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APPENDICES

APPENDIX A

Buffers and Reagents

1. 10 × Phosphate Buffer Saline (PBS) 1 Liter

NaCl	80	g
Na_2HPO_4	9.136	g
KCl	2	g
KH_2PO_4	2	g
Distilled water	900	ml

Adjusted the pH value to 7.3- 7.4 with 1M HCl, add distilled water to 1 liter and sterilized by autoclaving.

2. 1 × Phosphate Buffer Saline (PBS) 1 Liter

10 × PBS	100	ml
Distilled water	900	ml

Bring on sterilized by autoclaving.

3. 60% Percoll solution 0.5 Liter

Percoll (Stock solution)	300	ml
1 × PBS	200	ml

Mix well and stored at 4°C

4. 1% Paraformaldehyde in PBS 250 ml

Sodium cacodylate	2.68	g
Sodium chloride	1.90	g
Paraformaldehyde	2.95	g
1 × PBS	200	ml

Add the following while stirring, cool at room temperature , adjust the pH to 7.2-7.4 and raise the final volume to 250 ml. Store at 4°C in a foil wrapped.

5. Freezing solution for cryopreservative 80 ml

Bovine calf serum	20	ml
Dulbecco's solution	50	ml
DMSO	10	ml

Sterilized technique by filtering through a nito-cellulose membrane.

6. 20 × SSC Solution 250 ml

20 × SSC (Salt and Sodium Citrate)	66	g
Distilled water	200	ml

Adjusted the pH value to 5.3 with 10 N NaOH (about 6.5 ml),adjust final volume to 250 liter and filter through a 0.45 0.45 μm membrane filtration unit.

7. 1× SSC Solution 1 Liter

20 × SSC (pH 5.3)	100	ml
Distilled water	900	ml

Adjusted the pH value to 7.0 ± 0.2 with NaOH and sterilized filter through a 0.45 0.45 μm membrane filtration unit.

8. 1% formaldehyde solution 40 ml

37% formaldehyde solution	1	ml
1 × PBS	39	ml
$MgCl_2$	0.18	g

Mix well and stored at 4°C. Use with in 1 week.

9. Carnoy's Fixative 100 ml

Acetic acid	25	ml
Methanol	75	ml

Mix well and stored at -20°C.

10. 0.01 N HCl 100 ml

HCl 1 N 1 ml

Distilled water 99 ml

Mix well and stored at - 20°C for up to 3 month.

11. 1 M NaOH 100 ml

NaOH 4 g

Distilled water 100 ml

stored at room temperature.

12. 0.02% Pepsin in 0.01 NHCl 250 µl

Pepsin (Stock 10%) 0.4 µl

HCl 0.01 N 25 µl

Distilled water 225 µl

Make freshly each day before use.

13. Rnase – Dnase free (100 µg /ml) 500 µl

Rnase – Dnase free (500 µg /ml) 100 µl

Distilled water 400 µl

Make freshly each day before use

APPENDIX B

The Flow Cytometry (FACSCalibur ™,Becton Dickinson) Procedure for User's guide.

Before turning on the Flow Cytometry

1. Check the levels of the sheath reservoir should be no more than ¾ full. (sufficient for approximately 3 hours for run time)
2. Check the levels of the waste reservoir should contain approximately 400 ml of undiluted household bleach .(5% Sodium hypochlorite)

Fill the sheath reservoir before user turn on the Flow Cytometry.If the Flow Cytometry is already turned on , user will need to relieve the pressure from the sheath reservoir before fill it.

Starting up the FACSCalibur

Check the fluid levels of the sheath reservoir and the waste Reservoir .If necessary, fill the sheath reservoir and empty the waste reservoir and To ensure proper initialization between the cytometer and computer, turn on the FACSCalibur before turning on the computer

1. Turn on the FACSCalibur. The power switch is located in the lower – left conner of the instrument. Move the tube support arm to the right and remove the tube of distilled water from the sample injection port (SIP).
2. Make sure the FACSCalibur is pressurized. This can be done simply by making certain the sheath reservoir fits snugly beneath bracket. When the system is fully pressurized, the reservoir dose not move.
3. Check the sheath filter for trapped air bubbles. Vent the air from the filter if necessary. If bubbles are visible, gently tap the filter body with your finger to dislodge the bubbles and force them to the top . Push the roller in the pinchcock forward to allow the pressurized sheath fluid to force the air bubbles into the waste reservoir.
4. Clear the flow cell area of trapped air bubbles by press on switch the fluid

control dial to " PRIME" repeat 2-3 times as necessary until no bubbles are visible upon filling and display the fluid control dial to " STANDBY".

Starting up the Computer and Acquisition

1. Turn on the computer Macintosh system.
2. Check the operating state of the FACSCalibur , choose status from the flow cytometer menu. The displays the Status window, status is "*not ready*" that indicates the laser is warming up to 15 mW.(approximately 5 min.),the sheath reservoir is empty or the waste reservoir is full. If status is " *Ready*" that indicates a sample is on the SIP ,the tube support arm is centered and the fluid control dial is set to run.
3. Choose connect to Cytometry from the Acquire menu
4. Choose CellQuest software from the Apple menu. The CellQuest copyright message appears, followed by the CellQuest desktop displaying an untilled experiment window. All CellQuest software functions are performed in this window through the use of the tool palette and items within the menu bar. User may create plots for acquisition or analysis for experiment.
5. Optimization is the instrument adjustment procedure that optimally sets by choose the detectors, threshold, amplifiers and compensation for specific sample. when using CellQuest software
6. Install the sample tube on the SIP and quickly center the tube support arm under the tube and set the fluid control dial to RUN
7. If user want walking away from the system, set the fluid control dial to STANDBY to stop sheath consumption and reduce laser power. Install a tube containing no more than 1 ml of distilled water on the SIP and center the tube support arm. This prevents the sample injection tube from drying out. Follow this rule when the instrument is turn on or off.

Quality Control and Instrument Adjustment Before Using

Performing a quality control procedure is recommended to monitor the instrument and ensure that it is operating consistently from one day to the next.

1. Open FACSComp software, follow on running FACSComp.

2. Run Calibrite beads (white , red and green) in unstained and mixed tube
3. Each time user run FACSComp software, the instrument settings are saved to a file in the BD references folder.

System Daily Cleaning

FACSCalibur has been designed to require a minimum amount of maintenance for preserve the reliability of the instrument, basic preventive maintenance procedures must be performed daily cleaning when user shut down the instrument to clean the sample injection tube. This aim to prevent it from becoming clogged and to remove adhesive dyes.

1. With the tube support arm to the right , install a tube containing 4 ml of 5% Sodium hypochlorite on the SIP. Allow the vacuum to aspirate 2 ml of the solution.
2. Set the sample flow rate switch to HI, then center the tube support arm and allow the 5% Sodium hypochlorite to run for 5 min.
3. With the tube support arm to the right , install a tube containing 4 ml of distilled water on the SIP. Allow the vacuum to aspirate 2 ml of the water.
4. Center the tube support arm and allow the water to run for 5 min.
5. Set the fluid control dial to STANDBY.
6. The distilled water no more than 1 ml should remain on the SIP after the instrument has been turn off to prevent salt deposits from forming in the sample injection tube.

Shutting Down the FACSCalibur

1. Allow the FACSCalibur to remain in STANDBY for 5 min. before turn off the power. This gives the laser time to cool before shut down.
2. Turn off the FACSCalibur.
3. Exit the software. Choose Quit from the file menu. Quit all other applications before turning off the computer.
4. Shut down the computer, choose from the special menu.

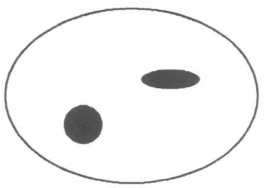
APPENDIX C

Evaluation of FISH Results

Probe signal Enumeration

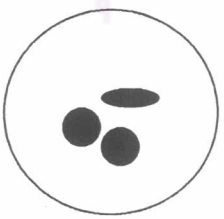
It is important to become familiar with the size, shape and fluorescence intensity of each of the DNA probe signals in order to accurately enumerate the hybridization results. The following information is provided to assist how to accurately enumerate probe signals. Using objectives with higher magnification (100X) , count the number of probe signals within lymphocyte boundary.

The following diagram illustrate correct interpretation of probe signals

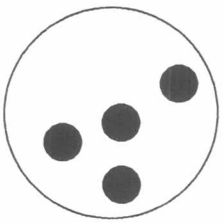


Count as 2 signals : one is very compact, the other is diffuse

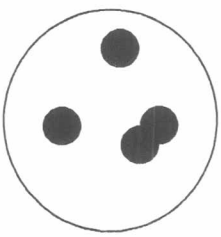
Count as 2 signals. One signal is split



Count as three signals.



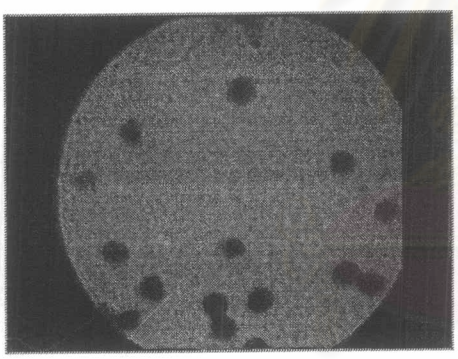
Count as four signals.



Count as three signals. One is split.

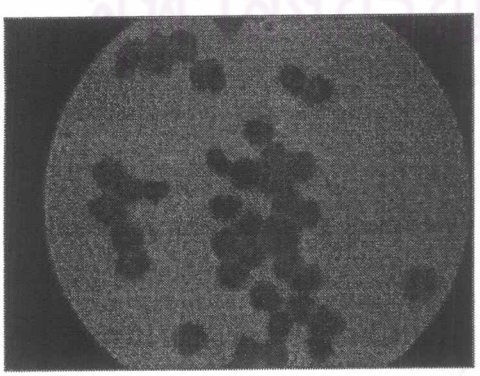
The Quality control of FISH method

Example of the positive control (Human lymphocyte) and the negative control (sheep lymphocyte) were smeared on slide for dye that compare both of the positive and negative control after hybridized with CEP 16 DNA probe.

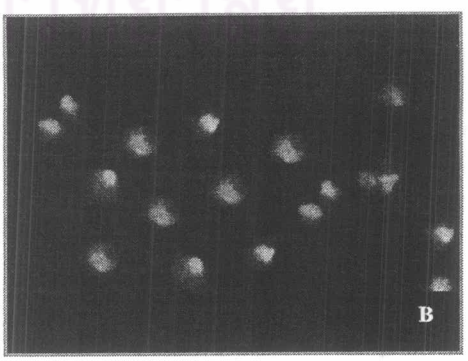


Positive control(Human lymphocyte) were smeared on slide for dye

Positive control(Human lymphocyte) were hybridized with CEP 16 DNA probe.

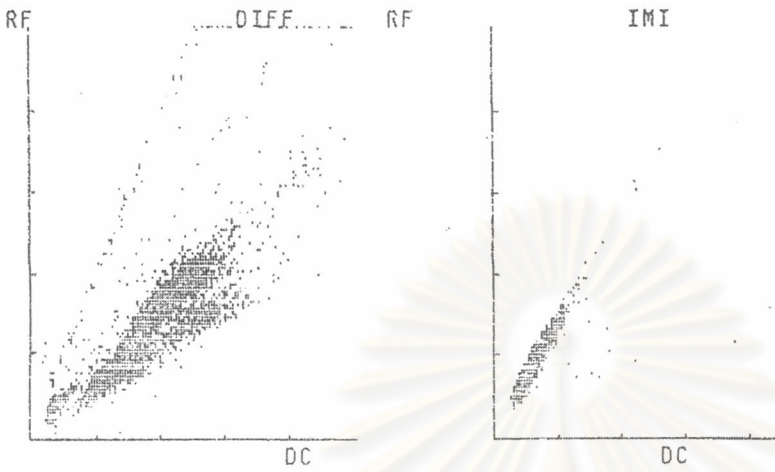


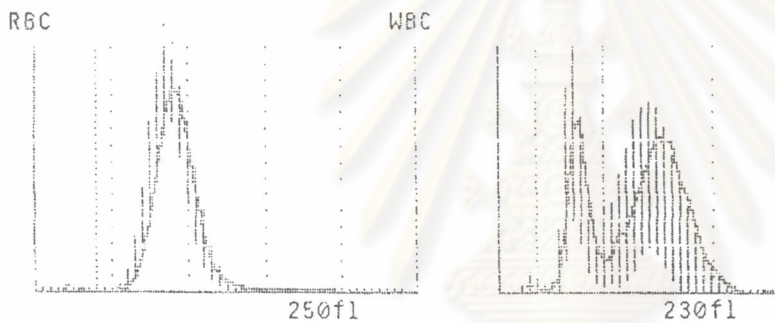
Negative control(Human lymphocyte) were smeared on slide for dye

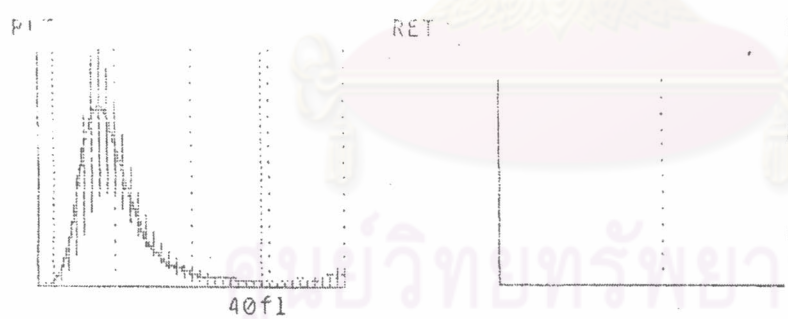


Negative control(Human lymphocyte) were hybridized with CEP 16 DNA probe.

Complete Blood count of human umbilical cord blood



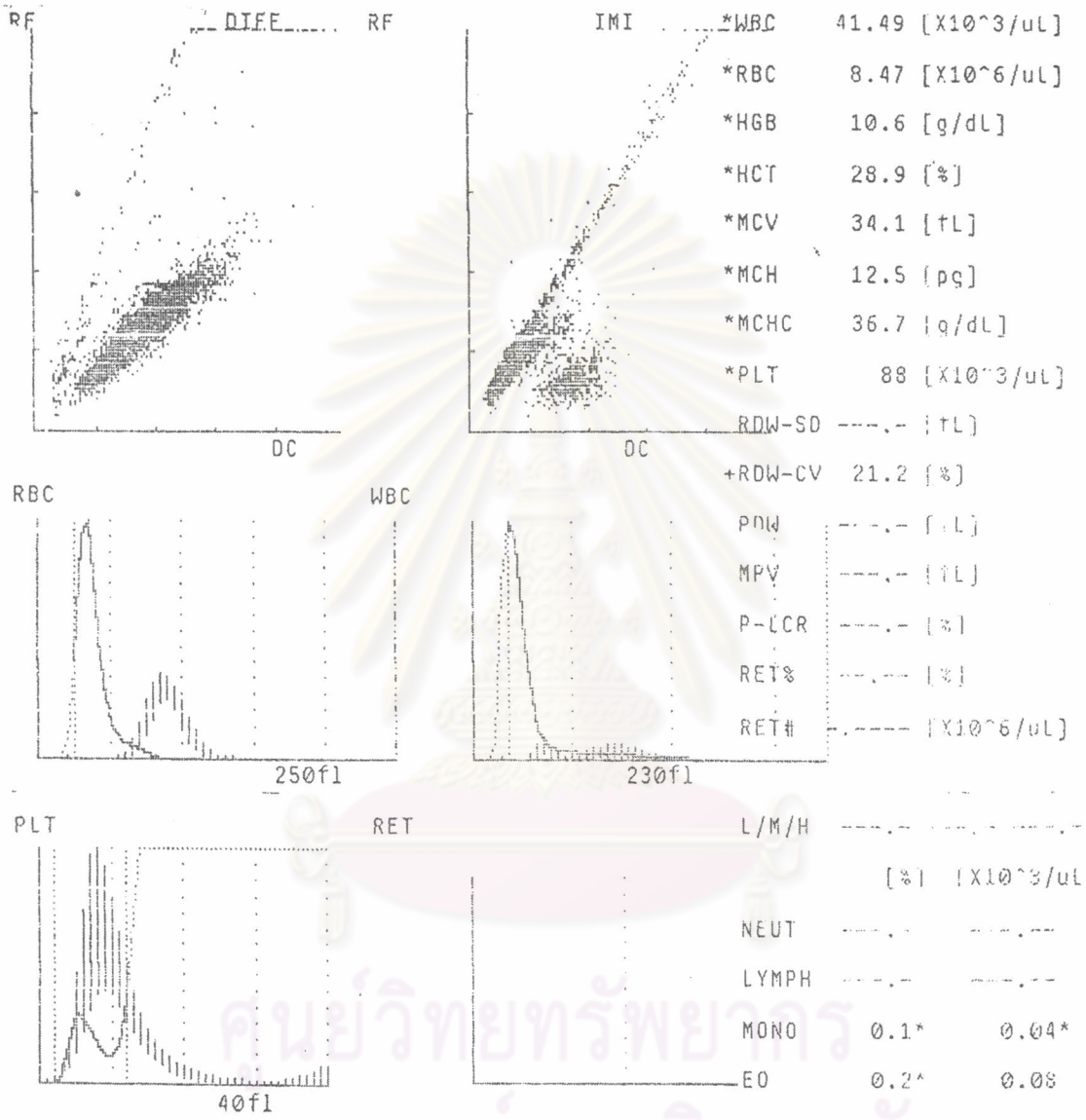




WBC	7.10	[X10^3/uL
RBC	3.98	[X10^6/uL
HGB	12.0	[g/dL]
HCT	36.1	[%]
MCV	90.7	[fL]
MCH	30.2	[pg]
MCHC	33.2	[g/dL]
PLT	247	[X10^3/uL]
RDW-SD	43.3	[fL]
RDW-CV	13.0	[%]
PDW	11.1	[fL]
MPV	10.1	[fL]
P-LCR	24.5	[%]
RET%	--.--	[%]
RET#	.----	[X10^6/uL]

L/M/H	--.-	--.-	--.-
	[%]	[X10^3/ul
NEUT	51.4	3.65
LYMPH	30.9	2.19
MONO	7.0	0.50
EO	10.3+	0.73+
BASO	0.4	0.03

Complete Blood count of Sheep blood



APPENDIX D

Results of statistic analysis

The SPSS version 9.0 window program were used for the statistic analysis of the percent of human lymphocytes in this objectives were paired t-test (Table) . The actual measured number of CD 45+ cells was compare to the predicted number of the concentration between human and sheep lymphocytes at all 5 ratio. (1:100, 1:500, 1:1,000, 1:5,000 and 1:10,000) by Flow Cytometry

A. The actual measured number of CD 45+ cells
   Ratio mixed human/sheep 1:100 t=1.839
   P=0.087

B. The actual measured number of CD 45+ cells
   Ratio mixed human/sheep 1:500 t=1.773
   P=0.098

C. The actual measured number of CD 45+ cells
   Ratio mixed human/sheep 1:1,000 t=-1.928
   P=0.074

D. The actual measured number of CD 45+ cells
   Ratio mixed human/sheep 1:5,000 t=-0.696
   P=0.498

E. The actual measured number of CD 45+ cells
   Ratio mixed human/sheep 1:10,000 t=1.839
   P=0.087

The actual measured number of CD 45+ cells was compare to the predicted number of the concentration between human and sheep lymphocytes at all 5 ratio. (1:100, 1:500, 1:1,000, 1:5,000 and 1:10,000) by FISH analysis.

A. The actual measured number of CD 45+ cells

Ratio mixed human/sheep 1:100 t=-4.847

P=0.000*

B. The actual measured number of CD 45+ cells

Ratio mixed human/sheep 1:500 t=-4.782

P=0.000*

C. The actual measured number of CD 45+ cells

Ratio mixed human/sheep 1:1,000 t=-5.446

P=0.000*

D. The actual measured number of CD 45+ cells

Ratio mixed human/sheep 1:5,000 t=-5.576

P=0.000*

E. The actual measured number of CD 45+ cells

Ratio mixed human/sheep 1:10,000 t=3.955

P=0.001*

* The mean of difference is significant at the lower 0.05 level.

The percent donor cells of human lymphocytes were detected at ratio 1:100,1:500,1:1,000,1:5,000and 1:10,000 in mixed experiment by Flow Cytometry and FISH analysis.

Ratio mixed human/sheep 1:100

Flow-FISH analysis of human/sheep 1:100 t = 7.065

P = 0.000*

Ratio mixed human/sheep 1:500

Flow-FISH analysis of human/sheep 1:500 t = 6.006

P = 0.000*

Ratio mixed human/sheep 1:1,000

Flow-FISH analysis of human/sheep 1:1,000 t = 2.197

P = 0.045*

Ratio mixed human/sheep 1:5,000

Flow-FISH analysis of human/sheep 1:5,000 t = -1.012

P = 0.327

Ratio mixed human/sheep 1:10,000

Flow-FISH analysis of human/sheep 1:10,000 t = 0.924

P = 0.371

* The mean of difference is significant at the lower 0.05 level.

The percent donor cells engraftment in chimeric lambs no. 40A3 and 41J2 at two times intervals(10 days and 1 month after birth) by Flow Cytometry analysis .

A. Chimeric lambs no. 40A3

Comparison between 10 days and 1 month after birth t= 1.600

P= 0.356

B. Chimeric lambs no. 41J2

Comparison between 10 days and 1 month after birth t= 5.027

P= 0.110

C. Comparison chimeric lambs

no. 40A3 and no. 41J2 at 10 days after birth t= -1.940

P= 0.330

D. Comparison chimeric lambs

no. 40A3 and no. 41J2 at 1 month after birth t= 3.025

P= 0.197

BIOGRAPHY

Miss Sukanya Suwanmaneerut was born on June 14 1976 in Bangkok, Thailand. She received the degree of Bachelor of Biology of Science in 1988 from faculty of science, Mahidol University, Bangkok, Thailand. She has enrolled at Chulalongkorn University in graduate program for the degree of master of science in medical science in 2003.