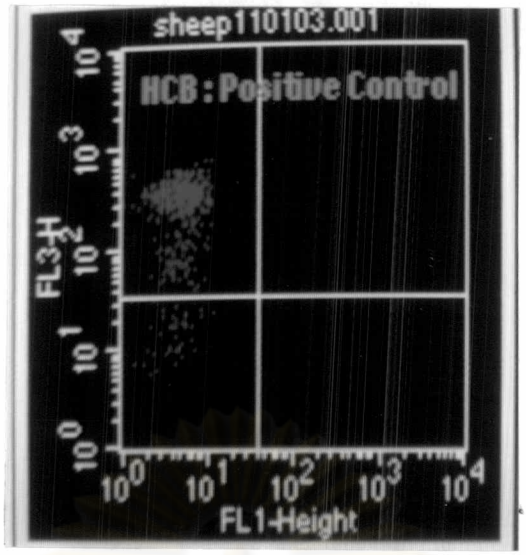
CHAPTER IV

RESULTS

1. Detection of donor cells *in vitro*

1.1 Flow Cytometry

Flow Cytometry analysis was performed on the mixing human – sheep lymphocyte by using monoclonal antibody against human lymphocyte marker (CD 45 PerCP). Artificially prepared mixtures of human and sheep lymphocytes were analyzed to establish the relative sensitivity of Flow Cytometry that can detect human cells from sheep cells in a series of 1:100, 1:500, 1:1,000, 1:5,000 and 1: 10,000 (Figure 21 A-E) included a positive control was prepared from unique human UCB lymphocyte and a negative control prepared from sheep lymphocyte as show in Figure 20 A and B. Both positive and negative controls were included in each examination.



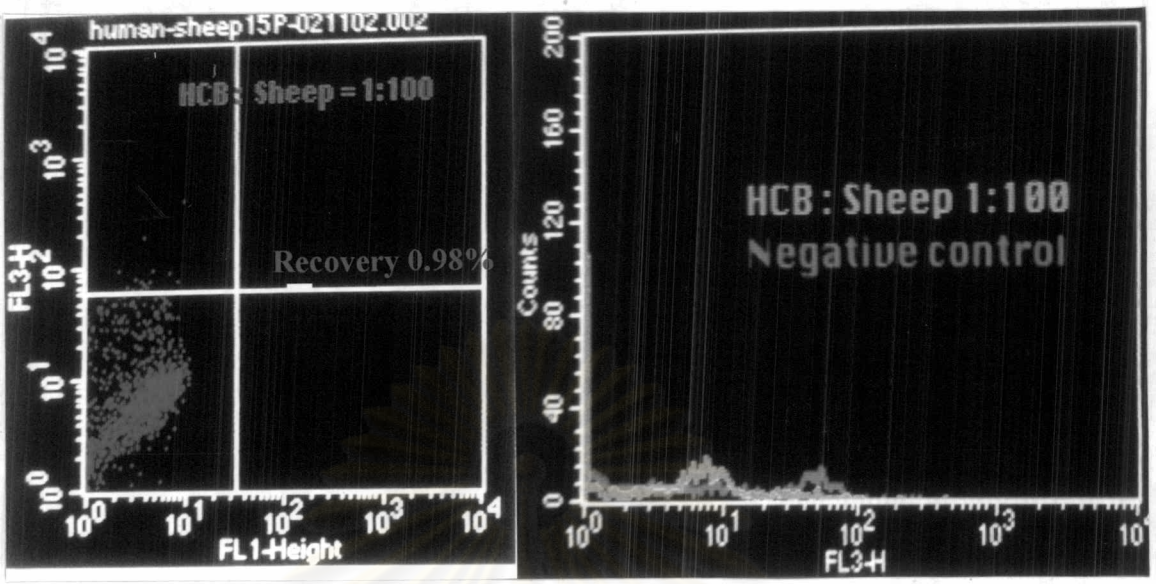
A) Human UCB :Positive control

Sheep : Negative control

B) Sheep : Negative control

Figure 20 Flow Cytometric analysis of control samples labeled with monoclonal antibody CD 45 specific for the human lymphocyte. A) The positive control was prepared from human UCB lymphocytes. B) Sheep lymphocytes alone were the negative control.

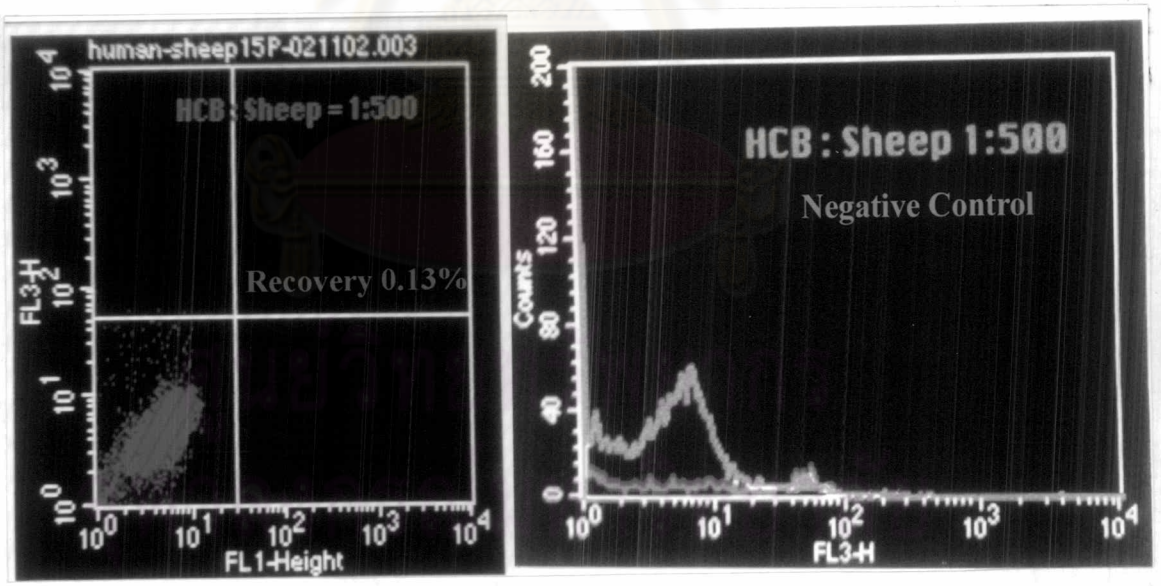
Flow Cytometry analysis Histogram analysis



A) Lymphocyte counted :1,634

Event in positive region : 16

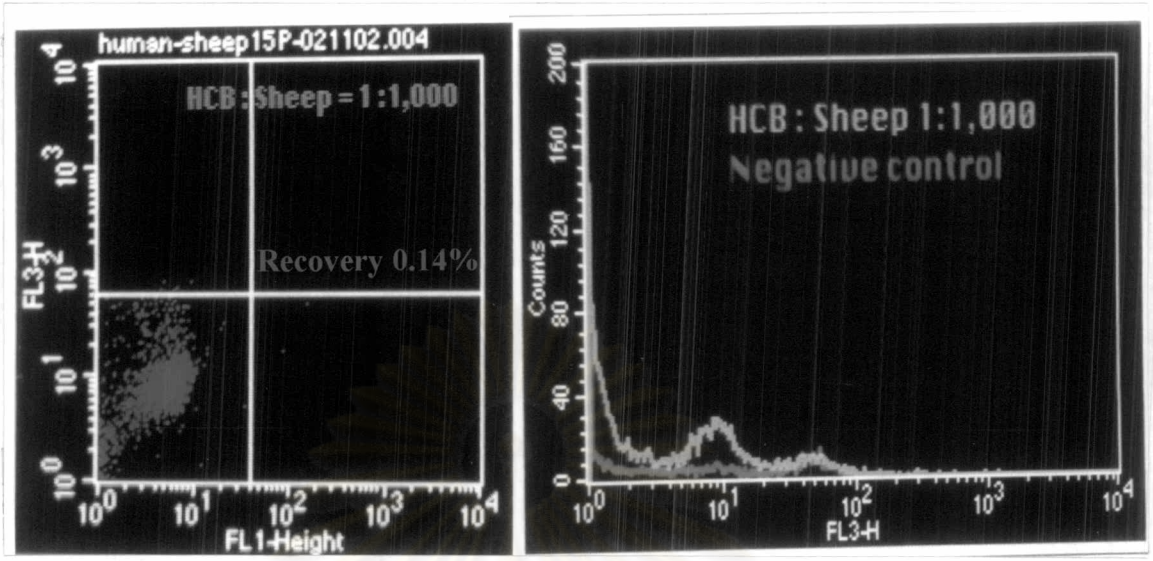
Flow Cytometry analysis Histogram analysis



B) Lymphocyte counted :4,724

Event in positive region : 6

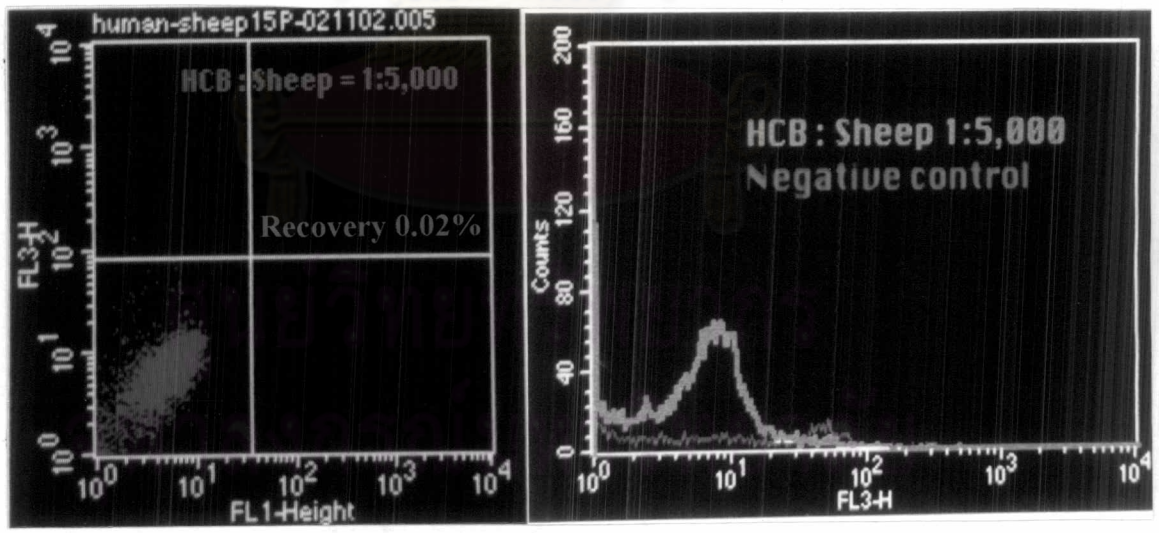
Flow Cytometry analysis Histogram analysis



C) Lymphocyte counted :3,633

Event in positive region : 5

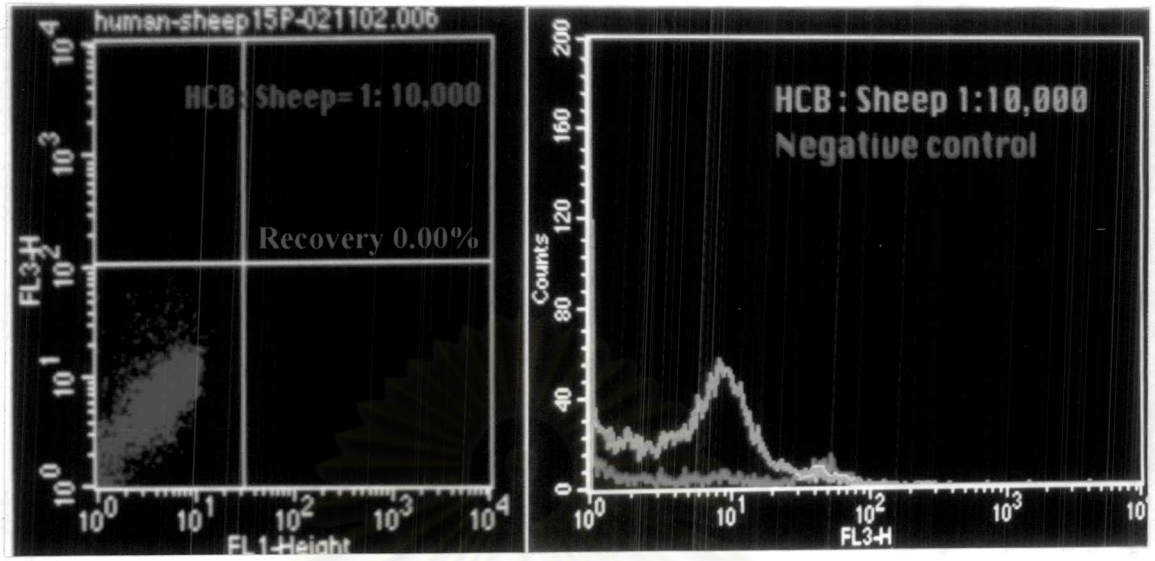
Flow Cytometry analysis Histogram analysis



D) Lymphocyte counted :5,291

Event in positive region : 1

Flow Cytometry analysis Histogram analysis



E) Lymphocyte counted :4,600

Event in positive region : 0

Figure 21 Flow Cytometric analysis of mixed human and sheep lymphocytes ratio of 1:100, 1:500, 1:1,000, 1:5,000 and 1:10,000 (A-E) respectively showed percentage of positive donor cells CD 45 lymphocyte (Left portion) and histogram analysis (Right portion) .

1.2 Fluorescence *in situ* hybridization (FISH)

Artificial mixtures of human and sheep lymphocytes ratio of 1:100, 1:500, 1:1,000, 1:5,000 and 1:10,000 were analyzed by FISH technique. They were viewed under a fluorescence microscope with higher magnification (100 X)(Figure 23 A-E respectively). Appropriate viewing and analysis were depended upon utilization of optimum dual – bandpass (DAPI/TRICT) and single bandpass filter set. The positive cells would show CEP 16 probe signal as the bright orange (spectrum aqua) two spots signal. A positive control slide was obtained from human UCB lymphocytes. (Figure 22A) .A negative control slide was prepared from sheep lymphocyte that shown in figure22 B. Both the positive and negative control were used in each experiment.

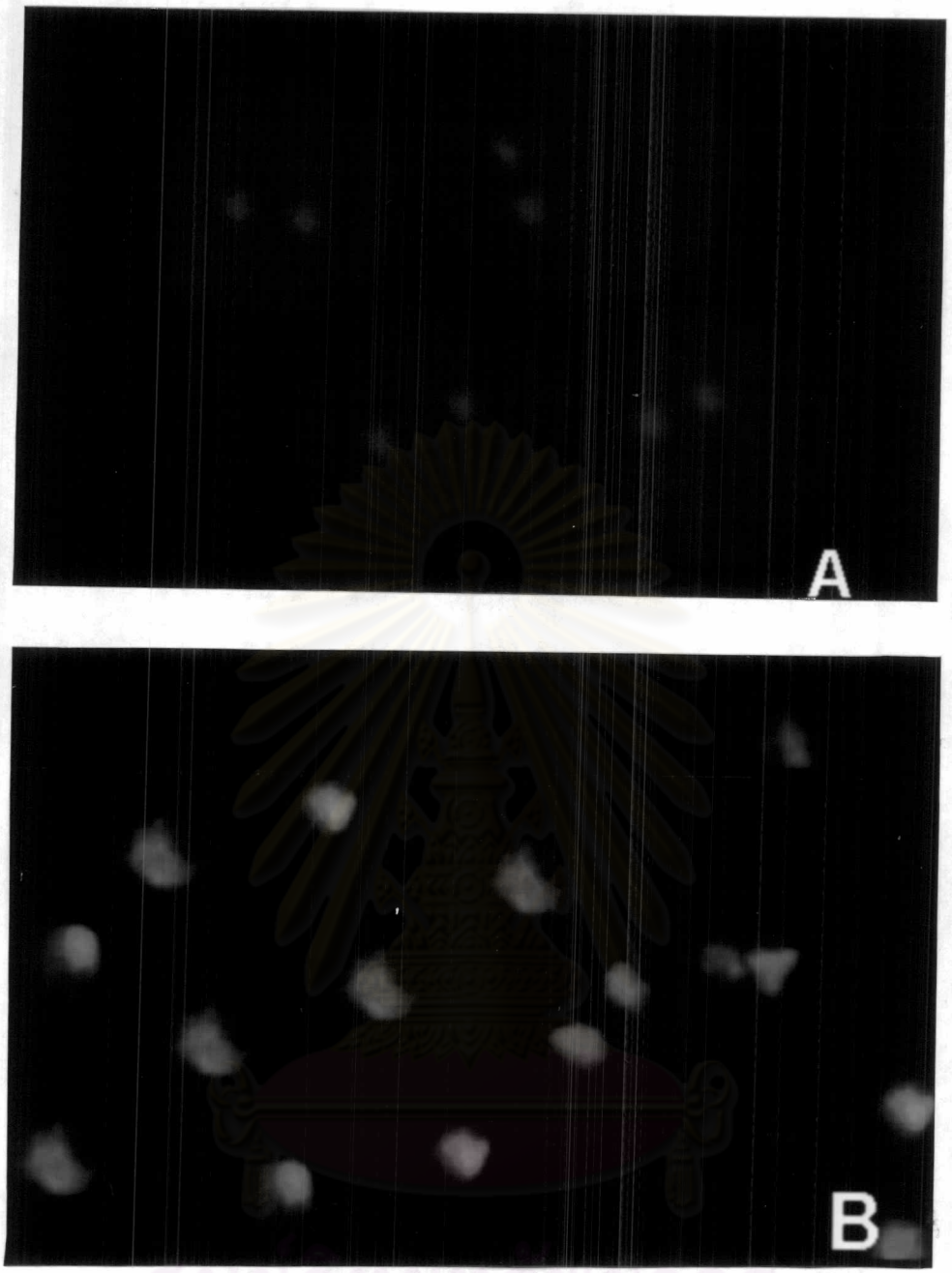
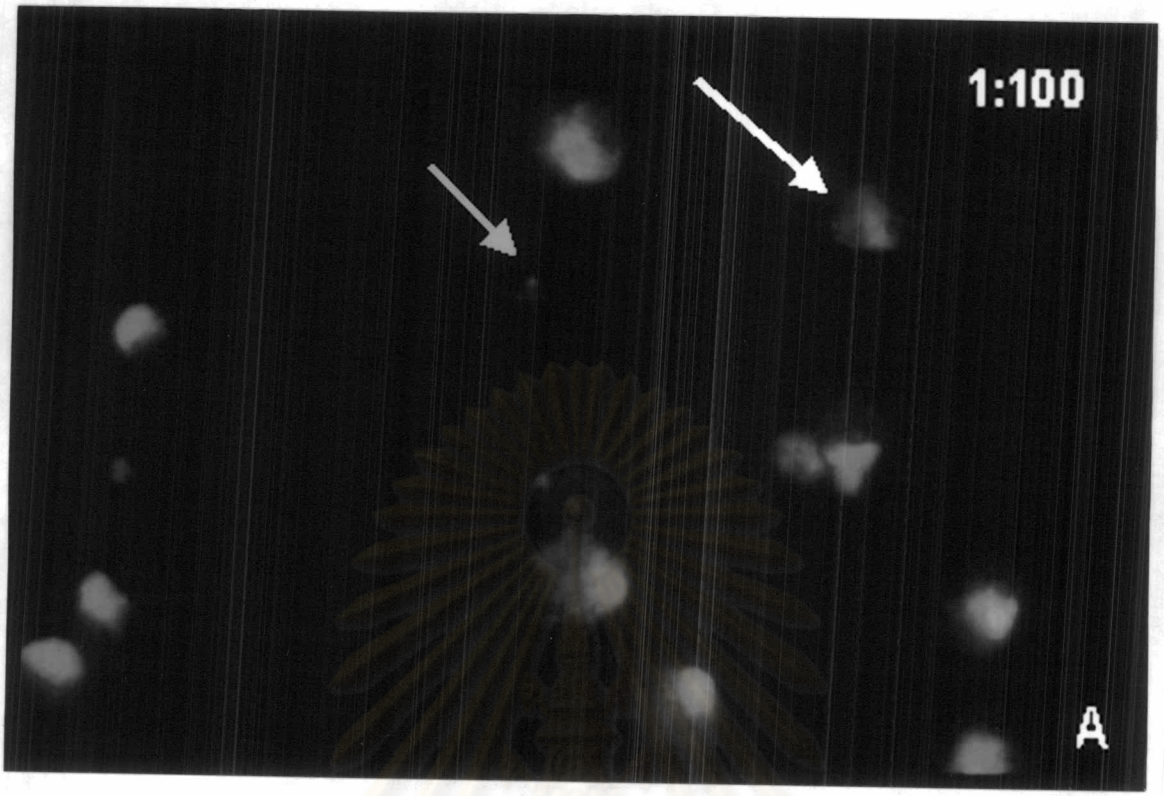
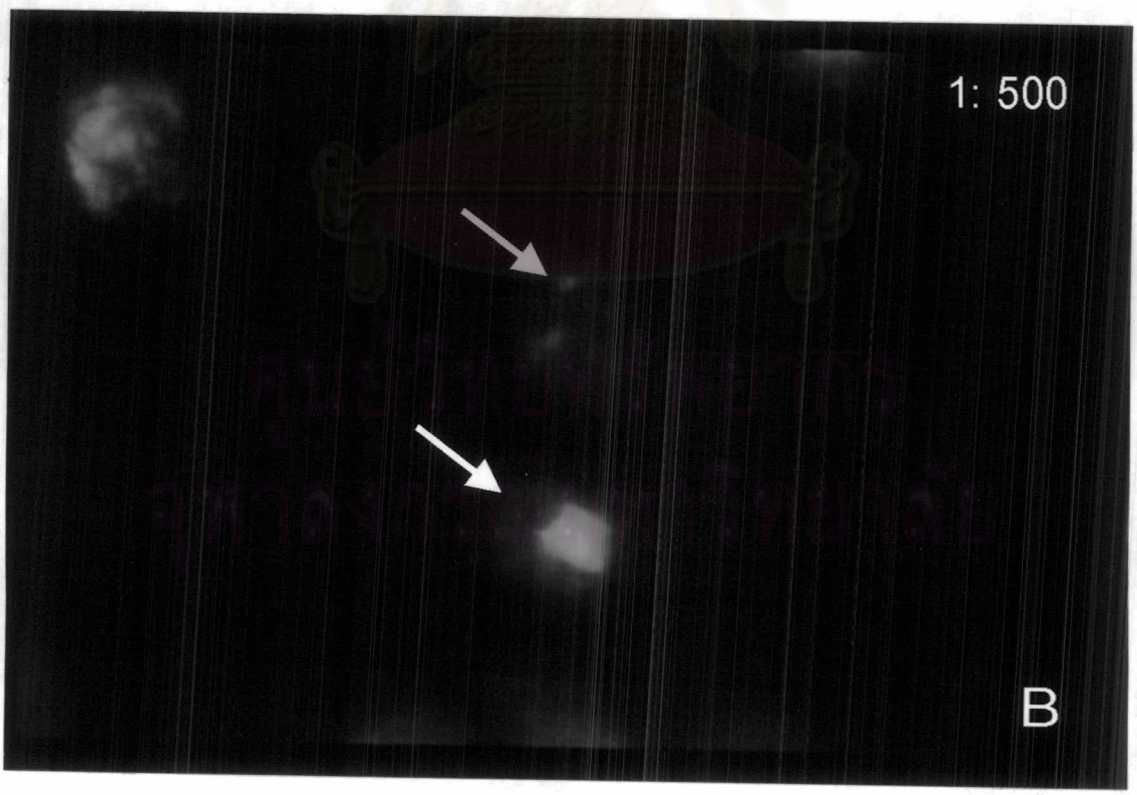
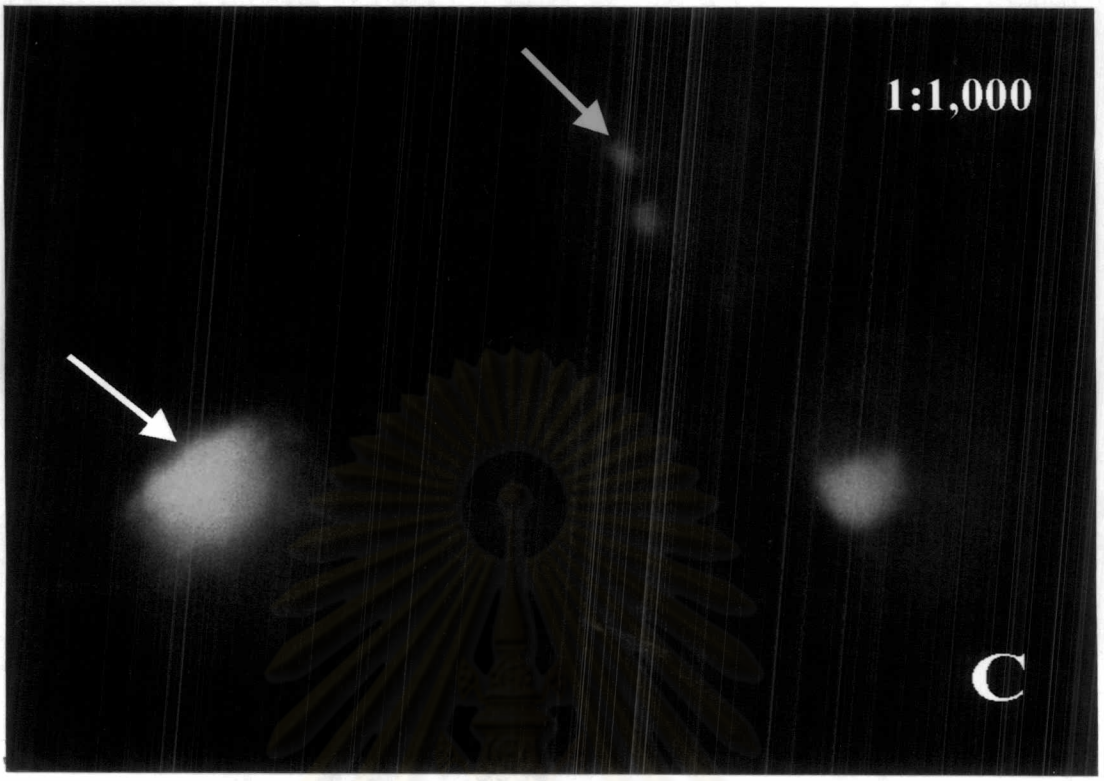


Figure22 FISH on a positive and negative control according to the hybridization technique by used CEP 16 DNA probe. A) A positive control was prepared from human umbilical cord blood lymphocytes. In this case the CEP16 DNA probe binds to major hybridization sites (the band 16q11.2,locus D16Z3 of human chromosome 16) that appears as two bright orange spots signal on the human lymphocytes. B) A negative control was obtained from sheep lymphocytes showed that no probe signal was detected, the nonspecific signals were due to non – specific binding of the negative sample.


1:100
A


1: 500
B


1:1,000
C


1: 5,000
D

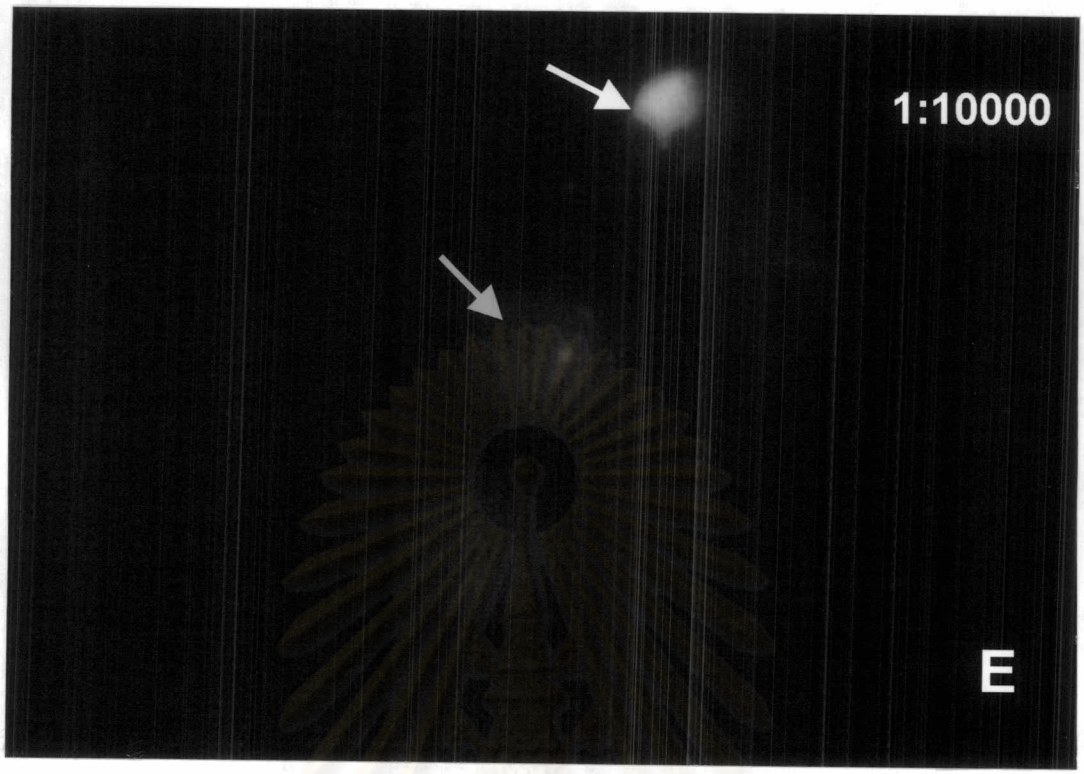


Figure 23 FISH on artificial mixtures of human and sheep lymphocytes were hybridized with CEP 16 DNA probe. The ratio of human and sheep lymphocytes are A) 1:100, B) 1:500, C) 1:1,000, D) 1:5,000, and E) 1:10,000. The yellow arrows indicated positive cells that are seen as the bright orange two spots probe signals within the field. In contrast, the negative cells were not seen as the same positive cell due to non- specific binding indicated by the white arrows.

2. Creation of human *In vivo* studies

Summarized are the end results of In utero transplantation of CD 34+ hematopoietic stem cells from human umbilical cord blood into 12 number of fetal sheep recipients.CD 34^{+} cells (2×10^{6} cells/ml) from human umbilical cord blood were repeatedly transplanted into fetal sheep at 48-54 days of gestation. Recipients were then serially examined for the evidence of engraftment after birth. A total of 12 sheep fetuses received injection with

human donor cells, one recipient was lost to study because of twin pregnancy sheep, 9 recipients were aborted before birth and only two recipients were born alive (Table 5) Thus, the remainder of two sheep born alive (No. 40A3 and 41J2 as show in figure 24 A- B) were analyzed for donor cell engraftment at 10 days and 1 month after birth by Flow Cytometry and FISH analysis.

Table 5: Summary of the end results of *in vivo* studies in sheep receiving human UCB transplantation In utero.

Results	Number of animals
In utero transplanted	12
Lost to study	1 (Twin pregnancy sheep)
Abortus of sheep pregnancy	9
Born alive	2
Evidence of engraftment	2

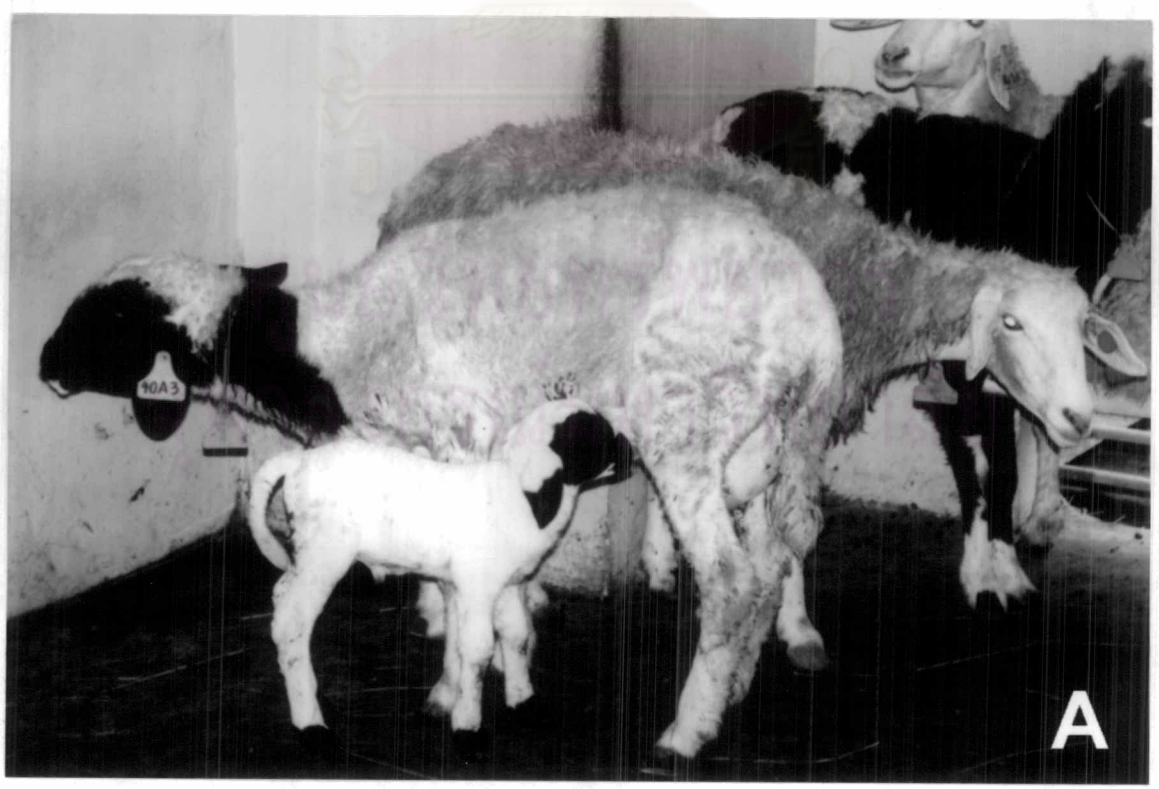


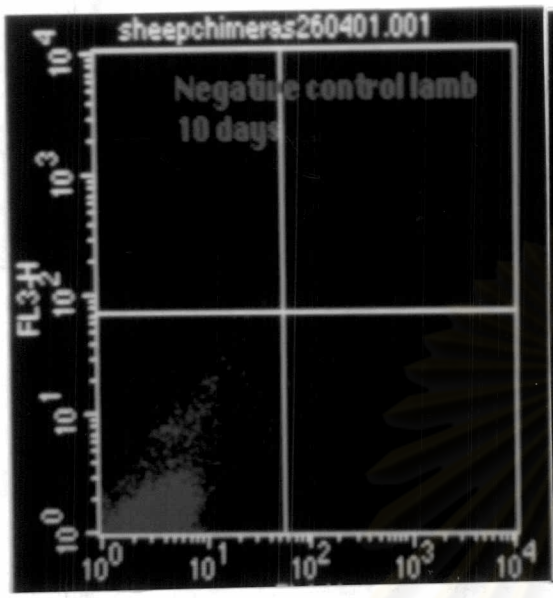
Figure 24 The picture of survival chimeras sheep at 1 month after birth. (A) no.40A3 and (B) no.41J2 were received human donor cells.

3. Detection of human donor cell engraftment *in vivo*

3.1 Flow Cytometry analysis

Human donor cell engraftment in two newborn lambs (no. 40A3 and no. 41J2) at 10 days and 1 month after birth were determined by Flow Cytometry of peripheral blood (PB). That was used monoclonal antibody against human lymphocyte marker CD 45 PerCP. Both sheep chimeras showed human donor cells in the PB at the level of lower than 1 percentage. Donor cells were detected from the PB of no.40A3 and 41J2 sheep chimeras at 10 days and 1 month at the level of 0.12 , 0.03, 0.50 , and 0.37% respectively as shown in Figure 25 and 27. In addition, Histogram or single parameter was analyzed using the same monoclonal antibody CD 45 PerCP to human lymphocyte, as describe in Figure 26 and 28 respectively.

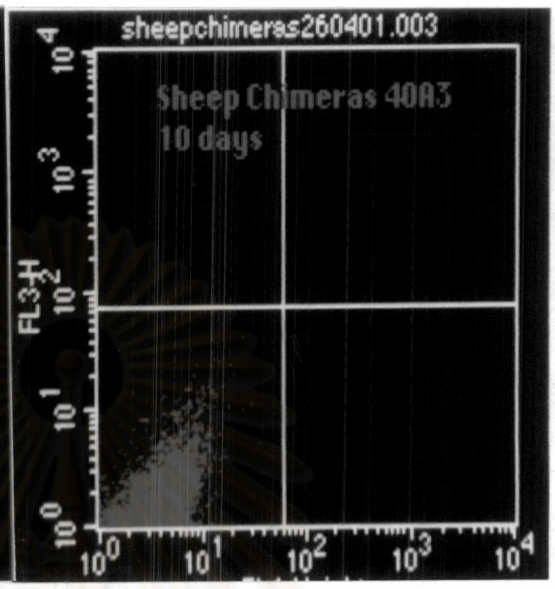
Negative control: Lamb 10 days



A) Lymphocyte counted: 6,212

Event in positive region: 1

Chimeric sheep 40A3 at 10 days



B) Lymphocyte counted : 5,323

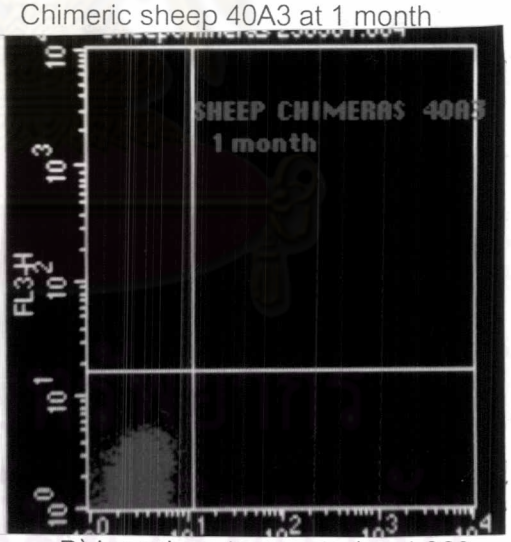
Event in positive region : 3

Negative control: Lamb 1 month

C) Lymphocyte counted : 2,640

Event in positive region : 1

Chimeric sheep 40A3 at 1 month



D) Lymphocyte counted : 4,960

Event in positive region : 2

Figure 25 Flow Cytometry analysis of donor cells in PB from chimeric sheep 40A3 A) Negative control: Lamb 10 days , B) Chimeric sheep 40A3 at 10 days , C) Negative control : Lamb 1 month and D) Chimeric sheep 40A3 at 1month.

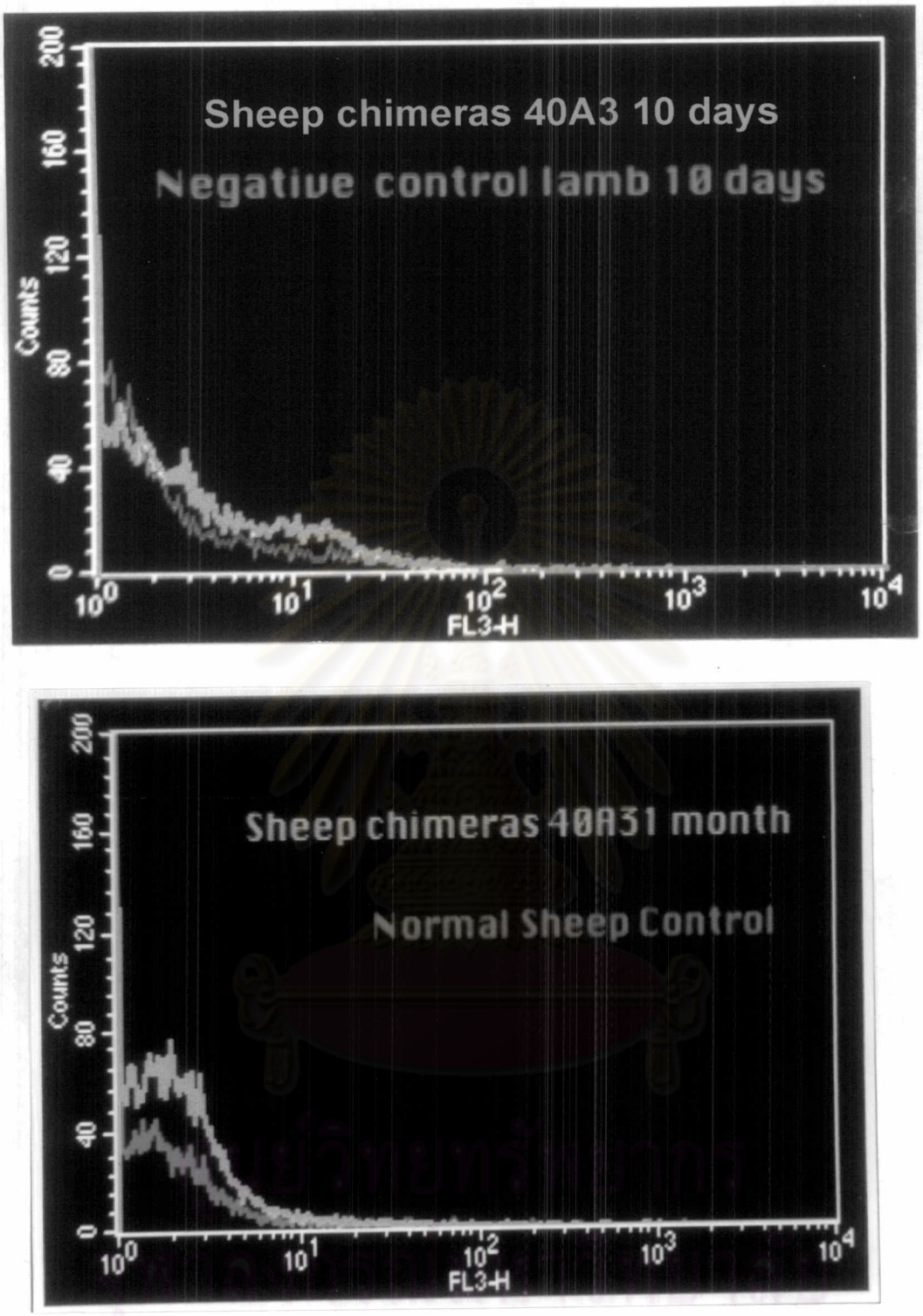
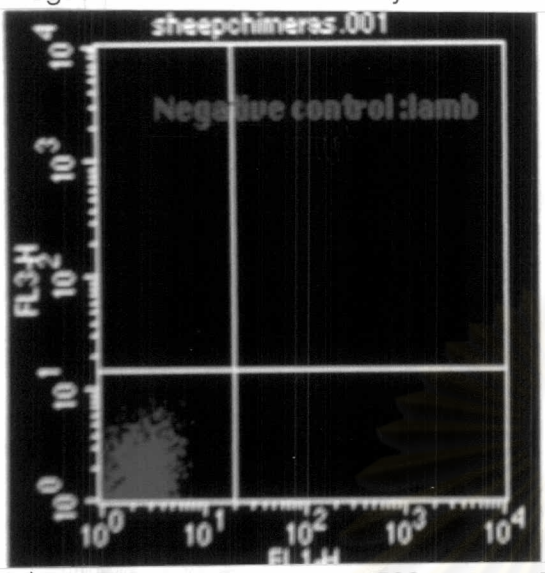


Figure 26 Histogram represented Above : Reactions was obtained with chimeric sheep no. 40A3 at 10 days and negative control (sheep lymphocytes) ,Below : Reactions was obtained with chimeric sheep no. 40A3 at 1 month and normal sheep control.

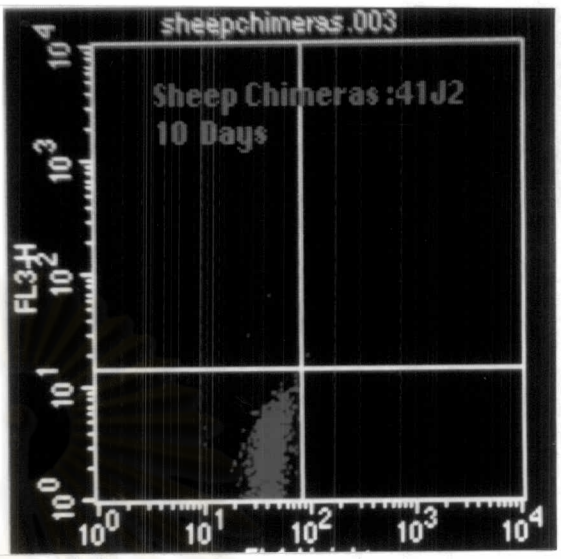
Negative control : Lamb 10 days



A) Lymphocyte counted : 1,509

Event in positive region : 0

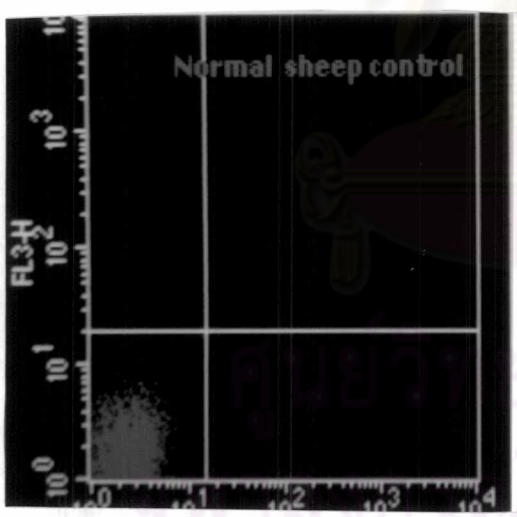
Chimeric sheep 41J2 at 10 days



B) Lymphocyte counted : 1,076

Event in positive region : 4

Negative control : Lamb 1 month



C) Lymphocyte counted : 2,640

Event in positive region : 1

Chimeric sheep 41J2 at 1month

D) Lymphocyte counted : 5,745

Event in positive region : 21

Figure 27 Flow Cytometry analysis of donor cells in PB from chimeric sheep 41J2 A) Negative control : Lamb 10 days , B) Chimeric sheep 41J2 at 10 days , C) Negative control : Lamb 1 month and D) Chimeric sheep 41J2 at 1month.

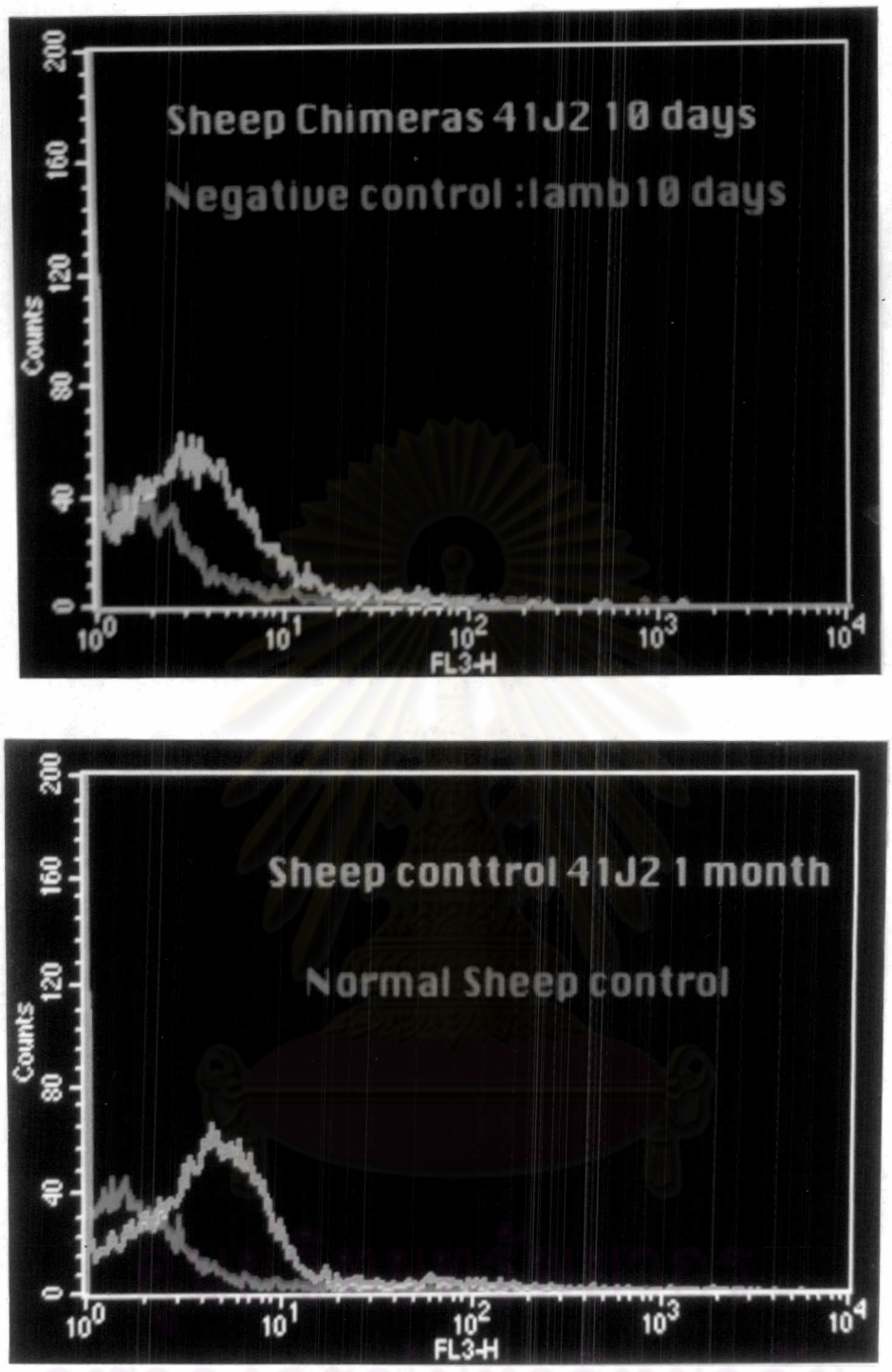


Figure 28 Histogram represented Above : Reactions was obtained with chimeric sheep no. 41J2 at 10 days and negative control (sheep lymphocytes) ,Below : Reactions was obtained with chimeric sheep no. 41J2 at 1 month and normal sheep control.

3.2 Fluorescence *in situ* hybridization (FISH)

FISH with human – specific CEP 16 spectrum aqua DNA probe demonstrated that xenogeneic sheep chimeras no. 40J2 and 40A3 were not achieved to detect human donor cell. Non – specific binding of all slide samples occurred from both sheep chimeras at 10days and 1 month as shown in Figure 29.

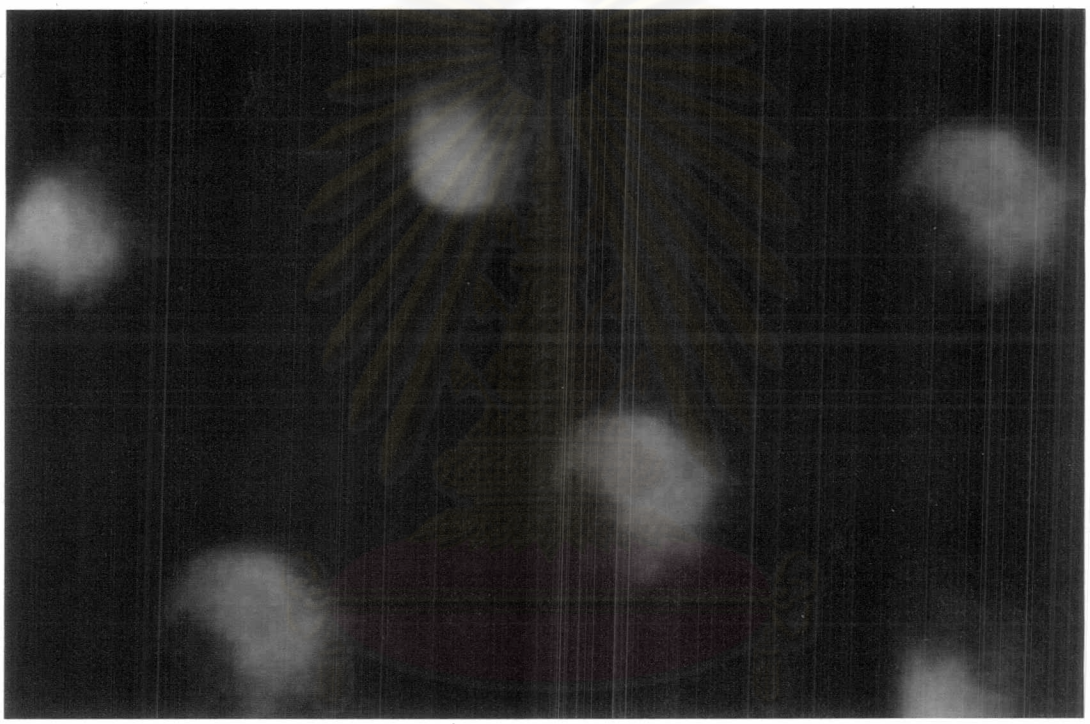


Figure 29 The picture of detection of human donor cells by FISH analysis from one of study chimeric sheep samples. All slide showed the presence of non – specific binding, that are seen at 100X higher magnification.

Statistical analysis

The detailed data on statistical analysis were presented in Appendix D

1. Percent of human lymphocyte in mixed experiment

1.1 Flow Cytometry

The percent of human lymphocyte in mixed experiments at 5 various dilutions between human and sheep 1:100, 1:500, 1:1,000, 1:5,000 and 1:10,000 had been detected by Flow Cytrometry analysis. The actual measured number of CD 45^{+} cells or human lymphocytes were compared with the predicted number of mixed human and sheep lymphocytes in each ratio for 15 experiments (n=15). The comparison of the actual measure value, predicted value and the coefficient of variance (CV) measured values in Flow Cytrometry analysis was shown in Table 6.

The results indicated that the actual measured number of human lymphocytes was comparable to the predicted number of human lymphocytes at all 5 ratio. (1:100, 1:500, 1:1,000, 1:5,000 and 1:10,000)(Figure 30). There were no significant difference at all experiment. (p = 0.087, 0.098, 0.074, 0.498 and 0.638 respectively, Appendix D) When the concentration was lower, These values of the actual measured number of CD 45+ cells were too low to be interpreted and not different from those obtained in negative control. The CV of measured number tended to be larger when concentration of CD45+ cells became lower.

Figure 31 gave the presentation of the mean $\pm$ standard deviation (SD) of CD 45 PerCP reactivity in mixed human and sheep lymphocytes at various dilutions (1:100, 1:500, 1:1,000,1:5,000 and1:10,000) and unique sheep lymphocytes as negative control in the bar graphic features. The reactivity of CD 45 PerCP antibody of lymphocytes in each dilution depended on the number of human lymphocytes, affinity of the antibody and the fluorochrome – to – antibody ratio. This result indicated the reactivity of the antibody was insufficient to discriminate between positive and negative samples when dilution of human lymphocytes became the lower.

Table 6: The percentage of counted lymphocytes data found in different dilutions (1:100, 1:500, 1:1,000, 1:5,000 and 1:10,000 taken from mixed human and sheep lymphocytes experiment by Flow Cytometry analysis.

Ratio human/ sheep lymphocyte	Percentage	Predicted value of Lymphocytes		Actual measured of lymphocytes		
		Human (EC)	Sheep (EC)	% human lymphocytes	% sheep lymphocytes	CV%
1:100	1%	1×10^4	1×10^6	1.96 ± 1.94	98.10	98.97
1:500	0.2%	2×10^3	1×10^6	0.41 ± 0.55	99.25	134.14
1:1,000	0.1%	1×10^3	1×10^6	0.11 ± 0.08	94.69	72.72
1: 5,000	0.02%	2×10^2	1×10^6	0.12 ± 0.12	99.15	100
1:10,000	0.01%	1×10^2	1×10^6	0.028 ± 0.06	98.77	214.29

* EC – Expected counts of true positive events.

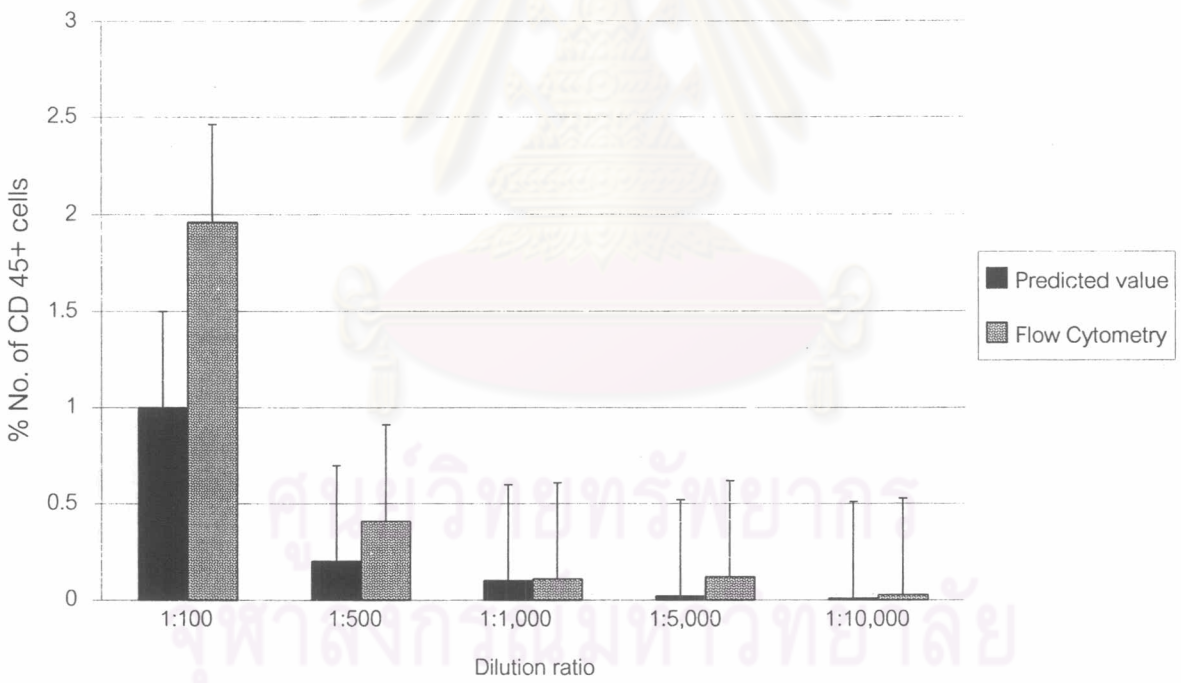


Figure 30 The percentage of the actual measured values of from mixed human and sheep lymphocytes experiments at various dilutions detected by Flow Cytrometry analysis.

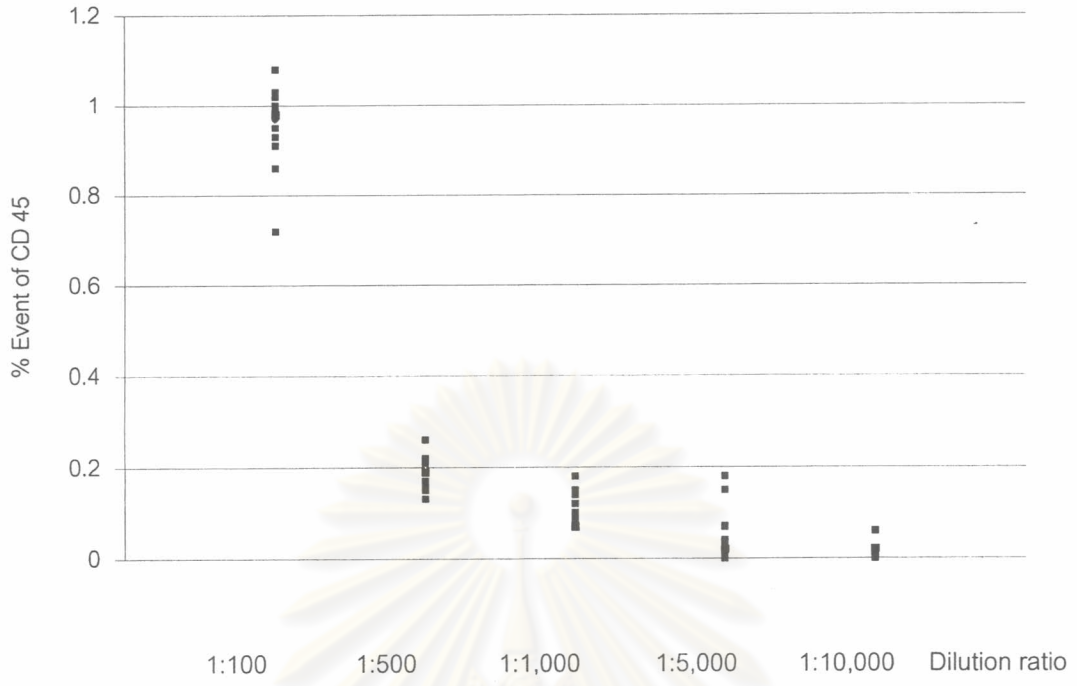


Figure 31 Mixed human and sheep lymphocytes experiments at various dilutions stained with CD 45 PerCP showed reactivity antibody in human /sheep lymphocytes dilution sample by Flow Cytometry analysis.

Mixed experiments performed by measuring between 0.01% and 1% of human lymphocytes by Flow Cytometry was shown in figure 32 to measure human lymphocytes after dilution. The absolute number of circulating gate human lymphocytes can be accurately measured by this method when the concentrations of human lymphocytes was higher. In contrast, the dilution experiments of lower concentration of CD 45 + cells yield a higher CV percentage.

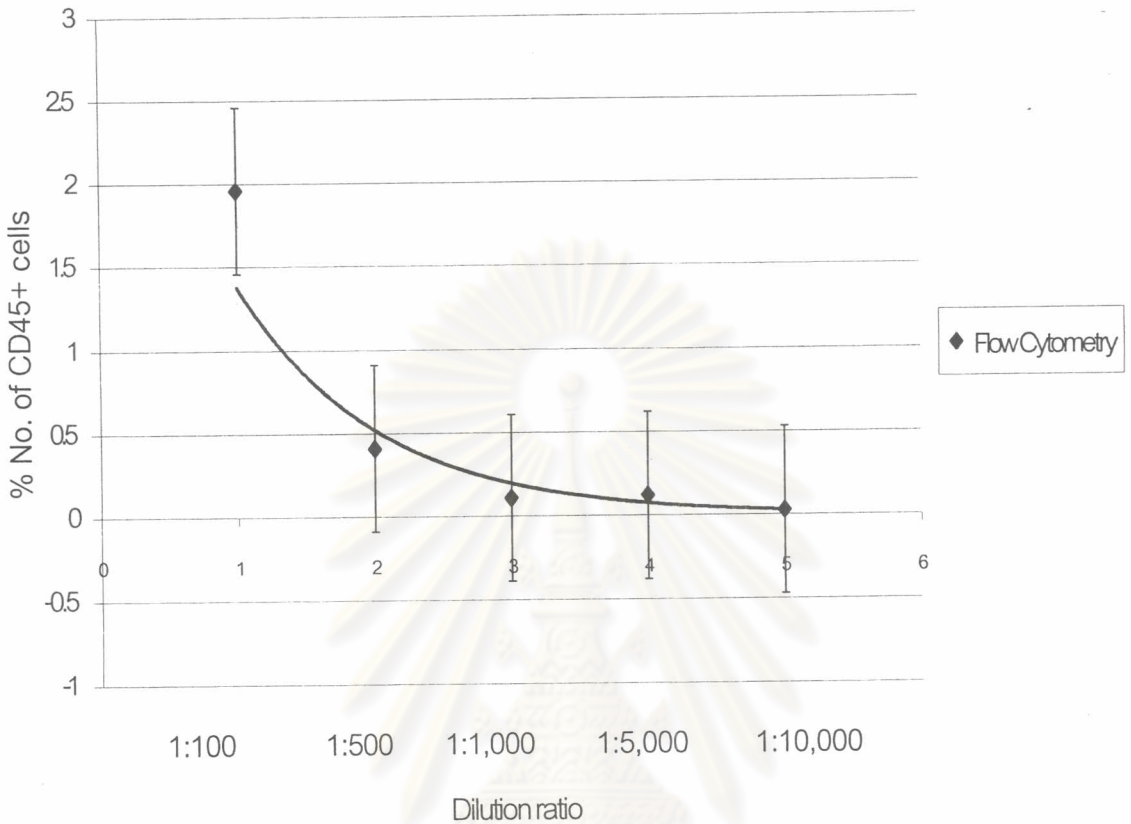


Figure 32 Measurement of human lymphocytes by Flow Cytometry was done cells after dilution. A known number of human cells was mixed with sheep lymphocytes as the recipient cells at various dilution (1:100 – 1:10,000) and the concentration of human lymphocytes assayed. This value was the compared with the predicted concentration of human lymphocytes. The results of mean values ± SD from experiment are shown.

1.2 Fluorescence in situ hybridization (FISH) analysis *in vitro*

The percentage of human lymphocytes in mixed human and sheep lymphocytes experiments at the same various dilutions had been detected by FISH analysis. The actual measured numbers of human lymphocytes were compared with the predicted number of mixed human and sheep lymphocytes in each dilution for 15 experiments. (n=15) The comparison was demonstrated of the actual measured value,

predicted value and the coefficient of variance (CV) measured values in FISH analysis as shown in Table 7.

The results indicated that the actual measured number of human lymphocytes were comparable to the predicted number of human lymphocytes at all 5 ratio. (1:100, 1:500, 1:1,000, 1:5,000 and 1:10,000) (Figure 33) There were significant different at all experiments (p= 0.000 but except 1:10,000 ,p=0.001, Appendix D) When the concentration of human lymphocytes was lower, the actual measured number of human lymphocytes tended to slightly lower than the predicted number. The CV of measured number were similar to those results of Flow Cytometry analysis, which tended to be larger when concentration of human lymphocyte became lower.

The bar graph in figure 34 presented a comparison of the counted target signals of human lymphocytes from mixed human and sheep lymphocytes at the same dilutions by FISH analysis. The reactivity CEP 16 probe signals were discriminated between positive (human lymphocytes) and negative.(sheep lymphocytes). The result showed that when the concentration was lower, the value of enumeration of positive cells was also lower.

Table 7: The percent age of counted lymphocytes data found in different dilutions (1:100, 1:500, 1:1,000, 1:5,000 and 1:10,000 taken from mixed human and sheep lymphocytes experiment by FISH analysis.

Ratio human/ sheep lymphocyte	Percentage	Predicted value of lymphocytes		Actual measured of CD 45^+ cell		
		Human (EC)	Sheep (EC)	% human lymphocyte	% sheep lymphocyte	CV%
1:100	1%	1×10^4	1×10^6	1.25 ± 0.68	98.68	13.74
1:500	0.2%	2×10^3	1×10^6	0.26 ± 0.58	99.70	11.96
1:1,000	0.1%	1×10^3	1×10^6	0.13 ± 0.52	99.85	21.09
1: 5,000	0.02%	2×10^2	1×10^6	0.02 ± 0.57	99.94	62.27
1:10,000	0.01%	1×10^2	1×10^6	0.03 ± 0.55	99.96	80.67

* EC – Expected counts of true positive events.

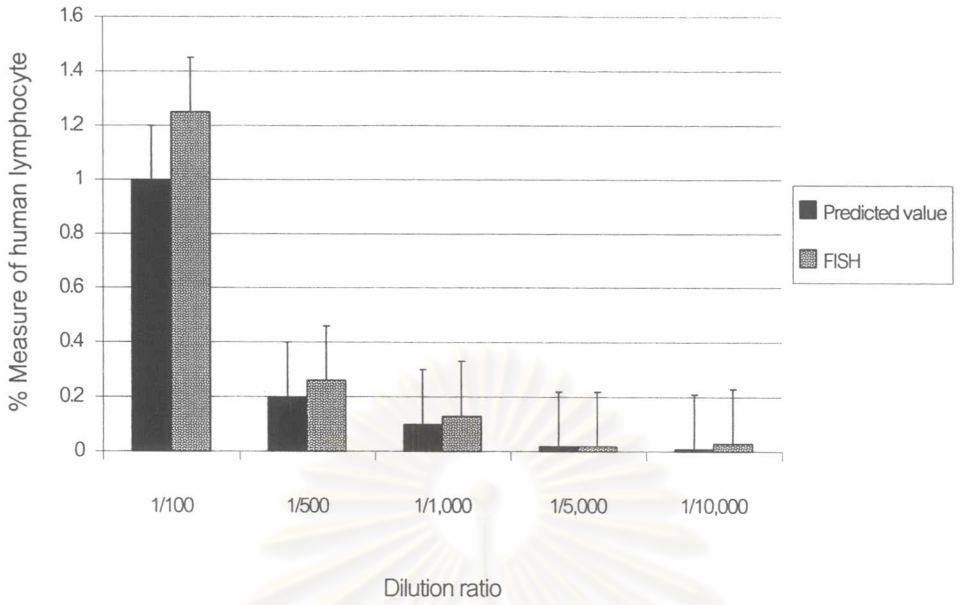


Figure 33 The percentage of the actual measured numbers of human lymphocytes from mixed experiment at various dilutions. Human lymphocytes were detected by FISH analysis after hybridization with CEP 16 probe.

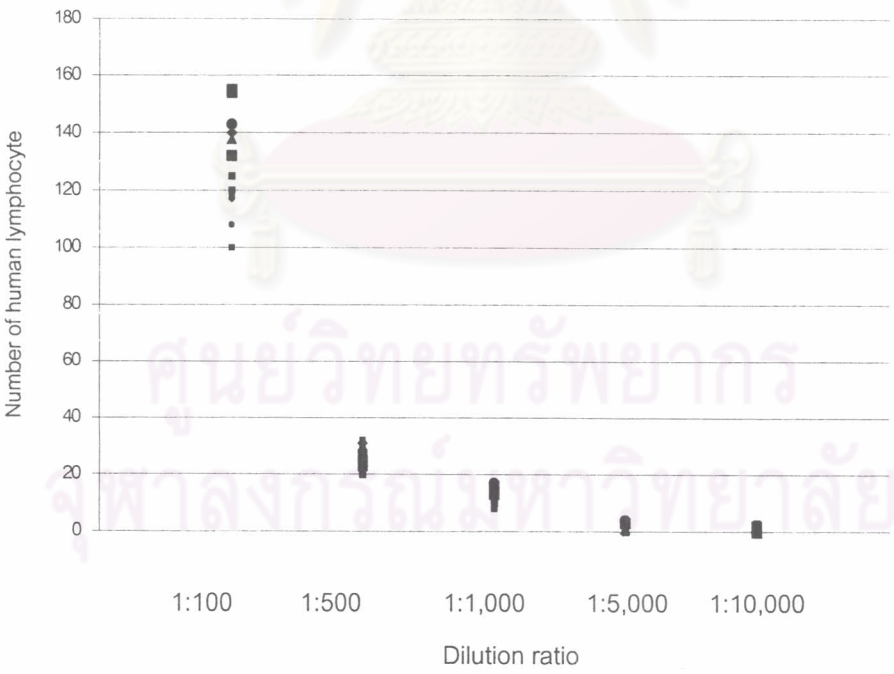


Figure 34 Mixed experiment between human and sheep lymphocytes at various dilutions were hybridized with CEP 16 probe. Count target signals of positive cells were demonstrated by FISH analysis.

The comparison of the actual measured number of human lymphocytes and predicted value of mixed human and sheep lymphocytes experiment between 0.01% and 1% dilutions was shown in Figure 35. The actual measured human lymphocytes was comparable to the predicted number of human lymphocytes when the concentration was higher. However, when the concentration was lower, The actual measured number of human lymphocytes tended to be slightly lower than the predicted number.

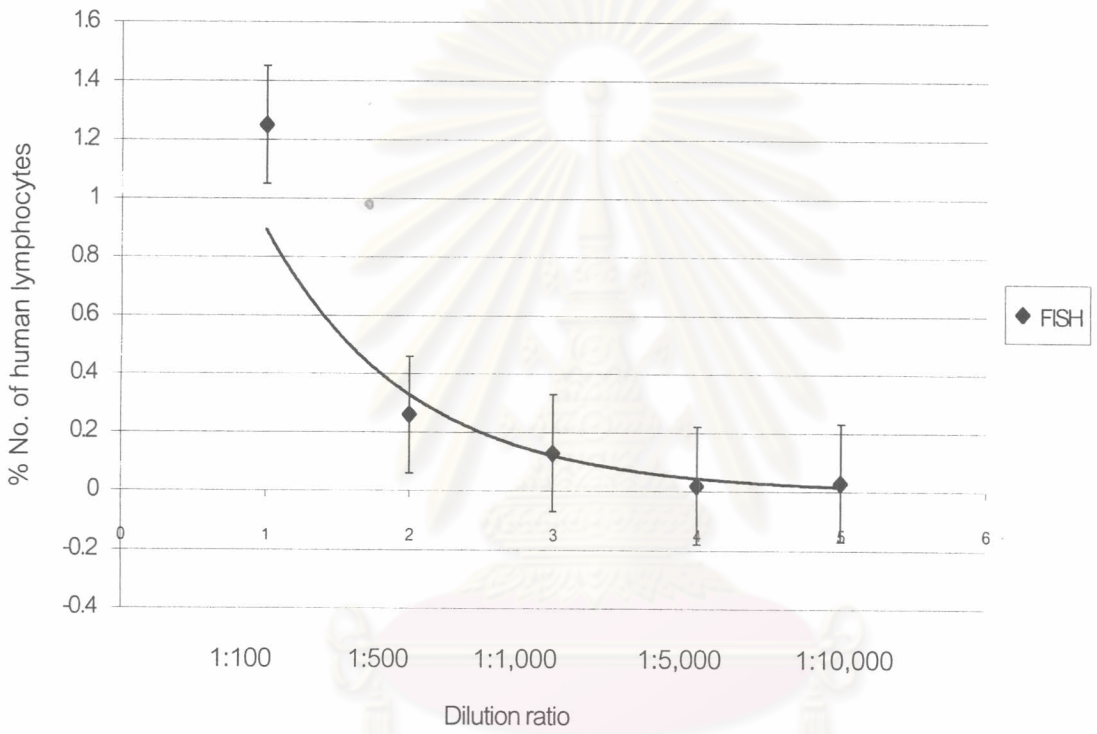


Figure 35 Measurement of percent human lymphocytes by FISH was done after dilutions. A known number of human lymphocytes were mixed with sheep lymphocytes at various dilutions (1:100 – 1:10,000) by FISH analysis.

1.3 Comparison of the number of human lymphocytes by Flow Cytometry and FISH analysis

The mean of percent human lymphocytes in mixed human and sheep lymphocytes from each dilutions and its matched different assays had been compared. The data was presented in Table 8 and as bar graph in figure 36 . The overall results demonstrated that the percentage of measured human lymphocytes at various dilutions by Flow Cytometry was lower than FISH assays at ratio 1:100, 1:500, 1:1,000 but except 1:5,000 and 1:10,000 with significant difference (p=0.000,0.000 and 0.045 respectively, Appendix D) In contrast, the remain ratios at 1:5,000 and 1:10,000 were without any statistical significance. (p = 0.329 and p = 0.371, Appendix D) The CV value in FISH analysis was less than Flow Cytometry analysis at all ratios. It was indicated that FISH analysis was more reproducible than Flow Cytometry analysis and gave lower differences. The concentration of human lymphocytes at various dilutions was assayed by Flow Cytometry analysis and compared with FISH analysis as shown in Figure 37.

Table 8 Comparison the percent human lymphocytes in mixed human and sheep lymphocytes experiments at various dilutions (1:100, 1:500, 1:1,000,1:5,000 and1:10,000) That were detected by Flow Cytometry and FISH analysis.

Ratio human/ Sheep lymphocytes	The Measurement of number of human lymphocytes (CD 45+ cells)			
	Flow Cytometry		FISH	
	% gate CD 45^{+}	% CV	% human lymphocyte	% CV
1:100 (1%)	1.96	98.97	1.25	13.73
1:500 (0.2%)	0.41	134.14	0.26	11.96
1: 1,000 (0.1%)	0.11	72.72	0.13	21.13
1:5,000 (0.02%)	0.12	100	0.02	62.41
1:10,000(0.01%)	0.028	1214.92	0.93	124.60

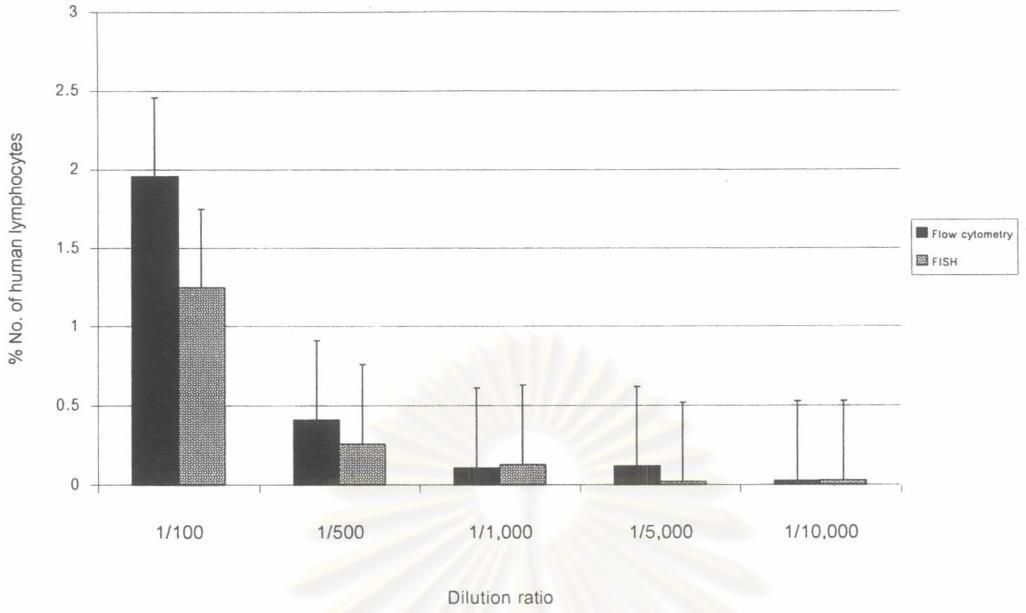


Figure 36 Comparison of the mean of percent human lymphocytes between Flow Cytometry and FISH analysis at various dilutions (1:100, 1:500, 1:1,000,1:5,000 and1:10,000).

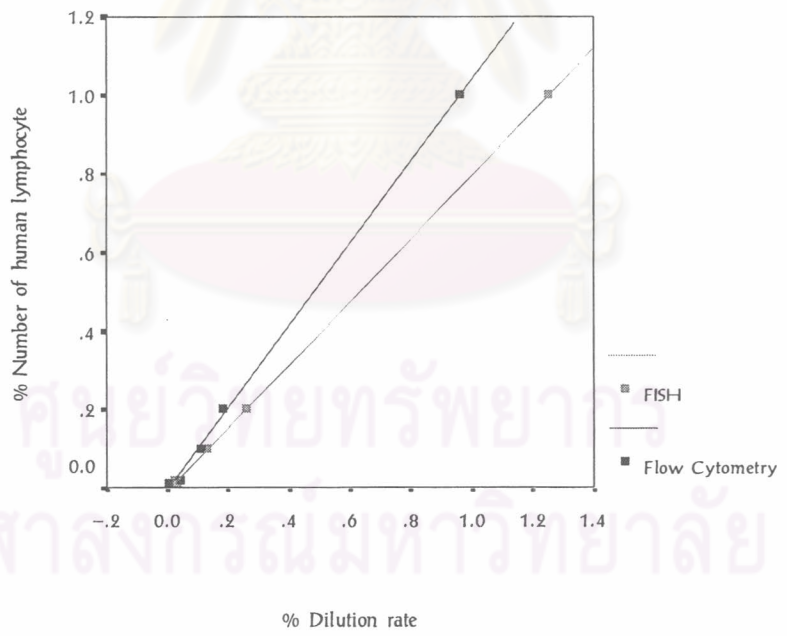


Figure 37 The detection of percent human lymphocyte (CD 45^{+}) by Flow Cytometry analysis was compared with that of percent human lymphocytes by FISH analysis.

2. Percent of donor cells (human lymphocytes) engraftment after In utero transplantation

In utero transplantation was done in 12 fetuses sheep who received human stem cells (CD 34^+) from umbilical cord blood. There were 10 sheep fetuses aborted and in two who were born healthy (code number of 40A3 and 41J2). The evidence of donor cells engraftment was detected by Flow Cytometry and FISH analysis from peripheral blood of then lambs at two time intervals: 10 days and 1 month after birth.

The results indicated that percent donor cells engraftment in chimeras lambs no. 40A3 and 41J2 was lower than 1% by Flow Cytometry analysis as shown in Table 9 . There was no difference between in two chimeras lambs at two times intervals (p = 0.356, 0.110, 0.330 and 0.197, Appendix D) In contrast, there was no cells exhibiting donor human lymphocytes by FISH analysis.

The bar graph in figure 38 gave the presentation of a comparison of the mean $\pm$ SD of percent donor cells in two chimeras lambs no. 40A3 and 41J2 at 10 days and 1 month detected in the peripheral blood by Flow Cytometry analysis. The slope of recovery donor cells in these lambs were slightly decreased as shown in Figure 39.

Table 9 : Percent of donor cells were shown in two chimeras lambs at two times intervals : 10 days and 1 month after birth. The donor cells were detected by Flow Cytometry analysis.

Code No. of chimeras lambs	Percent of donor cells (CD 45^+)		Code No. of chimeras lambs	Percent of donor cells (CD 45^+)	
	10 days	1 month		10 days	1 month
40A3	0.17%	0.03%	41J2	0.63%	0.37%
	0.06%	-		0.37%	-
$\bar{X}$	0.12	0.03	$\bar{x}$	0.50	0.37
SD	0.08	-	SD	0.04	-
CV	0.66	-	CV	0.08	-

Figure 38 Comparison of the mean ± SD of percent donor cells engraftment in two chimeras lambs at 10 days and 1 month were detected by Flow Cytometry analysis.

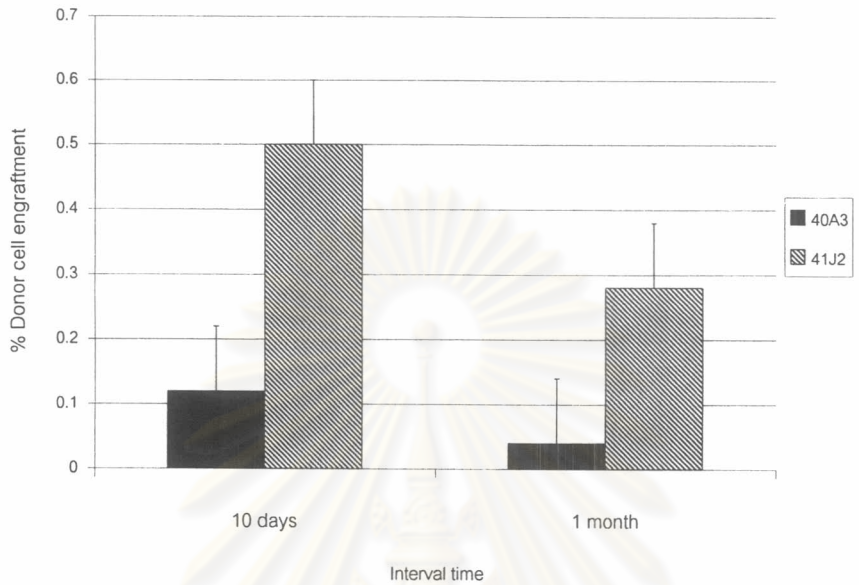


Figure 39 The percentage of donor cells in human – sheep chimeras taken from the blood lamb recipients no. 40A3 and 41J2 at 10 days and 1 month were detected by Flow Cytometry analysis.