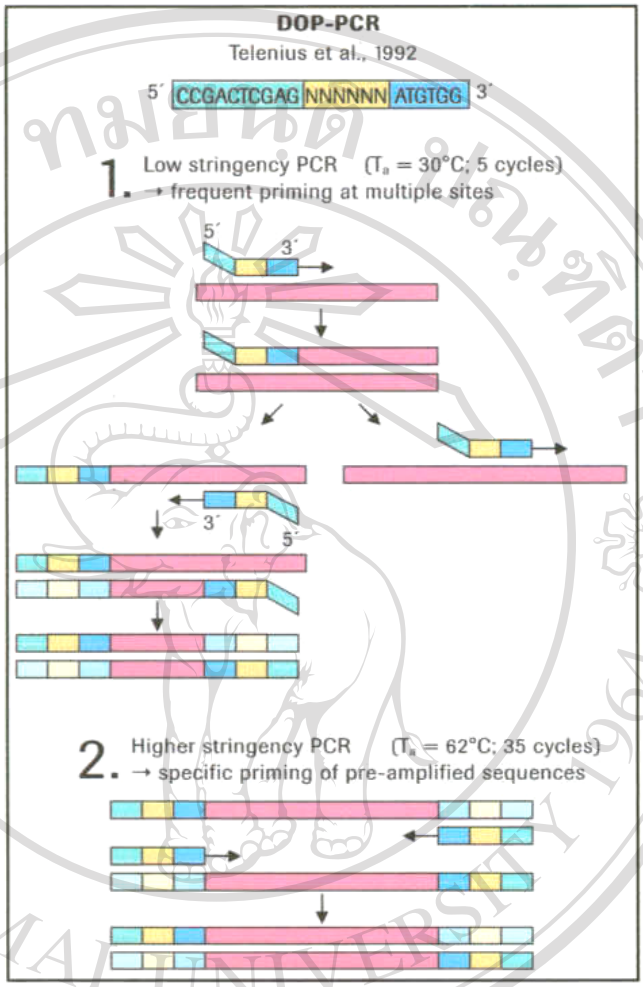


Figure 11 Schematic illustration of degenerate oligonucleotide primed polymerase chain reaction. (Modified from http://www.boehringer_mannheim.com/prod_inf/manvals/insitu/pdf/ISH95-103.pdf).

After 5-10 copies chromosomal materials were collected, the chromosomal DNA will be amplified by DOP-PCR reaction following the protocol of Engelen *et al*., 1998a with only minor modifications. The DOP-PCR experiments contained a negative control consisting of all PCR components except microdissected DNA, a positive control with 25 pg total human DNA and the microdissected chromosome in a collection drop. A preamplification step was performed in a Touch Down thermal cycle with heated lid for 20 cycles of 1 minute at 30ºC and 1 minute at 50ºC to

dissolve the dissected chromosome. Subsequently, 30 µl of the master mix containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.1 mg/ml gelatin, 2.5 mM $MgCl_2$, 100 µM of each dNTP, 4 µM universal primers and 1.5U ampliTaq Gold DNA polymerase (Perkin Elmer) was added to each sample. The universal primer 5'CCGACTCGAGNNNNNNATGTGG3' was used as suggested by Telenius *et al.* (1992). For initial denaturation the mixture was heated to 94ºC for 10 minutes and 93ºC for 3 minutes, followed by 8 cycles of 1 minute at 94ºC, 1 minute at 30ºC, 1 minute at 45ºC and 3 minutes at 72ºC and by 28 cycles of 1 minute at 94ºC, 1 minute at 56ºC, 3 minutes at 72ºC with a final extension step at 72ºC for 10 minutes.

1.4 Evaluation of DOP-PCR products

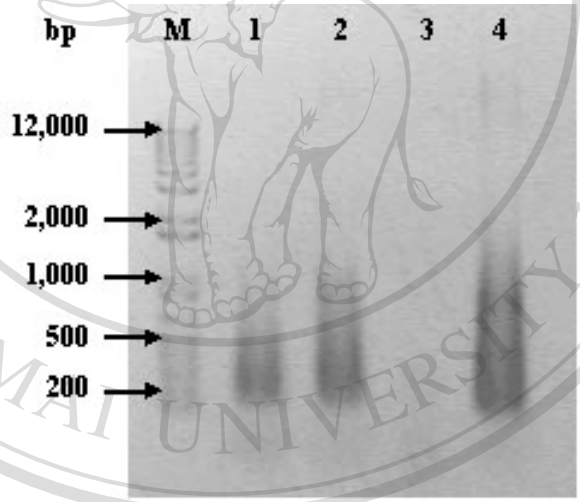


Figure 12 The primary DOP-PCR products after 1% agarose gel stained with ethidium bromide.

Lane M: 1 Kb plus DNA marker

Lane 1, 2: DOP-PCR products of 8 microdissected long arm of chromosome 21

Lane 3: negative control amplified with no template DNA

Lane 4: positive control (25 pg of DNA)

Size distributions of DOP-PCR-amplified DNA fragments in each the dissected chromosomes were measured by analyzing PCR products on 1% agarose gels after being stained with ethidium bromide. The 1 Kb Plus DNA LADDER is suitable for sizing linear double-stranded DNA fragments from 100 bp to 12 kb. Experiments were proceeded only if DNA synthesis was not apparent in the negative control. 1 µl was taken from the amplified PCR products and reamplification was done in another 50 µl collection drop. The secondary PCR reaction was done identical to that described above except for the preamplification step. The reaction was cycled for 10 minutes at 94ºC and 3 minutes at 93ºC, followed by 8 cycles of 1 minute at 94ºC, 1 minute at 30ºC, 1 minute at 45ºC and 3 minutes at 72ºC and by 28 cycles of 1 minute at 94ºC, 1 minute at 56ºC, 3 minutes at 72ºC with a final extension step at 72ºC for 10 minutes.

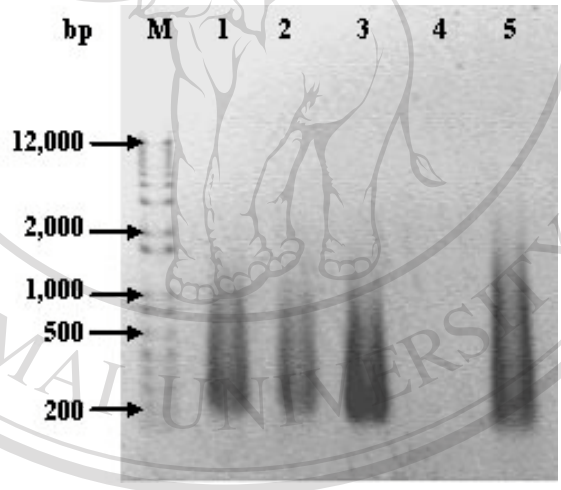


Figure 13 The secondary DOP-PCR products after 1% agarose gel stained with ethidium bromide.

Lane M: 1 Kb plus DNA marker

Lane 1, 2: reamplified DOP-PCR products of 8 microdissected chromosomal DNA from chromosome region 21q

Lane 3: the reamplification of the negative control from the previous reaction

Lane 4: negative control amplified with no template DNA

Lane 5: the reamplification of the positive control from the previous reaction

1.5 PCR product purification

The secondary PCR product was precipitated and purified by adding 3 M sodium acetate, 1 M $MgCl_2$ and cold 100% ethanol, then frozen at -70ºC for 1 hour, followed by centrifugating at 15000 rpm at 4ºC for 30 minutes. The pellet was washed with 500 µl cold 70% ethanol and after that, the supernatant was discarded and aspirated to dry the residual DNA in an 37ºC incubator. This purified PCR product was dissolved with a TE buffer (10 mM Tris-HCl, 1mM Na_2EDTA, pH 8.0) at 37ºC for 30 minutes in a water bath.

1.6 DNA labeling

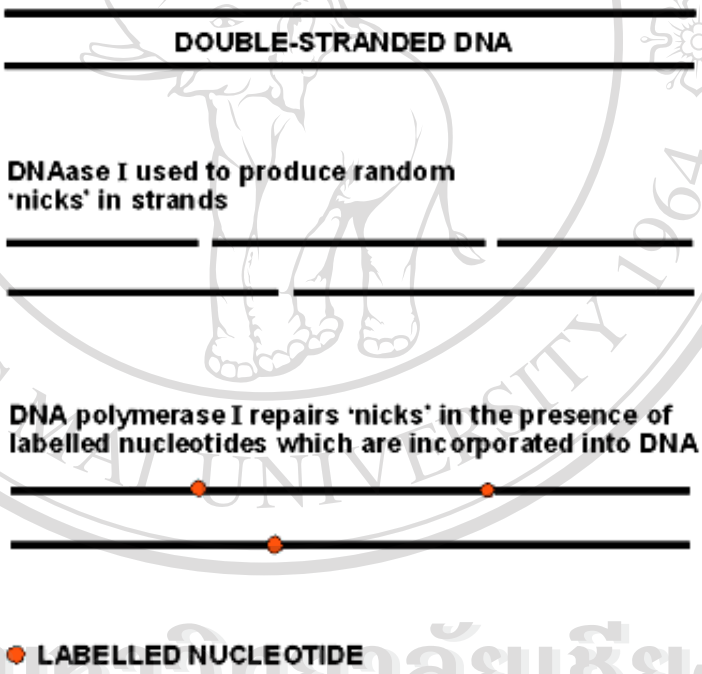


Figure 14 Representation of the nick translation technique.
(Modified from http://www2.westminster.ac.uk/~redwayk/lectures/probes.htm)

To prepare the probe, 2-4 µl of purified DNA were labeled with 4 µl of digoxigenin-11-dUTP or biotin-16-dUTP in a nick translation mix (DNA polymerase I, DNase I, digxoxigenin-11-dUTP/biotin-16-dUTP, dATP, dCTP, dGTP, dTTP and

optimized reaction buffer concentration in 50% glycerol) and sterile water that resulted in 20 µl of labeled product. Following the protocol of the supplier (Roche), the labeled product was incubated for 90 minutes at 15ºC and then was fixed by adding 1 µl of 0.5 M EDTA (pH 8.0) and heated to 65ºC for 10 minutes, to check the labeled DNA by gel electrophoresis and store it at -20ºC until ready to perform FISH.

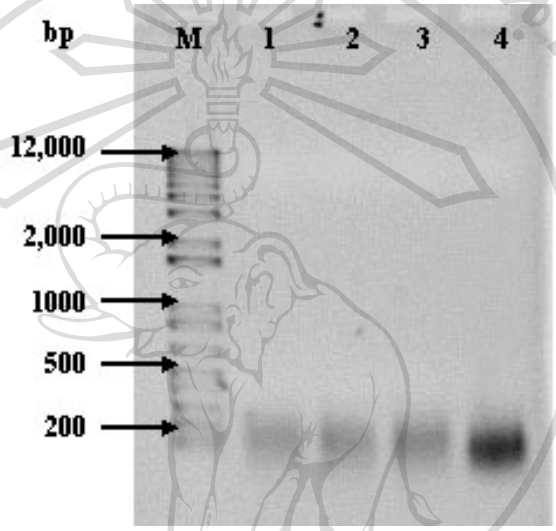


Figure 15 An 1% agarose gel stained with ethidium bromide demonstrating the size of DNA labeled as a probe with DIG-11-dUTP by nick translation.

Lane M: 1 Kb plus DNA marker

Lane 1, 2, 3: probes from chromosome region 21q

Lane 4: probe from human cot-1 DNA

2. Preparation of target DNA

2.1 Preparation of metaphase chromosome

Metaphases spread for FISH were prepared from phytohemagglutinin (PHA)-stimulated human lymphocytes and treated with colcemid and harvested as previously described. Metaphases were spread on a clean glass slide. Following rinsing with distilled water and dehydrated by an ethanol series for 1 minute each and stored at -20ºC until the time to perform FISH.

2.2 Preparation of uncultured amniocytes for interphase FISH

The 12 samples of 15-20 ml of amniotic fluid (AF) were retrospective FISH study with the conventional karyotyping. Maternal age varied from 36 to 43 years. Gestational age at the time of the procedure varied from 16 to 20 weeks. Ten ml of AF was used for standard karyotyping. The remaining AF was used for micro-FISH analysis. The bloody samples were excluded from this study to prevent maternal cell contamination.

Table 2 Information of amniotic fluid samples for FISH study

Case	maternal age (years)	week of gestation	cell pellet color
1	38	18	white
2	39	18	white
3	38	17	white
4	41	17	white
5	36	20	white
6	40	18	white
7	39	17	white
8	36	17	white
9	43	16	white
10	40	17	white
11	40	17	white
12	42	16	white

The interphase nuclei preparations from amniotic fluid were made using the method described by Klinger *et al*., 1992 and Lerner *et al*., 2001a with minor modifications. For each amniotic fluid sample usually 5-7 ml of clear amniotic fluid sample was centrifuged for 5 minutes at 2000 rpm and the supernatant was removed. The cell pellet was washed in 5 ml of PBS warmed to 37ºC. Following centrifugating, the pellet was treated with 5 ml of prewarm (37ºC) hypotonic solution (0.075 M KCl). After removing the supernatant, the cells were collected and placed directly onto slides coat with AES and incubated at 37ºC for 30 minutes. The

hypotonic solution was replaced by 200 µl of 30% 3:1 fixative and 70% 0.075 M KCl for 5 minutes at room temperature. About 5 drops of fresh fixative were dropped onto the cell area. Slides were briefly dried on a 60ºC hotplate and then rinsed in distilled water. Following ethanol dehydration, the slides were either hybridized or stored at -20ºC until required.

3. Fluorescence *in situ* hybridization (FISH)

3.1 Precipitation and denaturation of DNA probes

20-40 µl of Probes and 10 µl of human Cot-1 DNA [1 µg/µl] (Roche) were precipitated with 3 M sodium acetate, 1 M $MgCl_2$ and cold 100% ethanol, then frozen at -70ºC for 1 hour, followed by centrifugating at 15000 rpm at 4ºC for 30 minutes. The pellet was washed with 500 µl cold 70% ethanol after that, the supernatant was discarded and aspirated to dry the residual DNA at 37ºC incubator. Subsequently, 10 µl of hybridization mixture containing 50% formamide, 2xSSC, and 10% dextran sulfate was added to DNA probe. The DNA probe mixture was heated to 56ºC for 5 minutes and warmed at 37ºC for 30 minutes followed by denaturation at 80ºC for 10 minutes and rapidly chilling on ice for 10 minutes. Then, preannealing was done at 37ºC for 1-2 hours before hybridization.

3.2 Slides treatment and denaturation of the target DNA

The slides of the desired target cell (metaphase chromosome/interphase amniocytes) were washed in 2xSSC (standard saline citrate) for 10 minutes, treated with RNase solution (0.1 µg/µl) for 1 hour at 37ºC in a moist chamber and then washed three times in 2xSSC at room temperature for 5 minutes each. Subsequently, the slides were treated with 0.01% pepsin in 0.01 N HCl at 37ºC for 10 minutes and washed in 0.01 N HCl at room temperature for 5 minutes. The slides were fixed using 1% formaldehyde/50 mM $MgCl_2$ in PBS for 10 minutes at 37ºC, washed in PBS for 5 minutes before dehydration in 70%, 85%, and 100% ethanol for 1 minute each, and then air dried. The target DNA on the slide were denatured in 70% formamide/2xSSC at 75ºC for 5 minutes, dehydrated through a cold ethanol series and dried at 37ºC.

3.3 Hybridization and detection

The prepared denaturation of DNA probe (10 μl) was put onto the denatured target DNA. A cover slip (22x22 mm) was sealed in place with nail polish, and the slides were incubated overnight (16-20 hours) at 37ºC in a moist chamber.

After hybridization, the cover slips were removed and the slides were washed three times in 50% formamide/2xSSC at 42ºC for 1 minute, 0.1xSSC, and 0.1% tween 20 at 37ºC for 2 minutes each, followed by blocking nonspecific hybridization with 1% blocking in PBS at 37ºC for 30 minutes in a moist chamber and washed in PBS for 5 minutes. For fluorescent signals detection, the slides were incubated with 20 μ g/ml anti-digoxigenin-rhodamine (Roche) in 1% blocking in PBS or with 10 μg/ml avidin-fluorescein (Roche) in 1% blocking in PBS at 37ºC for 35 minutes. After that the slides were washed twice in 0.1% tween 20 for 2 and 4 minutes, washed again in PBS for 2 minutes, and dehydrated through an ethanol series. After the washing steps, slides were counterstained with 0.5 μg/ml DAPI (Sigma), rinsed in distilled water and dried at 37ºC incubator. Finally, the slides were covered with 7 μl of antifade solution (Vysis) under a cover slip (22x22 mm) which was then sealed with nail polish.

3.4 FISH analysis

The slides were analyzed under a fluorescent microscope (Zeiss Axioskop II) with a suitable filter set. The evaluation was done by analyzing hybridization signals of the dessected probe DNA to normal lymphocyte metaphase chromosome or interphase nuclei. The cross-hybridization was observed between painting probe of long arm of chromosome 21 and the t(21;21) chromosome that were hybridized to normal human peripheral lymphocyte metaphase chromosomes. For the application to prenatal diagnosis, chromosome 21 specific probes were used in uncultured amniocytes on 6 cases of normal interphase nuclei and 6 cases of trisomy 21 nuclei. The evaluation of signals was modified from Jobanputra *et al*. (2002). The nuclei were observed under x100 oil objective and a minimum of 50 nuclei or 20 metaphase spreads were scored for each probe. Nuclei free from any attached cytoplasm or cellular membranes showing 0, 1, 2, 3, 4 or 5 signals were selected for scoring. Only those signals, which were well embedded in the nucleus, were included for scoring.

Clumped or overlapping nuclei and nuclei with high background intensity or low signal intensity were not scored. Patchy and diffused signals were included in the evaluation only if they were well separated. Split-spot (i.e. signals in a paired arrangement) were scored as one signal only if the distance between the signals was less than the width of one of these signals; otherwise they were observed as two signals. The following criterion was chosen for the interpretation (adopted from Jobanputra *et al*., 2002; Luquet *et al*., 2002). On the basis of FISH scoring results the samples were considered to be informative, normal and abnormal. However final diagnosis was made only on the basis of karyotyping.

Results were defined as informative normal or disomy if ≥80% of the hybridized nuclei had two signals and informative trisomy 21 was defined if ≥70% of the nuclei had three signals.

Moreover, whenever at least 50 nuclei were enumerated for each probe. FISH was successful. When the number of nuclei observed was between 10 and 49, FISH was partially successful. In cases where less than 10 nuclei could be analyzed, the FISH was considered unsuccessful.