CHAPTER III

RESULTS

1. The quality of the DOP-PCR products

The representative gel in Figure 16 demonstrates that the PCR products from microdissected DNA results in a smear ranging from ~200-1,500 bp. Moreover, no amplification appeared in the negative control without template DNA (lane 3).

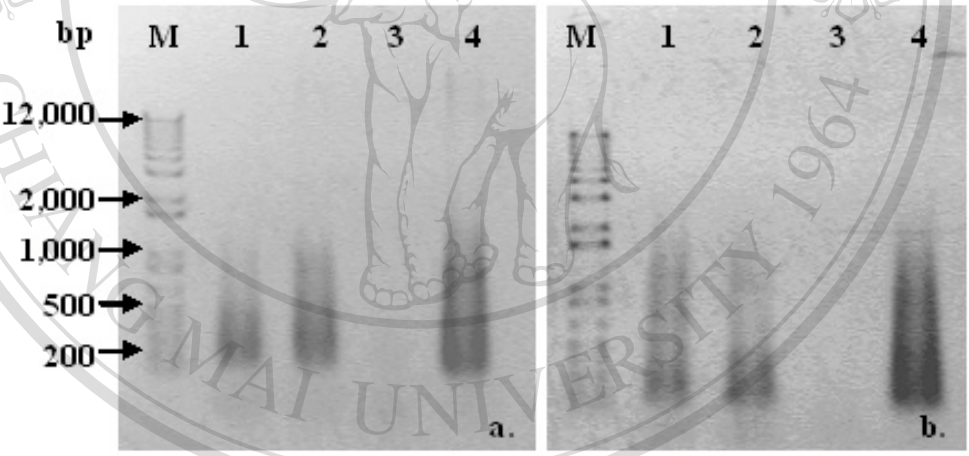

Figure 16 The primary DOP-PCR products (5 μl) from microdissection of (a) the normal chromosome 21q and (b) the t(21;21) chromosome were size fractionated on a 1% agarose gel and stained with ethidium bromide.

Lane M: 1 Kb plus DNA marker (conc. 0.1 μg/μl)
Lane 1, 2: DOP-PCR products of 8 microdissected chromosomes
Lane 3: negative control with no DNA contamination
Lane 4: positive control with 25 pg of DNA

In this study, only 1 μl of primary DOP-PCR reaction could be re-amplified several times. There are more secondary DOP-PCR products for both chromosomes

resulting in a smear ranging from ~200 to 1,500 bp. However, the endogenous contamination was appeared in the re-amplified previous negative control distinctly stronger than the PCR-products. We chose to disregard for this contamination and the experiment was continued to confirm the human origin of the amplified DNA from the dissected fragments.

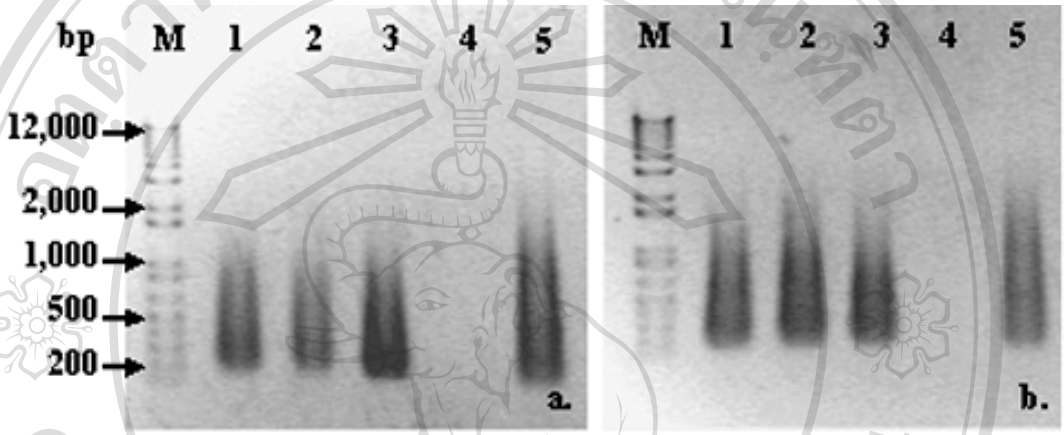

Figure 17 The secondary DOP-PCR products (5 μl) from the reamplified DNA microdissected of (a) the normal chromosome 21q and (b) the t(21;21) chromosome were size fractionated on a 1% agarose gel and stained with ethidium bromide.

Lane M: 1 Kb plus DNA marker (conc. 0.1 μg/μl)

Lane 1, 2: secondary DOP-PCR products

Lane 3: reamplified previous negative control

Lane 4: negative control with no DNA contamination

Lane 5: reamplified previous positive control

2. The size of DNA probes

The DOP-PCR product could be labeled by nick translation reaction. After the DNA was nicked with DNase I, the 5'→3' exonuclease activity of DNA polymerase I extend the nicks to gaps; then the polymerase replaced the excised nucleotides with labeled ones. The labeled DNA fragments were cut to an average of ~100-300 bp before hybridization.

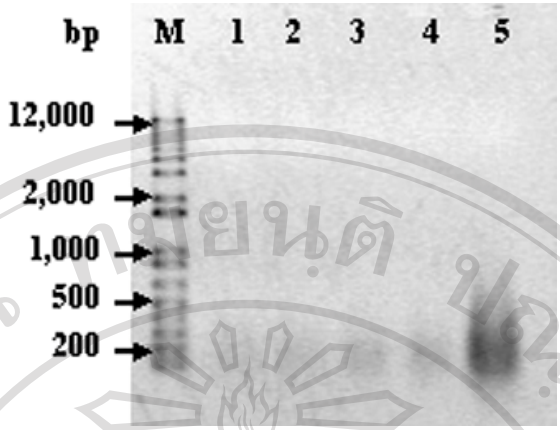

Figure 18 The DIG-11-dUTP labeled probe (1 μl) was size fractionated on a 1% agarose gel and stained with ethidium bromide.

Lane M: 1 Kb plus DNA marker (conc. 0.1 μg/μl)

Lane 1, 2: DNA probe from 21q chromosomal DNA

Lane 3, 4: DNA probe from t(21;21) chromosomal DNA

Lane 5: DNA probe from human cot-1 DNA

3. Determination of hybridization specificity

To confirm the origin of the amplified DNA from the primary and secondary DOP-PCR products, FISH analysis was performed using DNA probes from 21q and t (21;21) chromosomal DNA on normal metaphases with chromosome t(21;21)-derived probe and 21q-derived probe; it resulted in signals on the long arm of chromosome 21 and the centromeric region of two chromosomes of D-group. These chromosomes were counterstained with DAPI for evaluation and localize the metaphase chromosomes or interphase nuclei. This was clearify that it was the chromosome 13. The p arm of all acrocentric chromosomes (13-15, 21, and 22) were not hybridized corresponding to the study of Guan *et al*., 1994 but cross-hybridization was observed in the centromeric region between the 21 and 13 chromosomes using either a t(21;21) chromosome probe or a 21q chromosome probe. Because the α-satellites of chromosome 13 and 21 repeats exhibit almost 100% homology, the centromeric region could not distinguish between the two chromosomes. Even if both p arm and centromere were not dissected to amplify them, the cross-hybridization problem appears in all analyzing 20 metaphases with FISH signals.

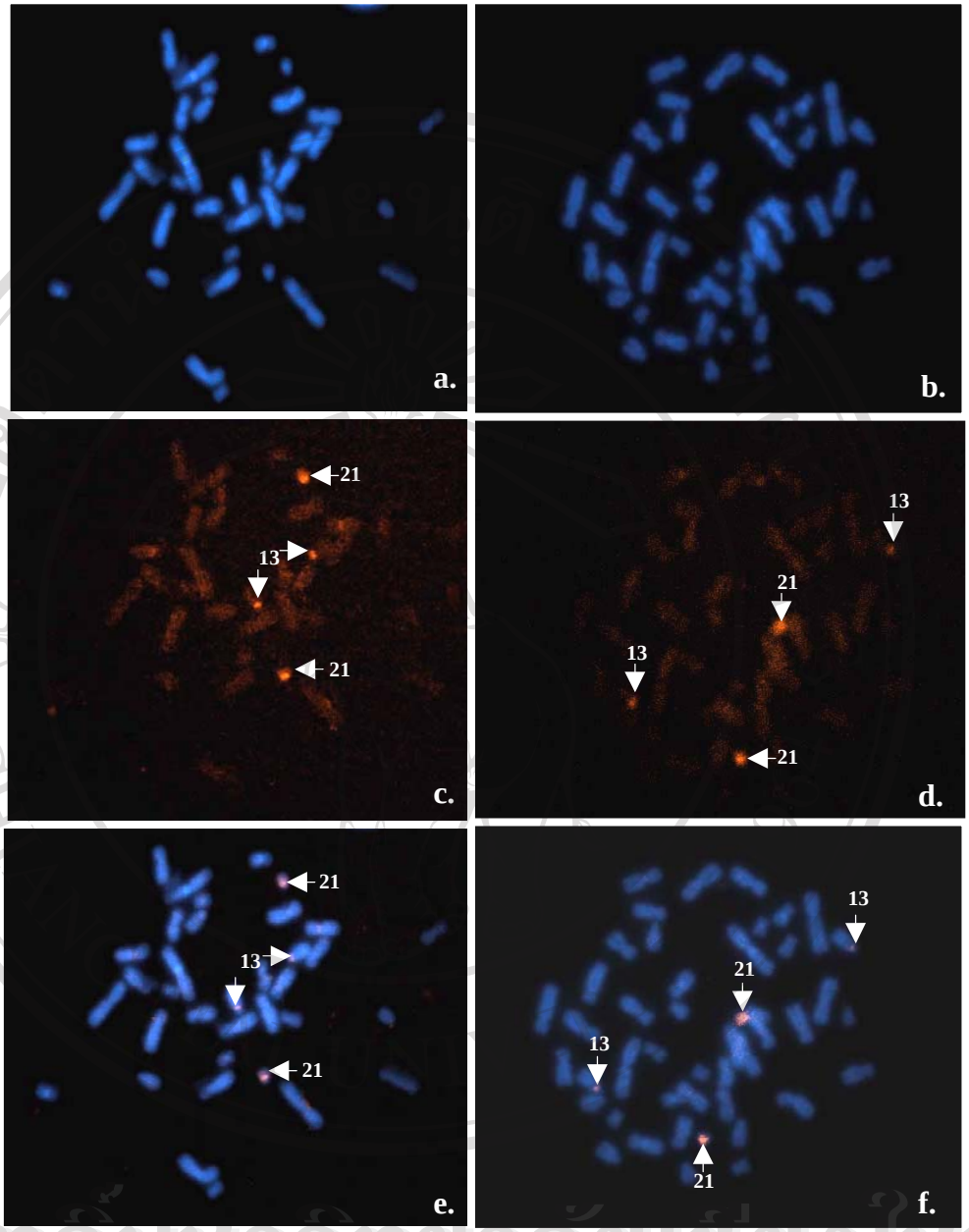

Figure 19 FISH with chromosome t(21;21) derived-probe on two different normal metaphase.

(a-b) DAPI counterstaining

(c-d) Probe was detected by Anti-digoxigenin-rhodamine

(e-f) Multichannals between DAPI and Cy3 (red) signals by AxioVision 3.1 program

The signals on chromosome 21 were more intense than chromosome 13.

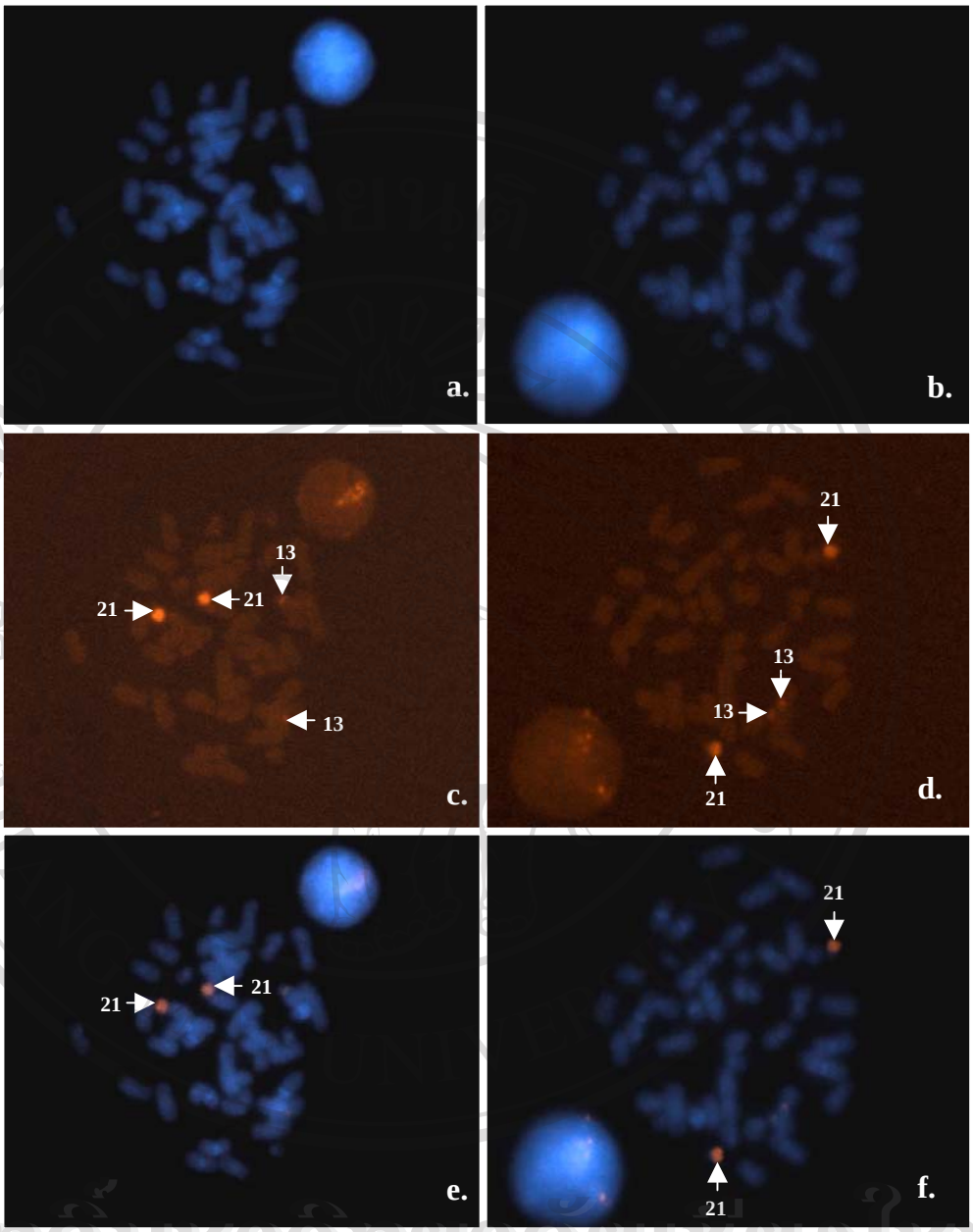

Figure 20 FISH with chromosome 21q derived-probe on normal metaphase chromosomes.

(a-b) DAPI counterstaining

(c-d) Probe was detected by Anti-digoxigenin-rhodamine

(e-f) Multichannals between DAPI and Cy3 (red) signals by AxioVision 3.1 program

The cross-hybridized chromosome 13 was diminished or less intense.

4. Interphase FISH results

Results of using the constructed probe on uncultured amniocytes were obtained in different types of signal expression; 0-5 signals, appeared as dot, patchy or diffused. Some dot signals are detected on nuclei that clear cytoplasm. Patchy or diffused signals were appeared mostly on nuclei with excess residual cytoplasm and debris so that many nuclei were excluded for scoring. For this reason, the number of nuclei for FISH analysis was insufficient.

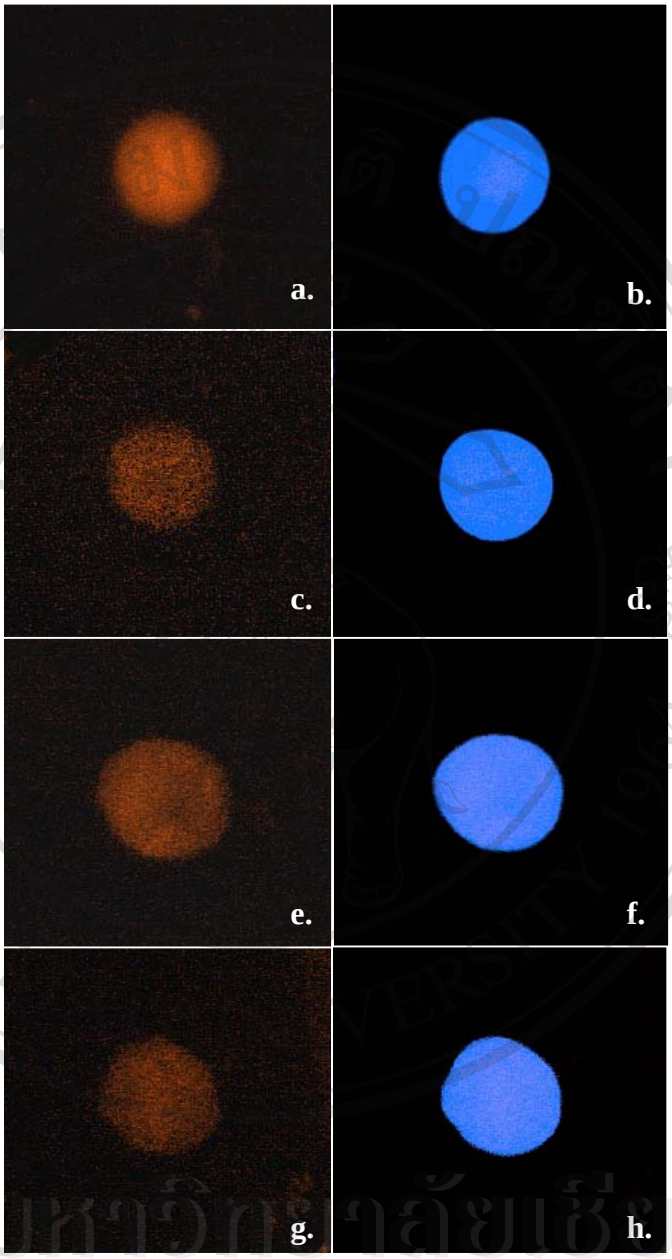

Figure 21 Interphase nucleus from uncultured amniocytes by FISH shows no signal.
(left; a, c, e, g) Probe was detected by Anti-digoxigenin-rhodamine
(right; b, d, f, h) Multichannals between DAPI (blue) and Cy3 (red) signals by AxioVision 3.1 program
(a, b and c, d) The two nuclei of normal cases
(e, f and g, h) The two nuclei of trisomy 21 cases

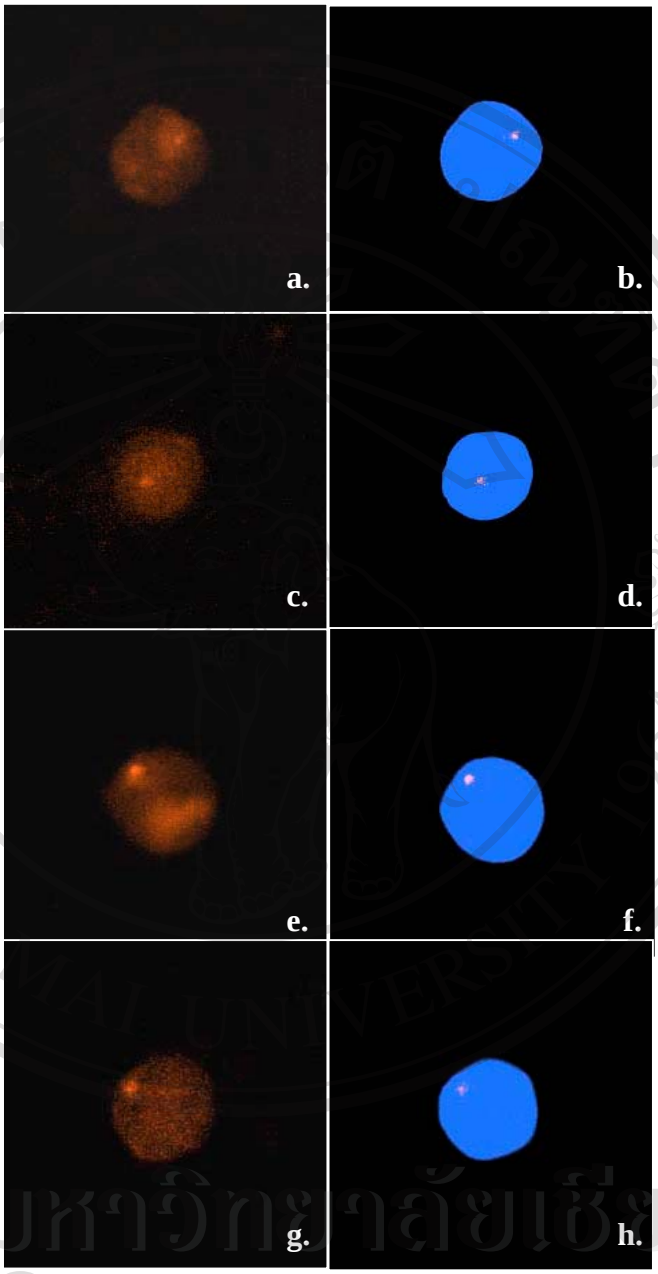

Figure 22 Interphase nucleus from uncultured amniocytes by FISH shows 1 signal.

(left; a, c, e, g) Probe was detected by Anti-digoxigenin-rhodamine

(right; b, d, f, h) Multichannals between DAPI (blue) and Cy3 (red) signals by AxioVision 3.1 program

(a, b and c, d) The two nuclei of normal cases

(e, f and g, h) The two nuclei of trisomy 21 cases

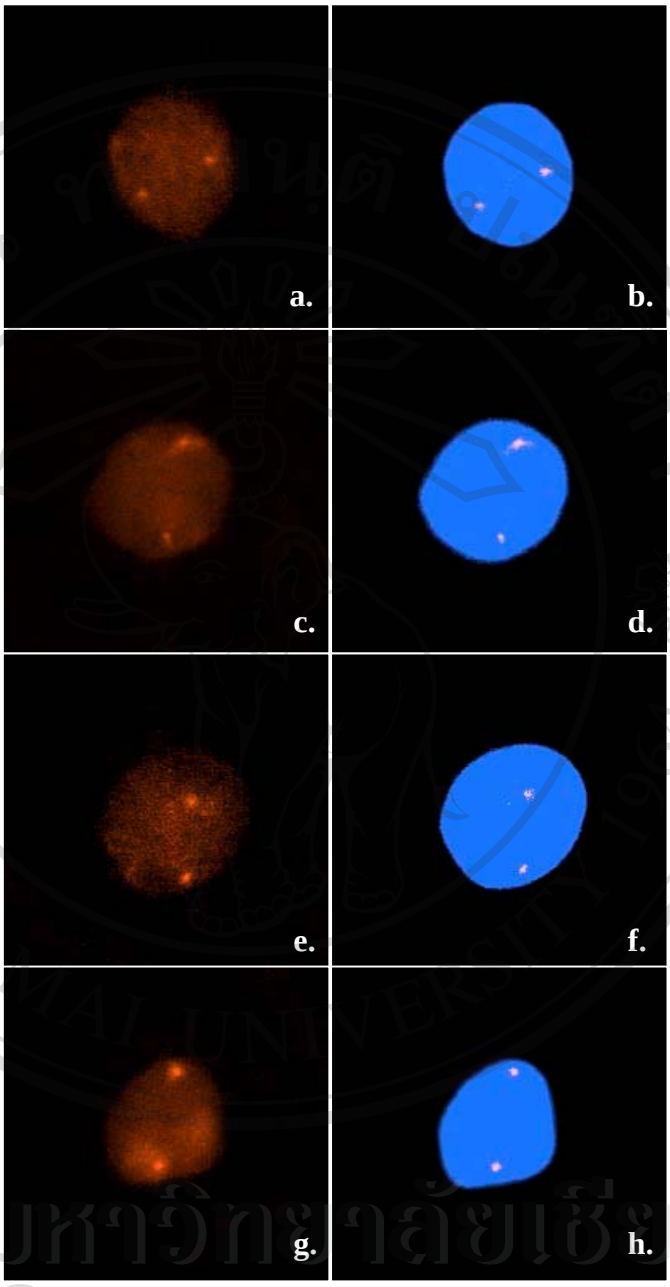

Figure 23 Interphase nucleus from uncultured amniocytes by FISH shows 2 signals.

(left; a, c, e, g) Probe was detected by Anti-digoxigenin-rhodamine

(right; b, d, f, h) Multichannals between DAPI (blue) and Cy3 (red) signals by AxioVision 3.1 program

(a, b and c, d) The two nuclei of normal cases

(e, f and g, h) The two nuclei of trisomy 21 cases

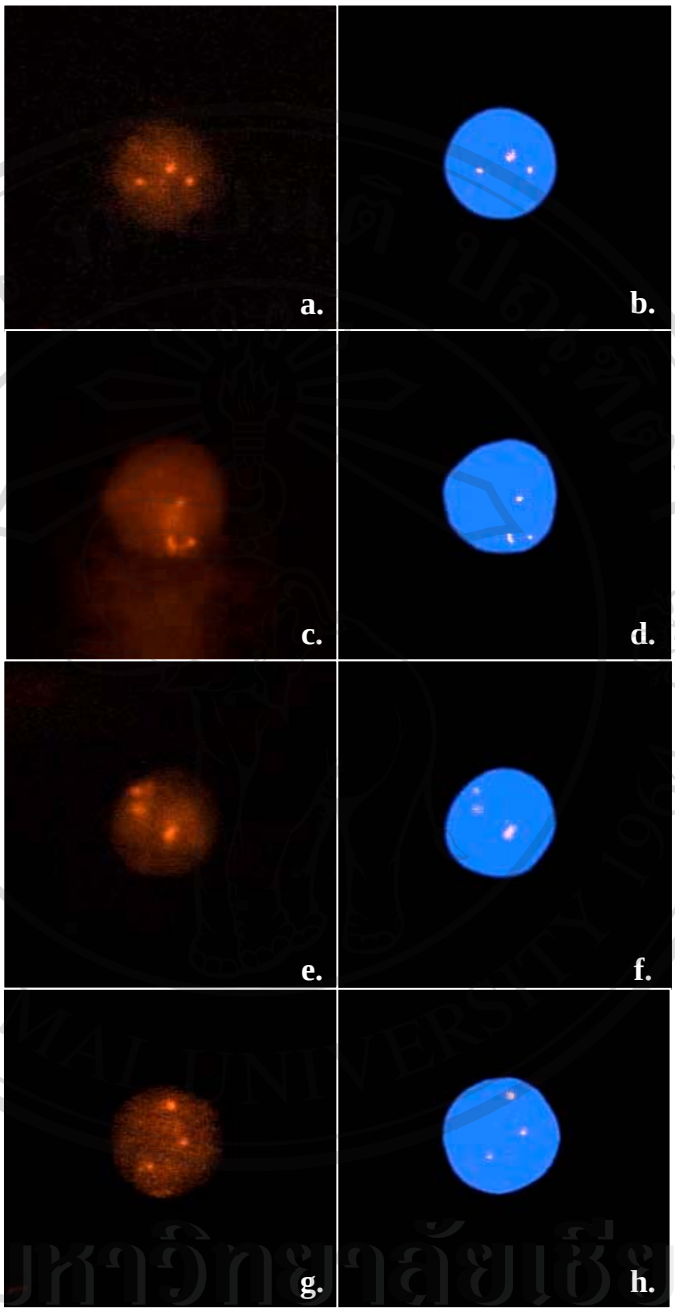

Figure 24 Interphase nucleus from uncultured amniocytes by FISH shows 3 signals.

(left; a, c, e, g) Probe was detected by Anti-digoxigenin-rhodamine

(right; b, d, f, h) Multichannals between DAPI (blue) and Cy3 (red) signals by AxioVision 3.1 program

(a, b and c, d) The two nuclei of normal cases

(e, f and g, h) The two nuclei of trisomy 21 cases

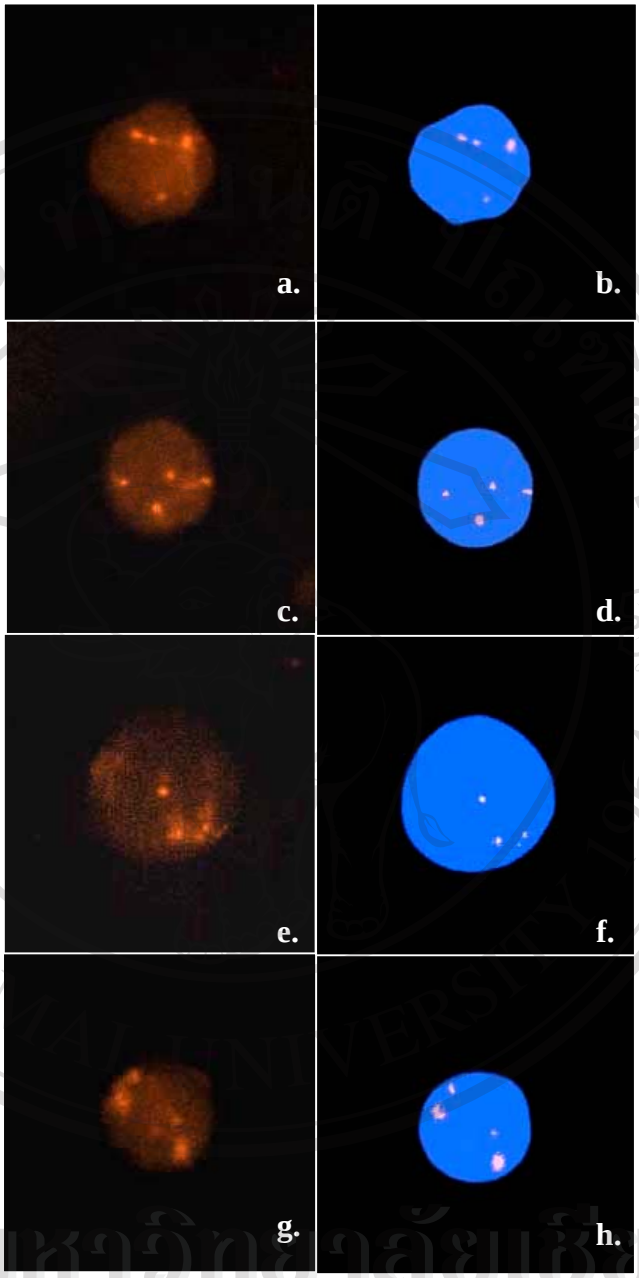

Figure 25 Interphase nucleus from uncultured amniocytes by FISH shows 4 signals.

(left; a, c, e, g)	Probe was detected by Anti-digoxigenin-rhodamine
(right; b, d, f, h)	Multichannals between DAPI (blue) and Cy3 (red) signals by AxioVision 3.1 program
(a, b and c, d)	The two nuclei of normal cases
(e, f and g, h)	The two nuclei of trisomy 21 cases

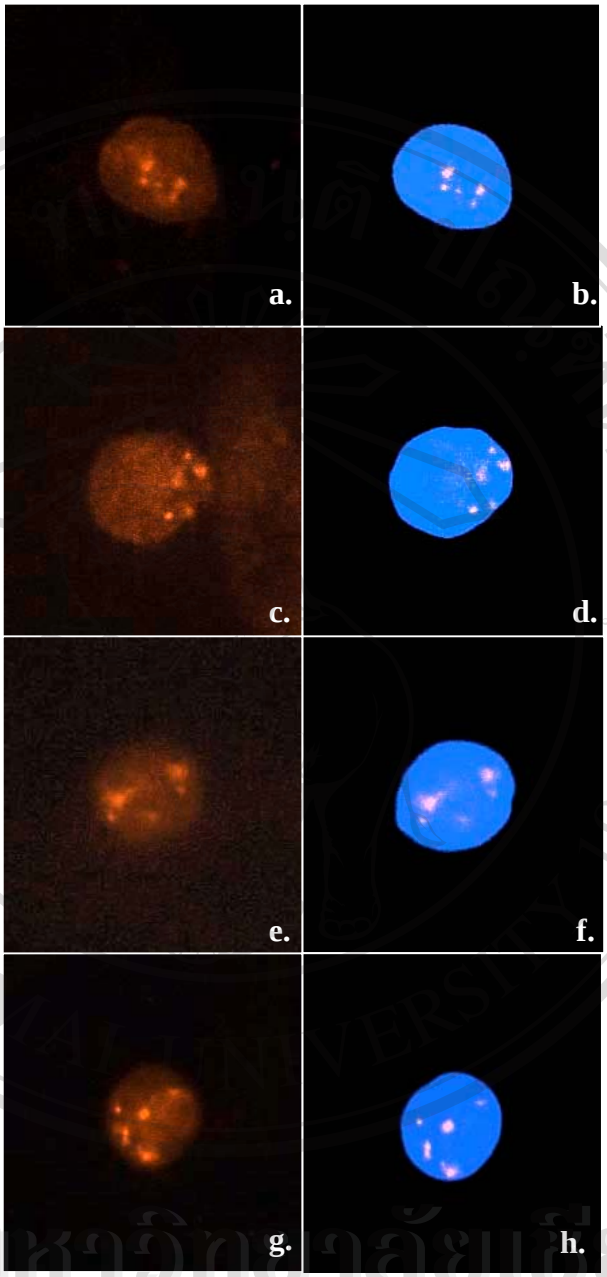

Figure 26 Interphase nucleus from uncultured amniocytes by FISH shows 5 signals.

(left; a, c, e, g) Probe was detected by Anti-digoxigenin-rhodamine

(right; b, d, f, h) Multichannals between DAPI (blue) and Cy3 (red) signals by AxioVision 3.1 program

(a, b and c, d) The two nuclei of normal cases

(e, f and g, h) The two nuclei of trisomy 21 cases

14 hybridizations were performed on 12 cases of uncultured amniocytes because of the insufficient by the nuclei in 2 cases. At least 47-50 nuclei for each case were investigated. This cut-off level was decreased from the analytic criteria. These could be decreased to 30 nuclei without loss of information according to Eiben *et al*. (1999) and Pergament *et al*. (2000a). There were 12 karyotyping that were expected to be detected using interphase FISH analysis, including six cases of normal and six cases of trisomy 21.

Table 3 Karyotype and interphase FISH finding in 12 amniotic fluid samples

		Hybridization signals (n)						
Case	nuclei scored (n)	0	1	2	3	4	5	karyotype
1	47	1	6	26	11	3	0	46,XY
2	48	1	11	25	10	1	0	46,XY
3	48	5	12	25	4	2	0	46,XX
4	49	0	8	32	7	2	0	46,XY
5	50	0	7	26	11	4	2	46,XY
6	50	4	8	27	8	3	0	46,XX
7	50	4	5	19	16	6	0	47,XX+21
8	50	1	2	17	28	2	0	47,XX,+21
9	50	0	1	16	26	4	3	47,XX,+21
10	50	2	4	14	21	5	4	47,XY,+21
11	50	0	3	15	26	5	1	47,XY,+21
12	50	1	7	13	22	7	0	47,XY,+21

Table 4 The percentage of the number of hybridization signals using 21q microdissected probe (case 1-6 were normal, case 7-12 were trisomy 21)

	Signal number					
Case	0	1	2	3	4	5
1	2.13	12.77	55.32	23.40	6.38	0.00
2	2.08	22.92	52.08	20.83	2.08	0.00
3	10.42	25.00	52.08	8.33	4.17	0.00
4	0.00	16.32	65.31	14.28	4.08	0.00
5	0.00	14.00	52.00	22.00	8.00	4.00
6	8.00	16.00	54.00	16.00	6.00	0.00
7	8.00	10.00	38.00	32.00	12.00	0.00
8	2.00	4.00	34.00	56.00	4.00	0.00
9	0.00	2.00	32.00	52.00	8.00	6.00
10	4.00	8.00	28.00	42.00	10.00	8.00
11	0.00	6.00	30.00	52.00	10.00	2.00
12	2.00	14.00	26.00	44.00	14.00	0.00

FISH was entirely successful (50 nuclei for each probe) in 8 cases (66.66%). In 4 cases (33.33%), FISH was partially successful actually 47-49 nuclei were enumerated. In cases that FISH was not successful, because either there were insuffient nuclei or no hybridization occurred the data not show for analysis. In karyotypically normal samples, an average of 55.14% of interphase nuclei showed two hybridization signals. In cases of trisomy 21, the average percentage of nuclei with three signals was 46.33%. All 12 cases could not be diagnosed because the percentage was lower than either 80% for informative normal or 70% for informative trisomy 21. Moreover, there are one false negative in case 7 in which 38% showed two signals whereas 32% as three signal.

However, the hybridization on uncultured amniocytes nuclei by using microdissected DNA probe was first described in this study. The expectation of as high a percentage as that of commercial probe could not be achieved. We decided to modify the informative criteria for normal or trisomy 21 diagnostic results only if the

hybridization resulted in maximum signals, and compared them to the conventional karyotyping.

Table 5 Comparison between FISH and karyotype in 12 cases

case	FISH results	karyotype
1	normal	46,XY
2	normal	46,XY
3	normal	46,XX
4	normal	46,XY
5	normal	46,XY
6	normal	46,XX
7	normal	47,XX,+21
8	trisomy 21	47,XX,+21
9	trisomy 21	47,XX,+21
10	trisomy 21	47,XY,+21
11	trisomy 21	47,XY,+21
12	trisomy 21	47,XY,+21

The accuracy of this study is shown in tables 6 and 7. There were 6 samples that were reported as disomic for chromosome 21 which were confirmed by cytogenetics. A result of trisomy 21 was obtained by FISH and was cytogenetically confirmed in 5 samples. There was 1 instance of false-negative results as defined by undetected presence of trisomy 21.

Table 6 Results of prenatal detection for chromosome 21 by FISH in 12 cases

	No. of informative results			
	True negative[a]	True positive[b]	False positive[c]	False negative[d]
chromosome 21	6	5	0	1

Note. (Described by Ward *et al*. 1993)

[a] Absence of aneuploidy for the tested chromosomes, as reported by FISH and confirmed by cytogenetics.

[b] Presence of aneuploidy for the tested chromosomes, as reported by FISH and confirmed by cytogenetics.

[c] Abnormal report, by FISH, which was shown to be normal on cytogenetics.

[d] Normal results by FISH, which were diagnosed as aneuploid for the tested chromosome by cytogenetics.

The total false-positive rate for chromosome 21 was 0% whereas the false-negative rate was 16.66%. For all detectable trisomy 21, the sensitivity was 83.33%, the specificity was 100% and the overall accuracy was 91.66% (table 7).

Table 7 Performance characteristics for informative FISH results

	Chrmosome 21
Sensitivity	(5/6) 83.33%
Specificity	(6/6) 100%
False-negative rate	(1/6) 16.66%
False-positive rate	(0/6) 0%
Overall accuracy	(6+5/12) 91.66%

Note. Modified from Ward *et al*. (1993)