CHAPTER III

RESULTS

3.1 Preparation of the three-color reagent

3.1.1 Purification of MT4 and MT14/3 mAbs

MT4 mAb was purified from MT4 hybridoma induced ascitic fluid by affinity chromatography using rat anti-mouse IgM sepharose 4B column. After purification process, the concentration of purified MT4 was determined by measurement the absorbance at 280 nm and protein concentration was calculated in accordance with 1 mg/ml solution of IgM has extinction coefficient as 1.18 (A_{280}). In this study, 0.58 and 1.14 mg of MT4 mAb were obtained from 1.6 and 1.2 ml of the starting ascitic fluid in first and second purification procedures.

MT14/3 mAb was purified from MT14/3 hybridoma induced ascitic fluid by affinity chromatography using protein A sepharose column. After purification process, the concentration of purified MT14/3 mAb was determined by measurement the absorbance at 280 nm and protein concentration was calculated in accordance with 1 mg/ml solution of IgG has extinction coefficient as 1.43 (A_{280}). Total amount of 0.104, 0.06, 0.512 and 0.664 mg of MT14/3 mAb were obtained from 0.4, 0.8 and 0.3 ml of the starting ascitic fluid in 4 preparations, respectively.

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3.1.2 Labeling of MT4 mAb with FITC

Purified MT4 mAb were then labeled with FITC using R ratio of 20 and 200. After labeling, F/P ratio was determined. It was found that F/P ratio was 0.3 and 0.59 when using R ratio of 20 and 200, respectively.

3.1.3 The activity and specificity of the purified antibodies

The activity of the FITC labeled MT4 mAb was determined by direct immunofluorescence. As shown in Figure 3.1, the purified MT4 mAb strongly reacted to CD4 expressing cells (CD4 lymphocytes). The FITC labeled MT4 mAb at F/P ratio of 0.59 showed higher positive reactivity compare to the F/P ratio of 0.3. The FITC-MT4 at F/P ration of 0.59 was selected for further experiment.

The activity of the purified MT14/3 mAb was determined by indirect immunofluorescence. As shown in Figure 3.2, all purified MT14/3 mAb preparations strongly reacted to monocytes.

The specificity of FITC labeled MT4 mAb against CD4 protein was assessed by direct immunofluorescence using CD4-DNA transfected COS cells as antigens. The FITC labeled MT4 mAb strongly reacted to CD4 expressing COS cells (figure 3.3) but not react to mock transfectants.

The specificity of purified MT14/3 against CD14 protein was also assessed by indirect immunofluorescence using CD14 transfected COS cells as antigens. As shown in Figure 3.3, the purified MT14/3 mAb strongly reacted to CD14 expressing COS cells but not react to mock transfectants.

These results demonstrated that the FITC labeled MT4 and the purified MT14/3 mAb could be used in further studies.

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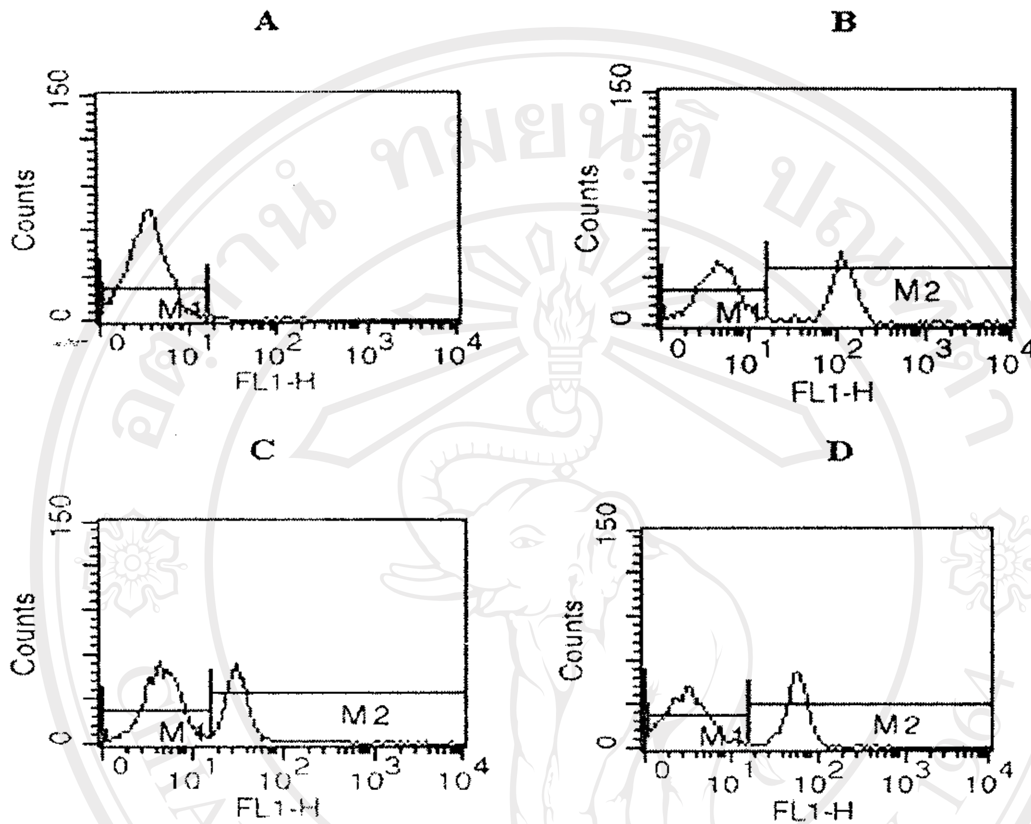

Figure 3.1 Immunofluorescence analysis of the reactivity of MT4 mAb after conjugated with FITC. PBMC were stained with 20 μg/ml of FITC labeled MT4 mAb which F/P ratio was 0.3 (C) and 0.59 (D). As control, PBMC were stained with MT4 ascitic fluid at dilution 1:250 as primary mAb then counterstained with FITC conjugated rat anti-mouse immunoglobulins at dilution of 1:20 (B) or stained with only conjugate only (A). The stained cells were analyzed by flow cytometry. Horizontal lines (M1) represent the background fluorescence of negative control cells (A).

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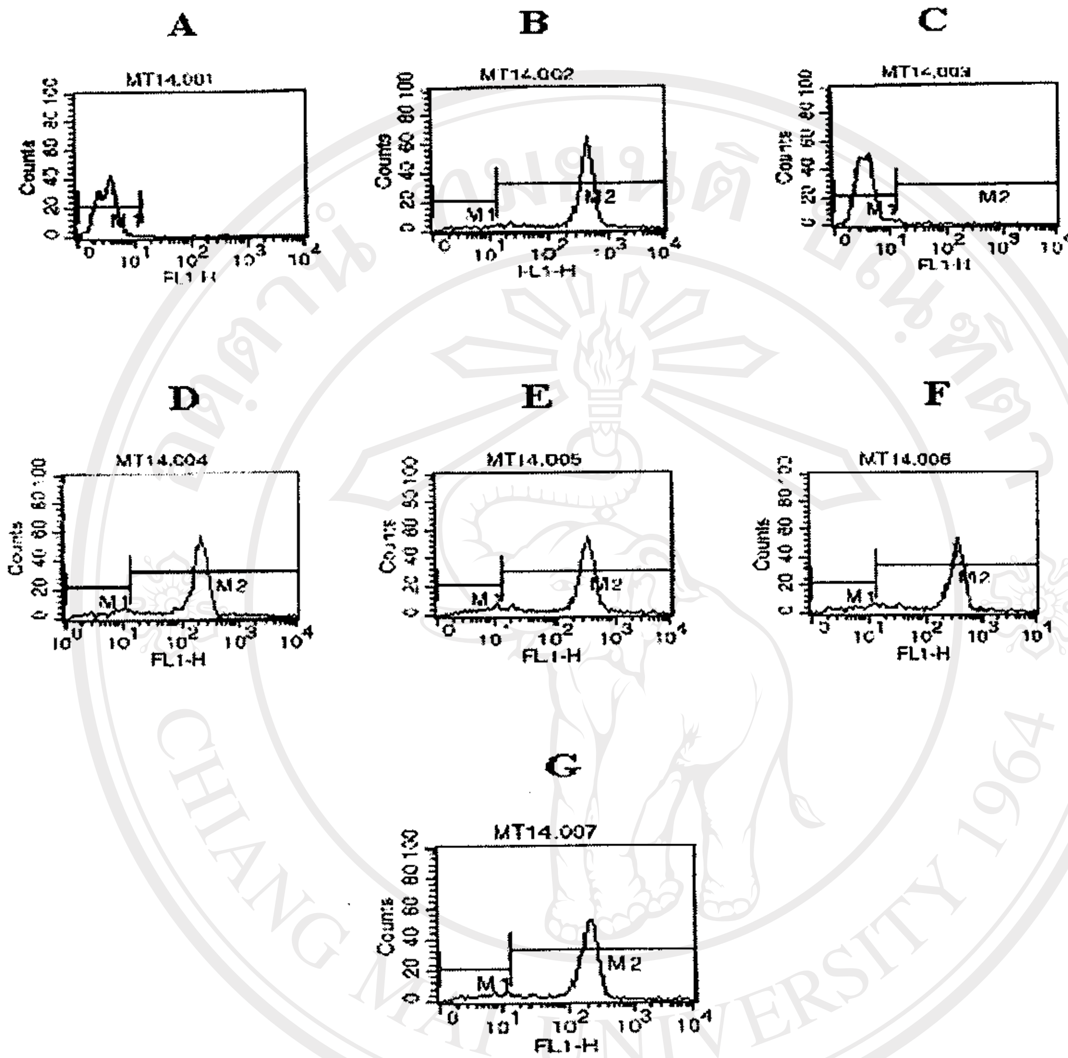

Figure 3.2 Immunofluorescence analysis of the reactivity of purified MT14/3 mAb. PBMC were stained with 20 μg/ml of four purified MT14/3 mAb preparations (D, E, F and G) and 20 μg/ml of purified MEM-18 mAb as positive CD14 control mAb (B). PBMC were stained with hybridoma induced ascitic fluid at dilution 1:250 as primary mAb and then counterstained with FITC conjugated rat anti-mouse immunoglobulins at dilution of 1:20 as negative control (C). The stained cells were analyzed by flow cytometry. Horizontal lines (M1) represent the conjugate control background (A).

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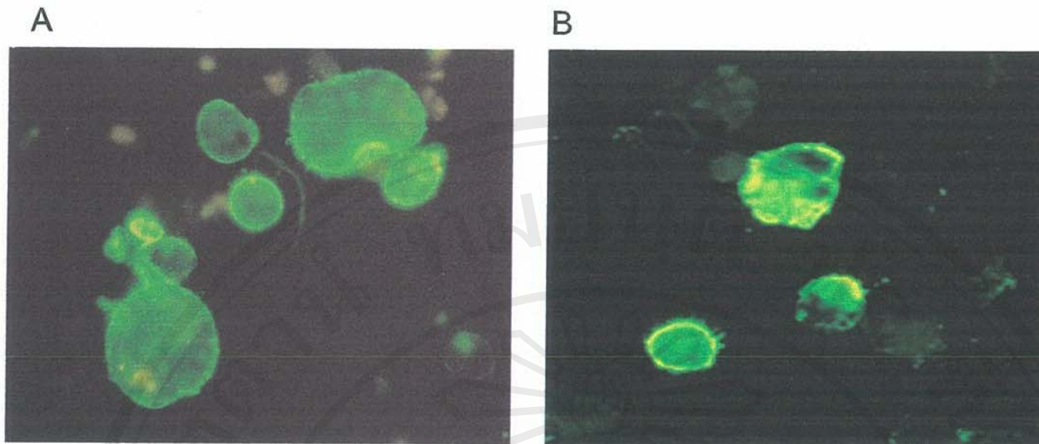

Figure 3.3 The reactivity of FITC labeled MT4 and purified MT14/3 mAbs to CD4 and CD14 proteins expressed on COS cells. COS cells were transfected with cDNA encoding CD4 protein and cDNA encoding CD14 protein. CD4-DNA transfected COS cells were stained with FITC labeled MT4 mAb (A). CD14-DNA transfected COS cells were stained with MT14/3 mAbs (B), then cells were counterstained with the FITC conjugated anti-mouse immunoglobulins at dilution of 1:20. The stained cells were analyzed by a fluorescence microscope.

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3.1.4 Optimal concentration of FITC labeled MT4, MT14/3, PE labeled anti-mouse IgG and PerCP-labeled CD45 mAb for development three color reagent

Whole blood obtained from different donors was stained with various concentrations (5, 10, 20 and 40 μg/ml) of FITC labeled MT4 at F/P ratio of 0.59 by direct immunofluorescence technique and membrane fluorescence of lymphocytes were analyzed by a flow cytometer. The FITC-MT4 mAb stained CD4 lymphocytes with various mean fluorescent intensity. The average of mean fluorescence intensity of each concentration was shown in Table 3.1. These results demonstrated that the optimal concentration of FITC labeled MT4 mAb for used to stain CD4 lymphocytes was 40 μg/ml.

Whole blood obtained from two donors were stained with various concentrations (25, 50, 100 and 200 μg/ml) of purified MT14/3 mAb and various dilutions (1:4, 1:8 and 1:16) of PE conjugated goat anti-mouse IgG. Membrane fluorescence of monocytes was analyzed by a flow cytometer. It was found that the average of mean fluorescence intensity of each concentration was varied from 237.74 to 2996.95 (Table 3.2). These results demonstrated that the optimal concentration of MT14/3 was 100 μg/ml and PE conjugated goat anti-mouse IgG was at dilution of 1:8 (Figure 3.4).

Whole blood obtained from four donors were stained with the various volumes (5, 10, and 20 μl) of PerCP conjugated anti-CD45 mAb by direct immunofluorescence technique and membrane fluorescence of cells were analyzed by a flow cytometer. It was found that the average of mean fluorescence intensity of positive lymphocytes was varied from 575.10 to 653.73, positive monocytes was varies from 349.36 to 400.52 and positive granulocytes was varies from 144.21 to

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187.88 (Table 3.3). These result demonstrated that the optimal concentration of PerCP conjugated anti-CD45 mAb was 20 μl (Figure 3.5).

3.2 The two-color flow cytometric analysis for enumeration of CD4 lymphocytes and CD8 lymphocytes by Simultest™ reagent

Whole blood samples were stained with Simultest™ reagent panel in four separate tubes. Leukogate tube was used to gate on lymphocyte population using dot plot displays of FSC (x-axis) versus SSC (y-axis) and established FL1 (x-axis) versus FL2 (y-axis) marker placement which were CD45-FITC versus CD14-PE. The Simulset software of Becton Dickinson automatically sets a lymphocyte analysis gate to eliminate most debris, monocytes, and granulocytes (Figure 3.6A). Isotype control tube was used to set fluorescence intensity markers using FL1 (x-axis) versus FL2 (y-axis) and gives an indication of nonspecific staining. Fluorescence markers should be set around the unstained population that appears as the cluster of events that are low in both yellow-green and red-orange fluorescence (Figure 3.6B). FITC labeled CD3/PE labeled CD4 tube and FITC labeled CD3/PE labeled CD8 tube were acquired and analyzed using the gates and markers established with leukogate tube and isotype control tube. Dot plot of FL1 (x-axis) versus FL2 (y-axis) were displayed in 4 quadrants (Q). The percentage of CD4 lymphocytes and CD8 lymphocytes were number of events in Q2 displayed in percent lymphocyte conversion automatically calculated with SimulSet software. An absolute count automatically calculated like wise by entering total white blood cells count (cells/μl) and the percentage of lymphocytes from a differential white blood cells count (Figure 3.7).

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Table 3.1 Titration of FITC labeled MT4 mAb for CD4 lymphocyte staining.

Concentration of FITC labeled MT4 (μg/ml)	Average mean fluorescence intensity	
	CD4 negative cell	CD4 positive cell
5	2.80	42.64
10	2.97	54.8
20	3.12	87.47
40	3.22	119.40

Whole blood were stained with various concentrations of FITC labeled MT4 mAb with F/P ratio of 0.59. After lysis of red blood cells, the stained cells were analyzed by a flow cytometer. Lymphocyte population was gated and determined for mean fluorescence intensity.

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Table 3.2 Titration of purified MT14/3 mAb and PE conjugated goat anti-mouse IgG for monocyte staining.

Concentration of purified MT14/3(μg/ml)	Dilution of PE conjugated anti-mouse IgG	Average mean fluorescence intensity	
		negative cells	Positive cells
25	1:4	12.18	237.87
25	1:8	9.51	237.74
25	1:16	10.31	279.87
50	1:4	13.91	833.97
50	1:8	13.53	895.17
50	1:16	12.02	705.90
100	1:4	13.86	1587.00
100	1:8	14.28	1451.81
100	1:16	11.19	852.82
200	1:4	15.34	2996.95
200	1:8	13.07	1858.66
200	1:16	12.12	1081.64

Whole blood specimens were stained with various concentrations of purified MT14/3 and PE conjugated goat anti-mouse IgG. After lysis of red blood cells, the stained cells were analyzed by a flow cytometer. Monocyte population was gated and determined for mean fluorescence intensity.

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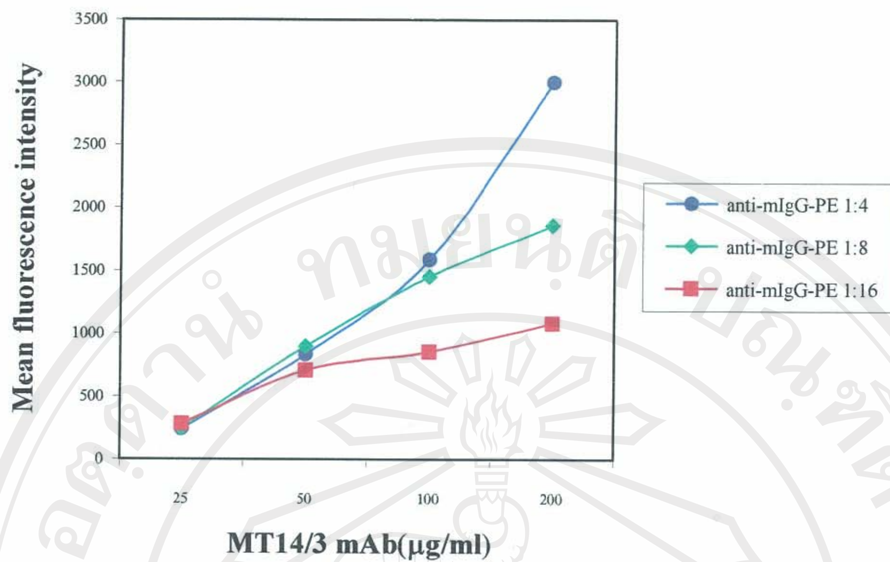

Figure 3.4 Average of mean fluorescence intensity of monocytes which were stained with various concentrations of purified MT14/3 and PE conjugated goat anti-mouse IgG.

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Table 3.3 Titration of PerCP conjugated anti-CD45 mAb.

Volume of PerCP conjugated CD45 mAb (μl)	Average mean fluorescence intensity		
	Granulocytes	Monocytes	Lymphocytes
Negative cell	11.49	6.56	4.65
5	144.21	349.36	575.10
10	169.83	384.65	638.97
20	187.88	400.52	653.73

Whole blood specimens were stained with the various volumes of PerCP conjugated anti-CD45 mAb. After lysis of red blood cells, the stained cells were analyzed by a flow cytometer. Lymphocyte, monocyte and granulocyte populations were gated and determined for mean fluorescence intensity.

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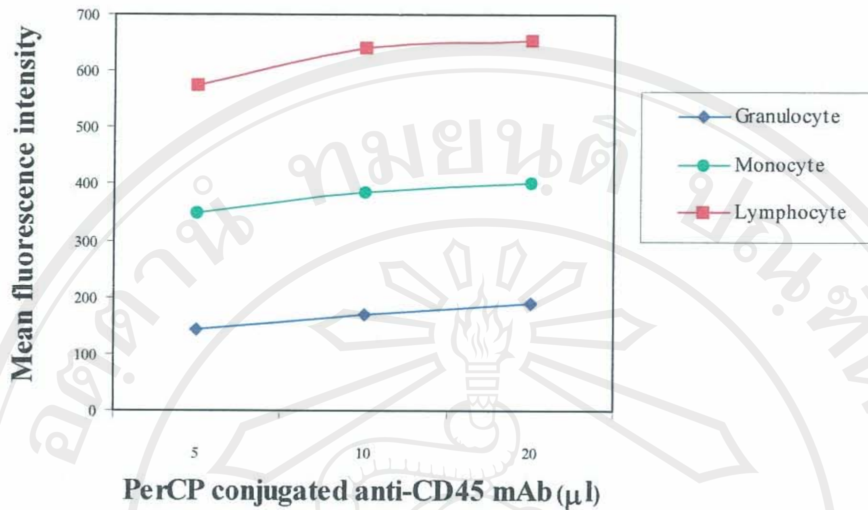

Figure 3.5 Average of mean fluorescence intensity of lymphocytes, monocytes and granulocytes were stained with various concentrations of PerCP conjugated anti-CD45 mAb.

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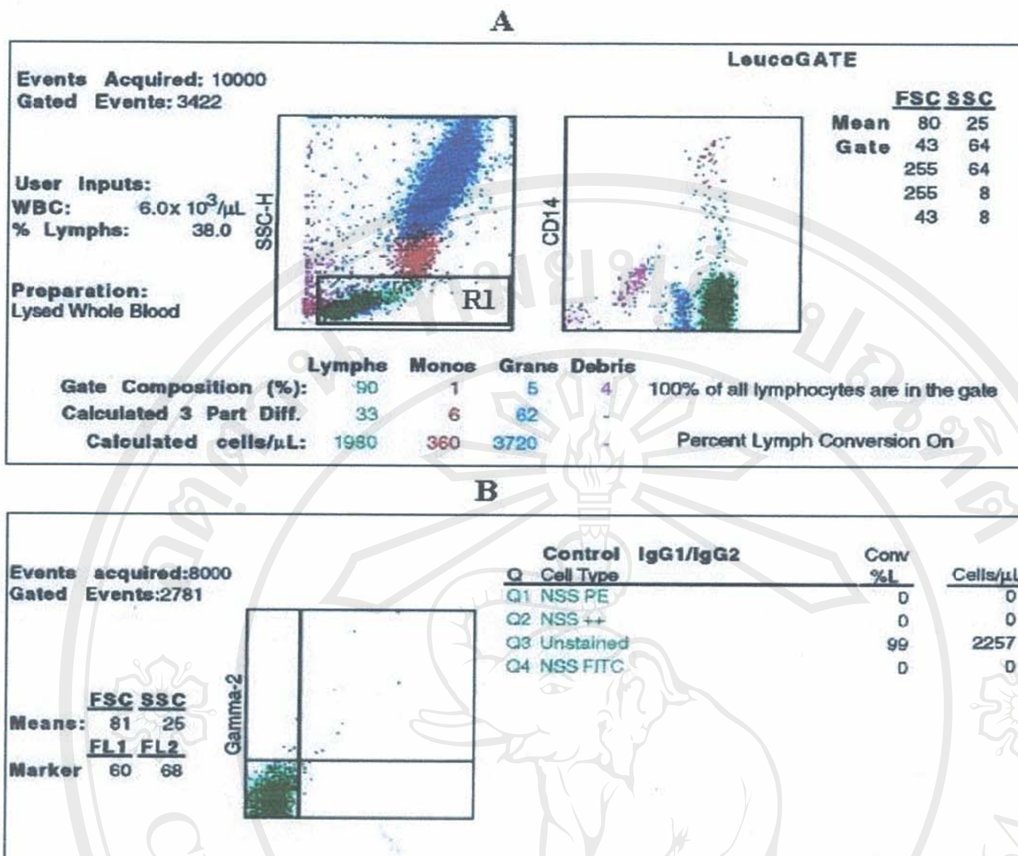

Figure 3.6 Flow cytometric analysis of whole blood sample stained with Simultest leukogate reagent (A), leukogate tube was used to reduce debris, monocytes, and granulocytes in the lymphocyte analysis gate (R1). Whole blood sample was stained with Simultest isotype control reagent (B). Isotype control tube was used to set fluorescence intensity markers and gives an indication of nonspecific staining.

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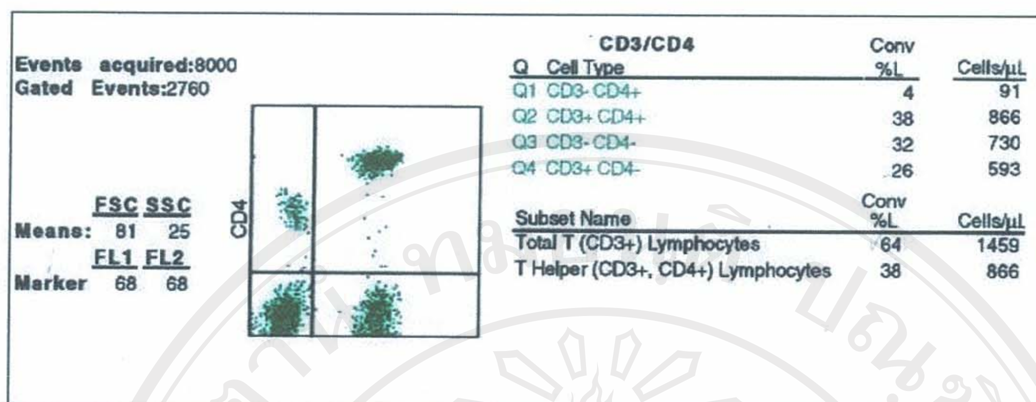

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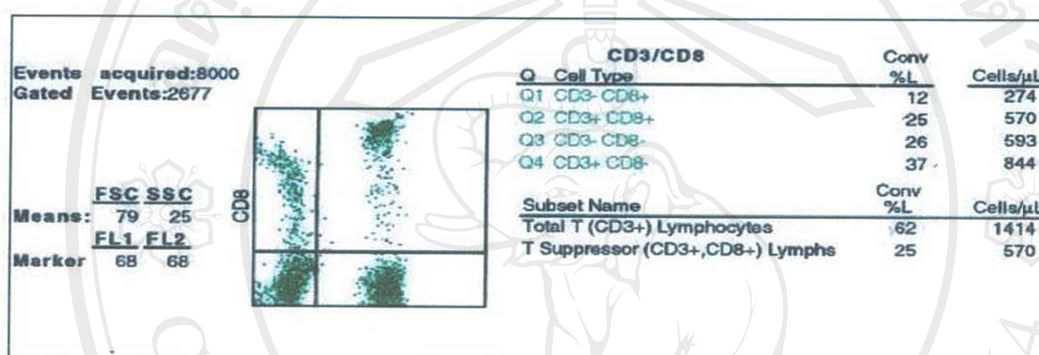

Figure 3.7 Flow cytometric analysis of CD4 lymphocytes (A) and CD8 lymphocytes (B). Whole blood samples were stained with Simultest CD3/CD4 reagent and Simultest CD3/CD8 reagent in separate tubes and then acquired and analyzed using the gates and markers established with leukogate tube and isotype control tube. The percentage of CD4 lymphocytes and CD8 lymphocytes were number of events in Q2 displayed in percent lymphocyte conversion automatically calculated with SimulSet software.

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3.3 The three-color flow cytometric analysis for enumeration of CD4 lymphocytes by developed three-color reagents

To develop a new method for enumering CD4 lymphocytes in blood samples using three-color immunofluorescence technique, the three-color immunofluorescence reagent which consisting of PerCP-CD45 mAb, FITC-CD4 mAb, CD14 mAb and PE-anti-mouse IgG conjugate were developed using the optimal concentration of antibodies that have been titrated. Single tube of whole blood specimen was stained with the developed three-color reagent, erythrocytes were lysed, and leukocytes were fixed, then analyzed by flow cytometer using CellQuest program. Dot plot of FSC (x-axis) versus SSC (y-axis) was displayed and established FL3 (x-axis) versus SSC (y-axis) which were CD45-PerCP versus granularity. By color-gating technique, the monocyte and granulocyte were gated as R1 and R2, respectively, using dot plot display of FSC versus SSC and then the lymphocyte population was gated as R3 using dot plot display of FL3 and SSC (Figure 3.8A and 3.8B). Fluorescence labeled cells in the gated lymphocytes were then determined for lymphocyte purity according to their fluorescence intensity by using FL3 (x-axis) and FL2 (y-axis) which were CD45-PerCP versus CD14-PE. By using this limited gating technique of R3, lymphocyte analysis gate was not contaminated with debris, monocytes, and granulocytes (Figure 3.8D). For obtaining CD4 lymphocyte count, fluorescence labeled lymphocytes in analysis gate were then determined according to their fluorescence intensity by using FL3 (x-axis) and FL1 (y-axis) which were CD45-PerCP versus CD4-FITC. Dot plot of FL3 versus FL1 was displayed in 4 quadrants, i.e., upper left (UL), upper right (UR), lower left (LL), and lower left (LR). The

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percentage of CD4 lymphocytes was number of events in UR displayed in percent of lymphocytes (Figure 3.8E and 3.8F).

An absolute number of CD4 lymphocytes (cells/μl) were calculated from total white blood cells count (cells/μl) and the percentage of lymphocytes (%L) from a differential white blood cell count as the following equation:

$$\text{Absolute number of CD4 lymphocytes} = \frac{\%\text{CD4 lymphocytes}}{100} \times \frac{\%\text{L}}{100} \times (\text{WBC})$$

To evaluate whether the developed reagent can be used for enumering CD4 lymphocyte count, five whole blood specimens were stained with the developed three-color reagent. The percentage of CD4 lymphocytes were compared with the results obtained from standard Simultest™ reagent kit. As shown in Table 3.4, the percentage of CD4 lymphocytes obtained from the developed reagent and standard Simultest™ reagent were very similar.

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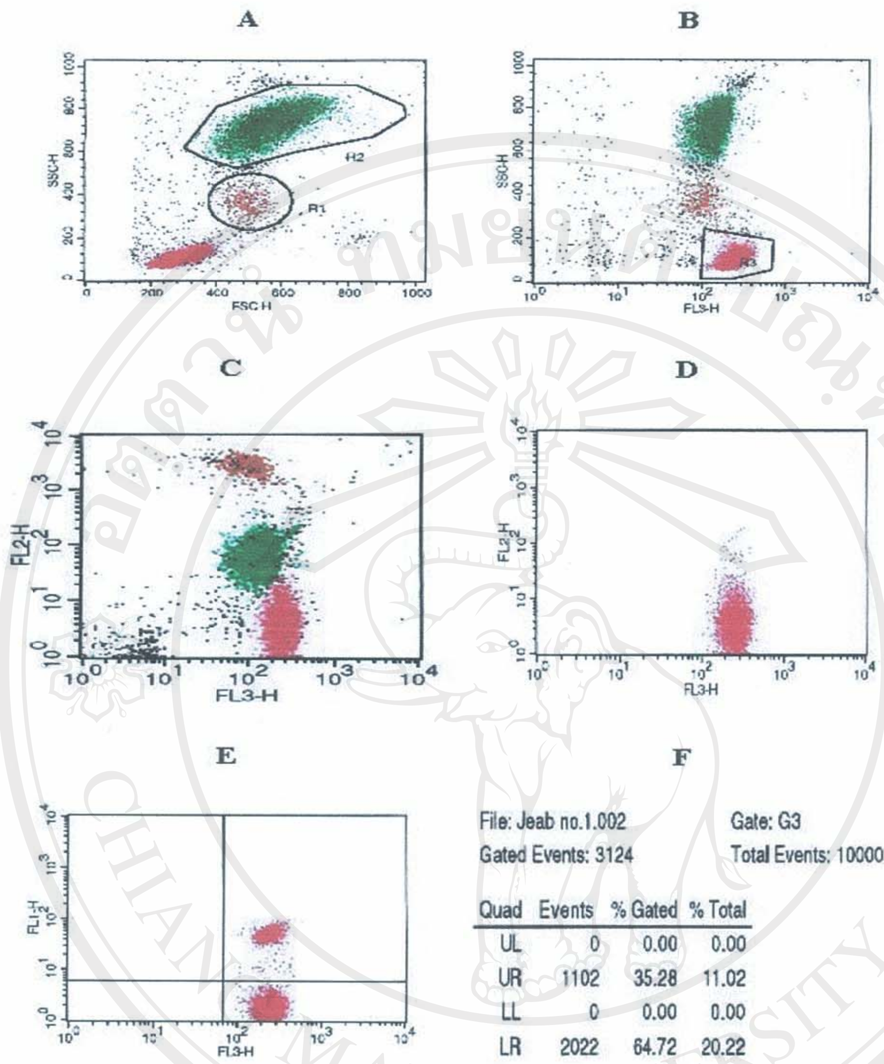

Figure 3.8 Flow cytometric analysis of CD4 lymphocytes using the developed three-color immunofluorescence reagents. Lymphocytes in the blood samples were gated according to FL3 and SSC (B, gate R3). The percentage of CD4 lymphocytes was determined by FL3 and FL1 (E). The number of events in UR (F) displayed as percentage of CD4 lymphocytes in analysis gate.

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Table 3.4 Percentage of CD4 lymphocyte determined by the developed three-color reagent and Simultest™ reagent kit.

Donor no.	%CD4 lymphocyte determined by developed three-color reagent	%CD4 lymphocyte determined by Simultest™ reagent kit
1	35	36
2	37	40
3	32	30
4	37	41
5	36	36

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3.4 Comparison of the developed three-color reagents and Simultest™ reagent

To validate the accuracy of the developed three-color reagent, CD4 lymphocytes from 30 healthy and 27 HIV infected persons were determined as percentage and absolute number by using the developed reagent and standard Simultest™ reagent kit. It was found that both percentage and absolute number of CD4 lymphocytes obtained from the two methods were similar with no statistically significant difference (Table 3.5, 3.6 and 3.7). Correlation plots comparing the percentage and absolute number of CD4 lymphocytes obtained from both methods were shown in Figure 3.9 and 3.10 respectively. Linear regression analysis resulted in a slope of 1.028 and 1.018, an intercept of –0.765 and –9.608 when the percentage and absolute number from the two methods were compared respectively while the correlation coefficient was 0.992 and 0.996 (Figure 3.9 and 3.10).

However, there were 10 blood samples, by using the standard Simultest™ reagent kit the obtained CD3, CD4 and CD8 lymphocyte count did not meet the quality control criteria (QC-criteria). These results may not accurate because of contamination from other cells in lymphocyte analysis gate. In contrast, by using three-color reagent, the purity of lymphocytes in the analysis gate was almost 100% (Table 3.8 and 3.9). When the percentage and absolute number of CD4 lymphocytes obtained from these 10 blood samples using both reagents were compared, the difference was therefore higher. As shown in Figure 3.11 and 3.12, linear regression analysis resulted in a slope of 0.987 and 0.967, an intercept of –1.403 and –15.383 while the correlation coefficient was 0.980 and 0.988 respectively.

Thus when the percentage and absolute number of CD4 lymphocytes obtained from 47 blood samples from the two methods which all sample test results met QC-

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criteria were compared both methods were very similar with no statistically significant difference. Correlation plots comparing the percentage and absolute number of CD4 lymphocytes obtained from both methods were shown in Figure 3.13 and 3.14 respectively. Linear regression analysis resulted in a slope of 1.033 and 1.025, an intercept of –0.550 and –6.662 when the percentage and absolute number from the two methods were compared respectively while the correlation coefficient was 0.995 and 0.998 (Figure 3.13 and 3.14).

These results indicated that the developed three-color reagent can be used to enumerate CD4 lymphocytes in blood samples equivalent to the commercial reagent. The developed three-color reagent can also overcome the disadvantages of the standard two-color antibodies method, and reduce the volume of whole blood used for analysis.

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Table 3.5 Percentage of CD3 lymphocytes, CD4 lymphocytes and CD8 lymphocytes determined by Simultest™ reagent kit and percentage of CD4 lymphocyte counts obtained by the developed three-color reagent from 30 healthy persons.

Donor no.	Simultest™ reagent			3-color reagent
	%CD3 lymphocyte	%CD4 lymphocyte	%CD8 lymphocyte	%CD4 lymphocyte
N1	68	37	27	39
N2	45	30	11	31
N3	75	38	31	39
N4	69	38	29	38
N5	74	29	36	29
N6	78	42	34	42
N7	75	37	31	35
N8	68	31	30	31
N9	66	39	23	39
N10	71	45	23	46
N11	71	38	27	36
N12	65	38	26	39
N13	67	40	26	41
N14	63	27	29	26
N15	69	45	22	48
N16	64	39	20	41
N17	87	40	42	40
N18	68	33	32	30
N19	63	32	29	33
N20	73	40	28	41
N21	62	34	23	38
N22	71	34	31	33
N23	59	42	13	45
N24	74	38	32	39
N25	69	31	29	32
N26	71	35	29	31
N27	76	39	38	38
N28	72	42	23	42
N29	62	27	27	30
N30	74	37	31	30

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Whole blood specimens were stained two sets with Simultest™ reagent kit and the developed three-color reagent then Simultest set was analyzed by SimulSet software as described in 3.2 and the 3-color set was analyzed by using CellQuest program as described in 3.3

Table 3.6 Percentage of CD3 lymphocytes, CD4 lymphocytes and CD8 lymphocytes determined by Simultest™ reagent kit and percentage of CD4 lymphocyte counts obtained by the developed three-color reagent from 27 HIV infected persons.

Donor no.	**Simultest™ reagent**			**3-color reagent**
	%CD3 lymphocyte	**%CD4 lymphocyte**	**%CD8 lymphocyte**	**%CD4 lymphocyte**
H1	63	34	27	33
H2	64	29	29	32
H3	64	6	52	5
H4	61	25	32	27
H5	61	2	59	2
H6	89	1	89	1
H7	63	11	47	12
H8	47	4	42	3
H9	89	26	55	23
H10	71	2	55	2
H11	61	11	45	10
H12	49	1	41	2
H13	34	5	29	6
H14	63	18	39	21
H15	69	15	52	16
H16	64	11	0	11
H17	67	18	45	18
H18	52	14	38	14
H19	60	2	53	1
H20	67	5	61	0
H21	72	32	43	30
H22	78	2	73	0
H23	75	21	53	21
H24	81	1	76	0
H25	65	2	61	0
H26	60	17	43	15
H27	73	48	23	50

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Whole blood specimens were stained two sets with Simultest™ reagent kit and the developed three-color reagent then Simultest set was analyzed by SimulSet software as described in 3.2 and the 3-color set was analyzed by using CellQuest program as described in 3.3

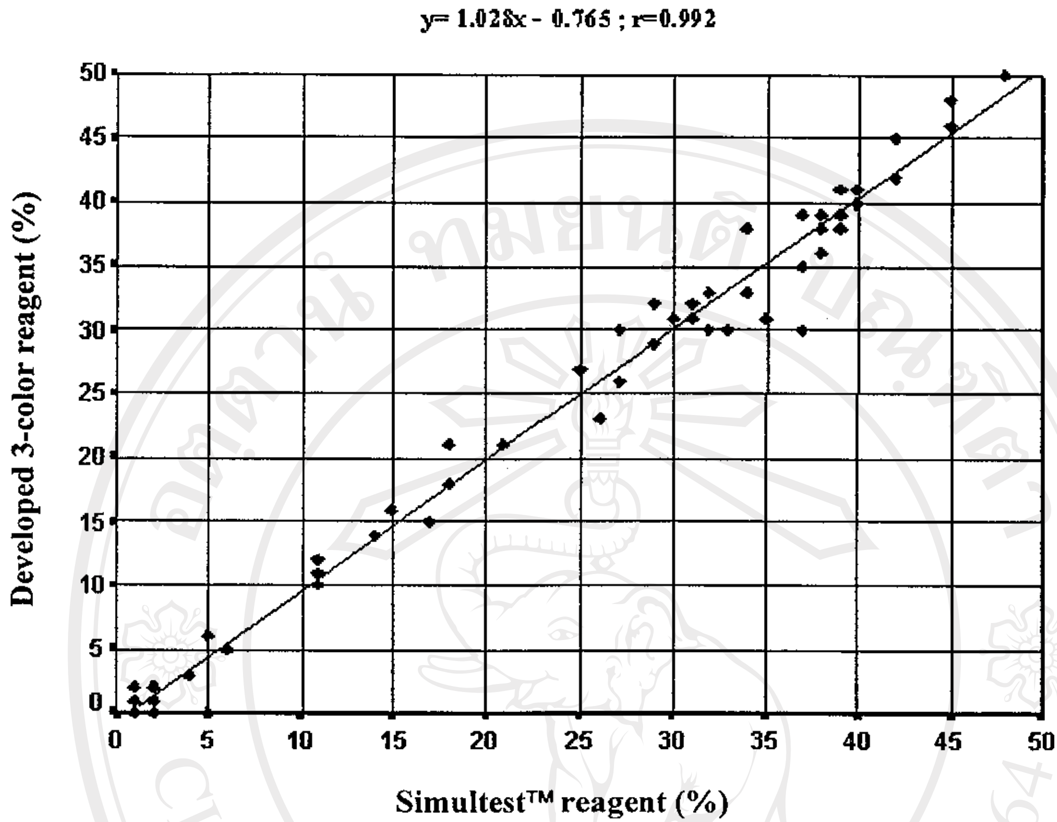

Figure 3.9 Correlation plot of percentage of CD4 lymphocytes from 57 blood samples. Blood samples were stained with the developed three-color and commercial Simultest™ reagent.

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Table 3.7 Absolute CD4 lymphocyte count (cells/µl) determined by Simultest™ reagent kit and the developed three-color reagents from 30 healthy persons (N1-N30) and 27 HIV infected persons (H1-H27).

Donor no.	Simultest™ reagent	3-color reagent	Donor no.	Simultest™ reagent	3-color reagent
N1	636	671	H1	438	425
N2	1060	1095	H2	587	648
N3	677	695	H3	68	57
N4	1291	1291	H4	348	376
N5	699	699	H5	18	18
N6	1235	1235	H6	32	32
N7	932	882	H7	218	238
N8	683	683	H8	53	40
N9	710	710	H9	674	596
N10	1103	1127	H10	15	14
N11	765	725	H11	133	121
N12	839	861	H12	11	21
N13	1638	1679	H13	48	58
N14	492	474	H14	376	439
N15	1539	1642	H15	400	427
N16	614	646	H16	239	239
N17	935	935	H17	284	284
N18	541	491	H18	306	306
N19	661	681	H19	29	14
N20	1156	1185	H20	57	0
N21	542	605	H21	487	456
N22	1010	980	H22	22	0
N23	1120	1200	H23	466	466
N24	1202	1234	H24	13	0
N25	804	829	H25	20	0
N26	1147	1016	H26	299	264
N27	955	931	H27	1104	1150
N28	1200	1199			
N29	437	486			
N30	740	600			

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Whole blood specimens were stained two sets with Simultest™ reagent kit and the developed three-color reagent then Simultest set was analyzed by Simulset software as described in 3.2 and the 3-color set was analyzed by using CellQuest program as described in 3.3

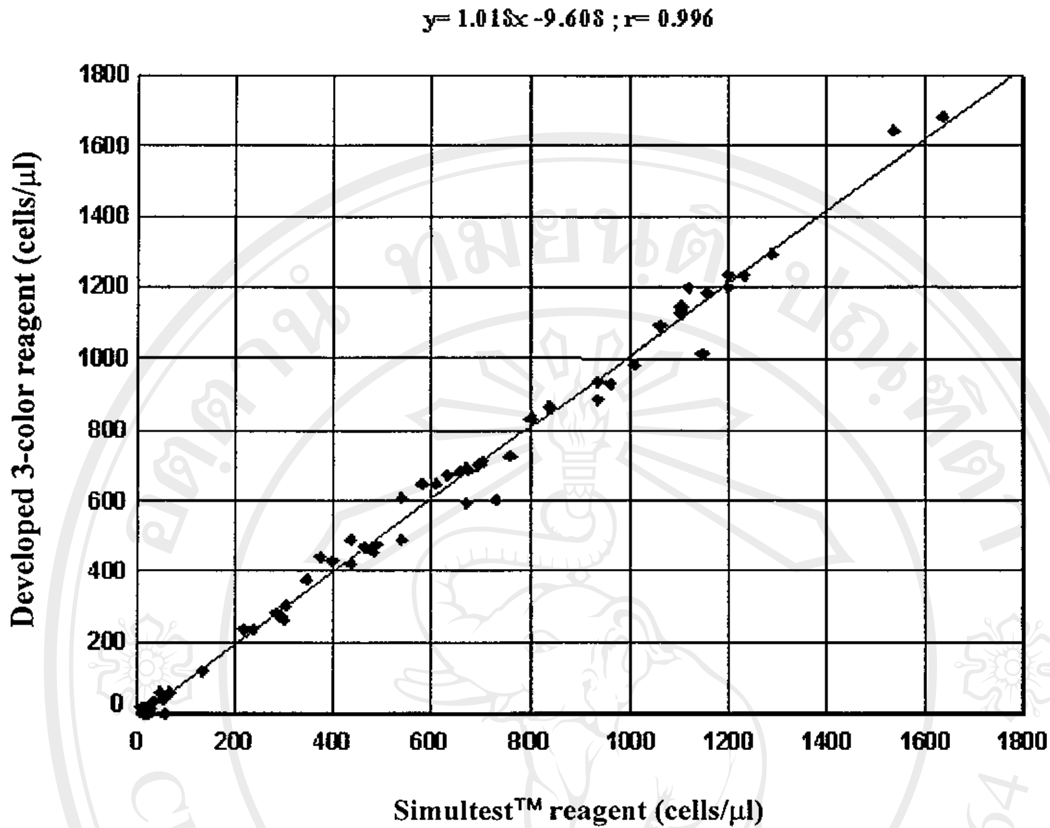

Figure 3.10 Correlation plot of absolute CD4 lymphocyte counts from 57 blood samples. Blood samples were stained with the developed three-color and commercial Simultest™ reagent.

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Table 3.8 Percentage of analysis gate composition (purity) and total lymphocytes (recovery) determined by Simultest™ reagent kit from 10 blood samples which did not meet the QC-Criteria.

Donor no.	**Analysis gate composition (%)**				**Total Lymphocytes in gate (%)**
	Lymphocytes	**Monocytes**	**Granulocytes**	**Debris**	
N7	70	0	2	28	9
N18	63	1	1	35	97
N23	72	1	2	25	94
N26	67	0	1	32	94
N35	77	0	2	20	92
H3	89	2	1	8	96
H4	41	1	0	57	89
H6	64	1	1	34	95
H18	88	1	11	2	97
H20	85	1	1	13	90
Quality Control criteria	**≥85 (optimally >90)**	**≤3**	**≤6**	**≤10**	**≥ 90 (optimally >95)**

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Table 3.9 Percentage and absolute CD4 lymphocyte counts (cells/μl) determined by Simultest™ reagent kit and the developed three-color reagent from 10 blood samples which all sample test results did not meet the QC-Criteria.

Donor no.	**%CD4 Lymphocyte**		**Absolute CD4 Lymphocyte**	
	Simultest™	**3-color**	**Simultest™**	**3-color**
N7	37	35	932	882
N18	33	30	541	491
N23	42	45	1120	1200
N26	35	31	1147	1016
N30	37	30	740	600
H3	6	5	68	57
H4	25	27	348	376
H6	1	1	32	32
H18	14	14	306	306
H20	5	0	57	0

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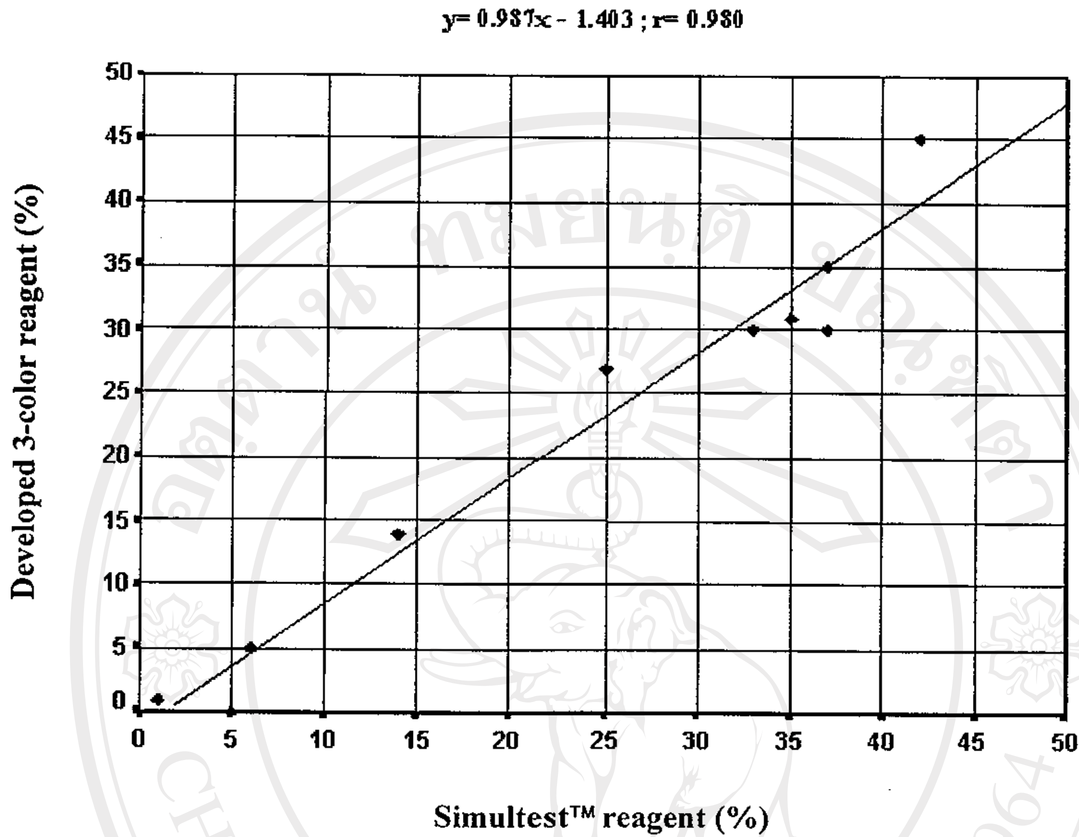

Figure 3.11 Correlation plot of percentage of CD4 lymphocytes from 10 blood samples, which all sample test results did not meet the QC-Criteria. Blood samples were stained with the developed three-color and commercial Simultest™ reagent.

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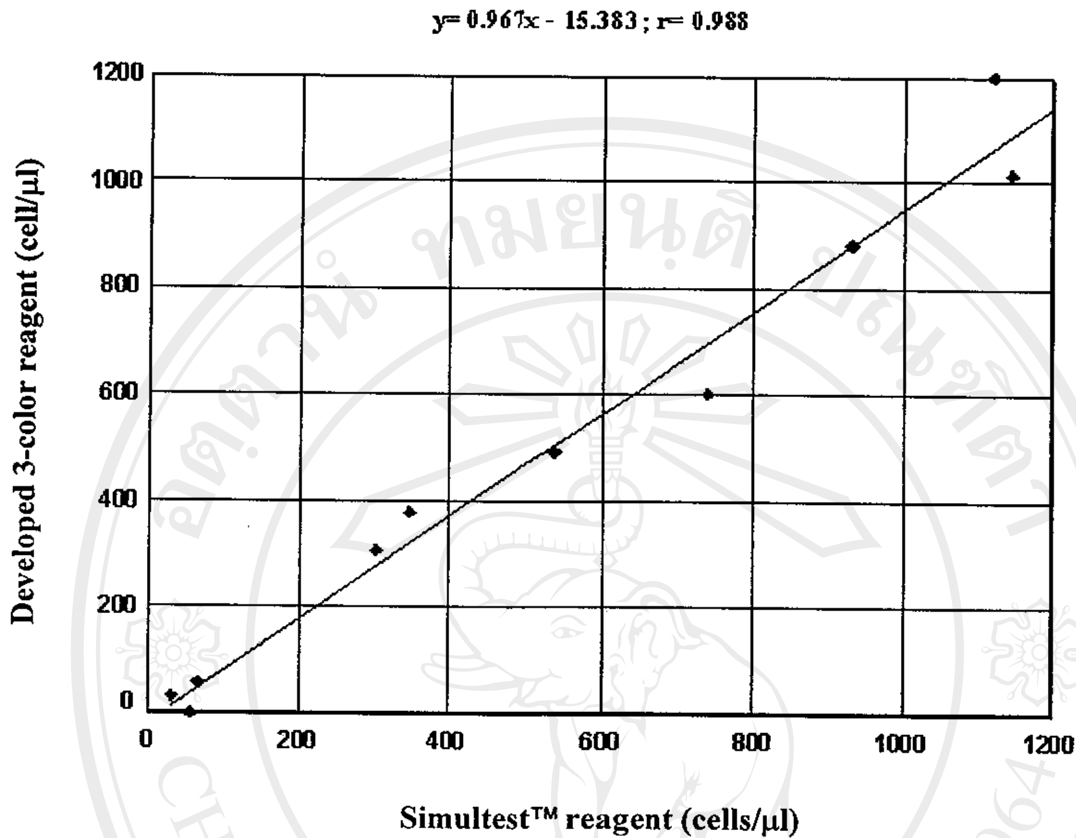

Figure 3.12 Correlation plot of absolute CD4 lymphocyte count from 10 blood samples, which all sample test results did not meet the QC-Criteria. Blood samples were stained with the developed three-color and commercial Simultest™ reagent.

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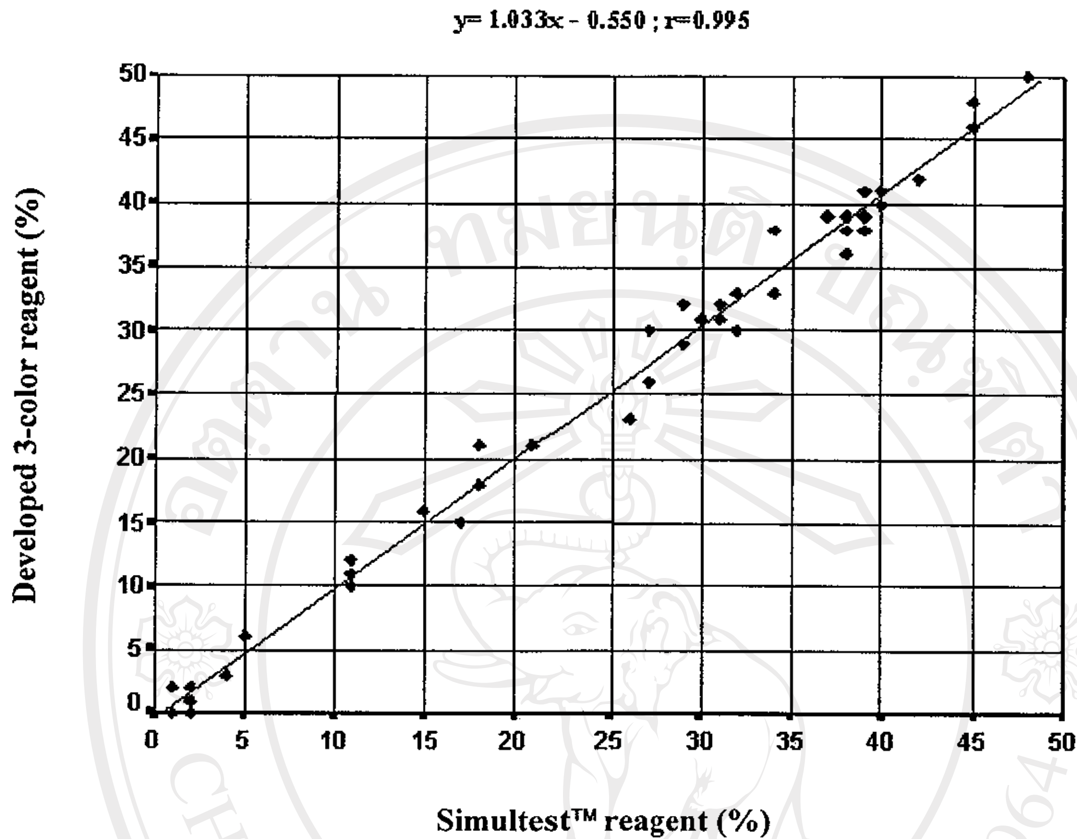

Figure 3.13 Correlation plot of percentage of CD4 lymphocytes from 47 blood samples which all sample test results met the QC-Criteria. Blood samples were stained with the developed three-color and commercial Simultest™ reagent.

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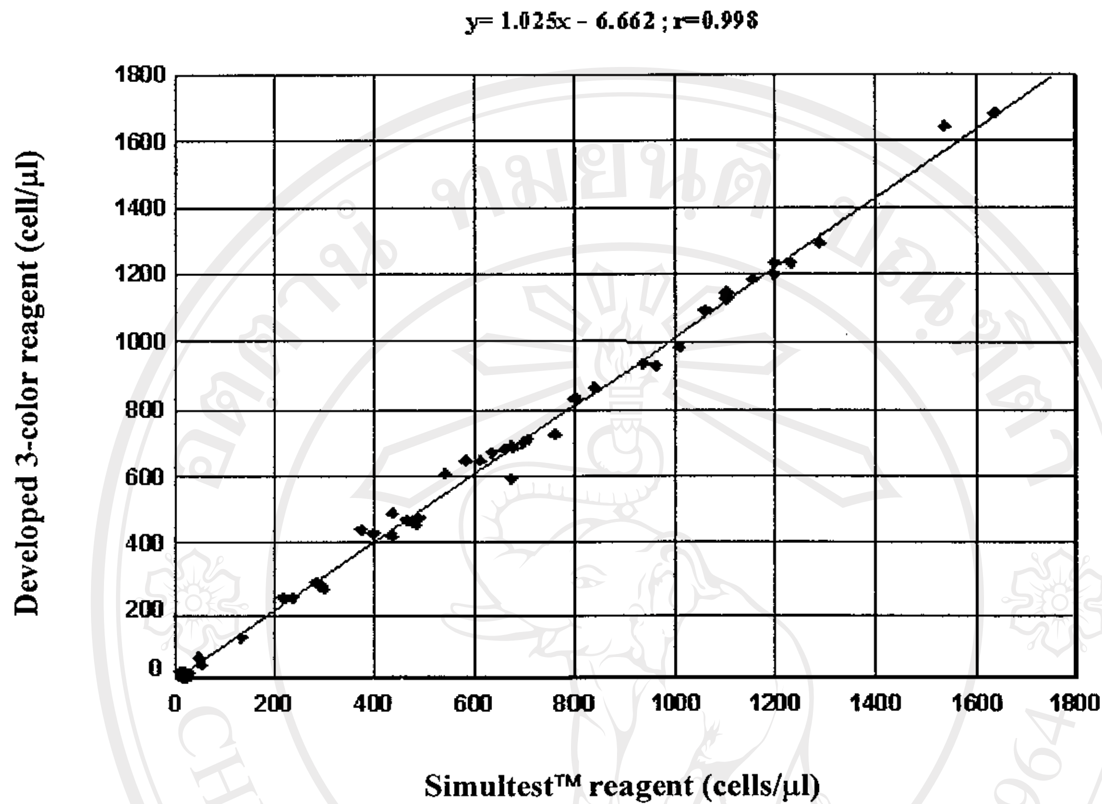

Figure 3.14 Correlation plot of absolute CD4 lymphocyte count from 47 blood samples which all sample test results met the QC-Criteria. Blood samples were stained with the developed three-color and commercial Simultest™ reagent.

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3.5 Preparation of red blood cell lysing solution

In flow cytometry it is imperative that leukocytes be analyzed free from interference by erythrocytes. In the past, this has been accomplished by density gradient separation or by red blood cell lysis with several washing steps. However, it has been clearly demonstrated that centrifugal washing may alter the remaining cellular distribution (35).

This study attempted to produce new lysing solution for lysing human red blood cells following direct immunofluorescence staining with monoclonal antibodies for flow cytometric analysis. Many solutions were prepared such as 1%ammonium oxalate, 0.83%ammonium chloride, ammonium chloride tris buffer, hypotonic ammonium chloride, 0.83%ammonium chloride containing 3%formaldehyde (3%formaldehyde-NH_4Cl) and PBS containing 0.83%ammonium chloride and 3%formaldehyde (3%formaldehyde-NH_4Cl-PBS). These solutions were used to lyse red blood cells in lysis step of staining as was described in materials and methods. As demonstrated in Figure 3.15, cellular distributions of each lysing solution were different when analyzed by flow cytometry with Simulset program. 3% formaldehyde-NH_4Cl and 3%formaldehyde-NH_4Cl-PBS lysing solutions were better than others and selected for further evaluation.

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3.6 Comparison of the developed red blood cell lysing solution with commercial FACS™ lysing solution.

3.6.1 Evaluation of 3%formaldehyde-NH_4Cl lysing solution

Lymphocyte subset as CD4, CD8 and CD3 lymphocytes of 20 healthy persons were determined using 3%formaldehyde-NH_4Cl lysing solution. Percentage

and absolute number of CD4, CD8 and CD3 lymphocytes were compared with the results obtained from standard Simultest™ reagent kit which red blood cell were lysed by FACS™ lysing solution. As shown in Table 3.10 and 3.11, both percentage and absolute number of CD4, CD8 and CD3 lymphocytes obtained from the prepared and commercial lysing solutions were similar with no statistically significant difference. A correlation plots comparing the percentage and absolute number of CD4, CD8 and CD3 lymphocytes obtained from both solutions were shown in Figure 3.16 to 3.21 respectively. Linear regression analysis resulted in a slope of 0.976, 0.928 and 0.905, an intercept of 0.934, 0.944 and 6.034 when percentage of CD4, CD8 and CD3 lymphocytes from the two lysing solution were compared respectively while the correlation coefficients were 0.940, 0.930 and 0.934 (Figure 3.16, 3.18 and 3.20). Linear regression analysis resulted in a slope of 0.990, 0.847 and 0.968, an intercept of 9.278, 70.992 and 41.737 when absolute number of CD4, CD8 and CD3 lymphocytes from the two lysing solution were compared respectively while the correlation coefficients were 0.990, 0.951 and 0.987 (Figure 3.17, 3.19 and 3.21). These results indicated that the home made 3%formaldehyde-NH_4Cl lysing solution can be used to enumerate CD4, CD8 and CD3 lymphocytes in blood samples equivalent to those given by the commercial FACS™ lysing solution.

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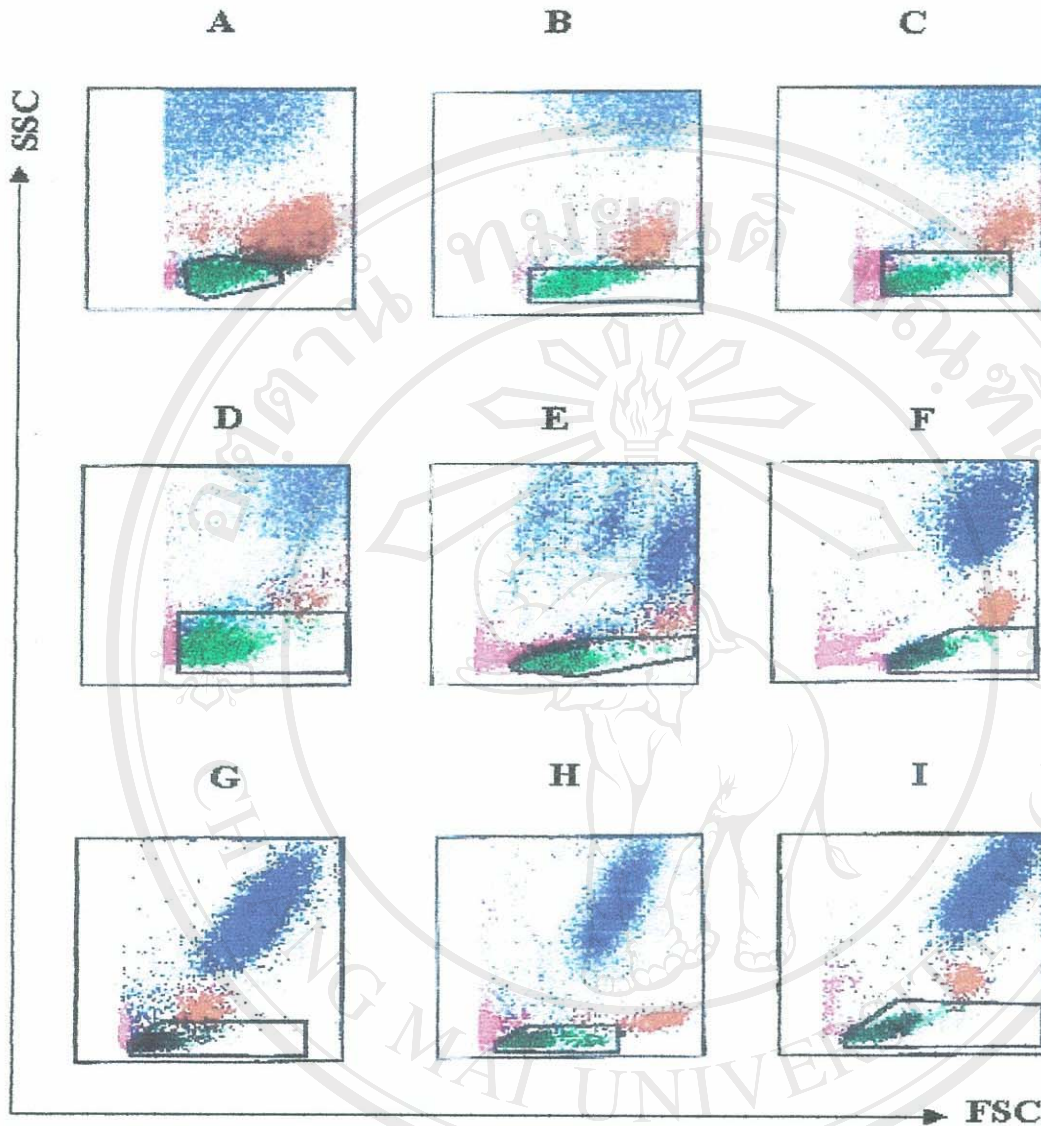

Figure 3.15 Comparison of cellular distribution of blood samples which red blood cells were lysed by nine lysing solutions.

A= Sterile distilled water
B= 1%ammonium oxalate
C= 0.83%ammonium chloride
D= ammonium chloride tris buffer
E= Hypotonic ammonium chloride
F= OptiLyse® B
G= 3%formaldehyde-NH_4Cl
H= 3%formaldehyde-NH_4Cl-PBS
I= FACS™

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Table 3.10 Percentage of CD4, CD8 and CD3 lymphocytes determined by FACS™ and 3%formaldehyde-NH_4Cl lysing solutions from 20 healthy persons.

Donor no.	FACS™ lysing solution			3%formaldehyde-NH_4Cl solution		
	%CD4	%CD8	%CD3	%CD4	%CD8	%CD3
N1	37	27	68	35	28	66
N2	30	11	45	32	11	47
N3	38	31	75	39	32	76
N4	38	29	69	36	29	69
N5	29	36	74	31	37	74
N6	42	34	78	42	26	77
N7	37	31	75	33	25	67
N8	31	30	68	32	26	65
N9	39	23	66	40	23	65
N10	45	23	71	44	22	69
N11	38	27	71	35	27	70
N12	38	26	65	38	26	64
N13	40	26	67	39	23	62
N14	27	29	63	26	28	63
N15	45	22	69	47	21	74
N16	39	21	64	43	21	69
N17	40	43	87	41	43	86
N18	32	27	63	31	27	63
N19	40	28	73	41	28	75
N20	27	28	62	28	28	62

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Whole blood specimens were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl lysing solutions. The stained cells were analyzed by Simulset software.

Table 3.11 Absolute number of CD4, CD8 and CD3 lymphocytes (cells/μl) determined by FACS™ and 3%formaldehyde-NH_4Cl lysing solutions from 20 healthy persons.

Donor no.	FACS™ lysing solution			3%formaldehyde-NH_4Cl solution		
	CD4	CD8	CD3	CD4	CD8	CD3
N1	636	464	1169	602	482	1127
N2	1060	389	1572	1132	389	1661
N3	677	552	1327	695	570	1345
N4	1291	985	2344	1223	985	2327
N5	699	868	1772	748	892	1784
N6	1235	1000	2279	1235	746	2249
N7	932	781	1877	832	630	1676
N8	683	661	1487	705	573	1432
N9	710	419	1192	728	419	1174
N10	1103	564	1728	1078	539	1678
N11	765	544	1429	705	544	1399
N12	839	574	1424	839	574	1402
N13	1638	1065	2723	1597	942	2539
N14	492	529	1140	474	511	1149
N15	1539	752	2342	1607	718	2513
N16	614	315	1008	677	331	1079
N17	935	982	2021	958	1005	2010
N18	661	599	1291	640	558	1291
N19	1156	809	2095	1185	809	2153
N20	437	437	996	454	454	1005

Whole blood specimens were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl lysing solutions. The stained cells were analyzed by Simulset software.

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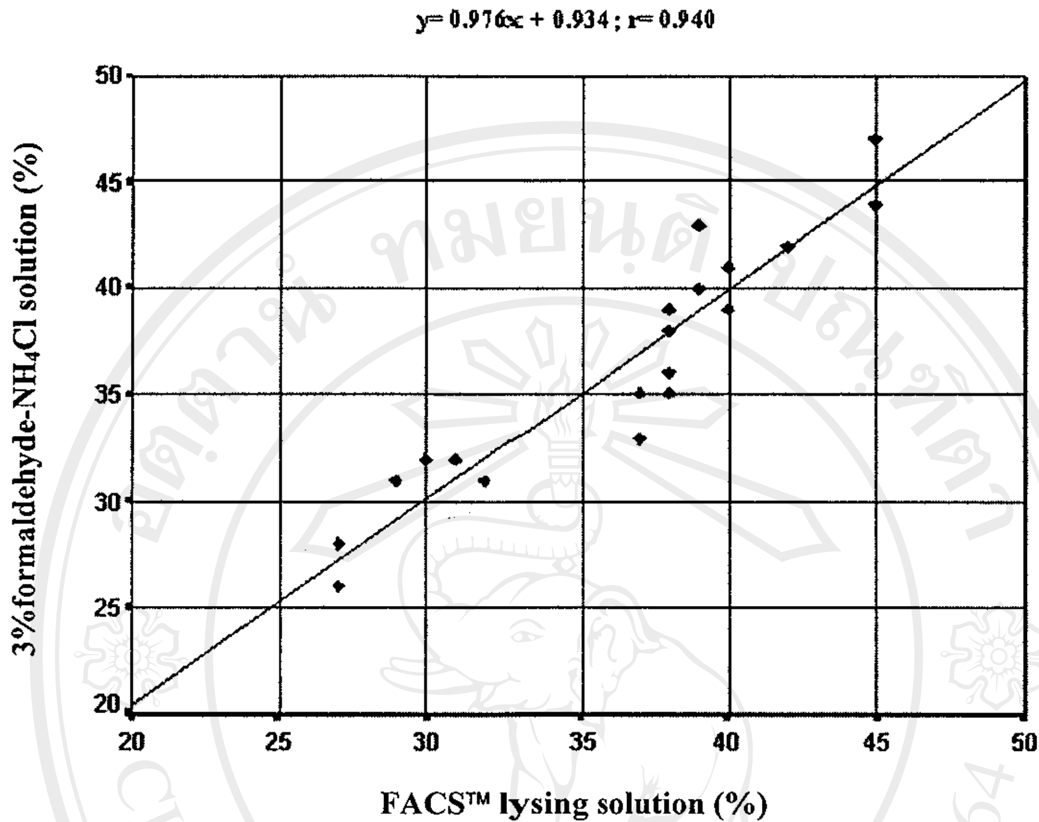

Figure 3.16 Correlation plot of percentage of CD4 lymphocytes from 20 blood samples. Blood samples were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl lysing solutions.

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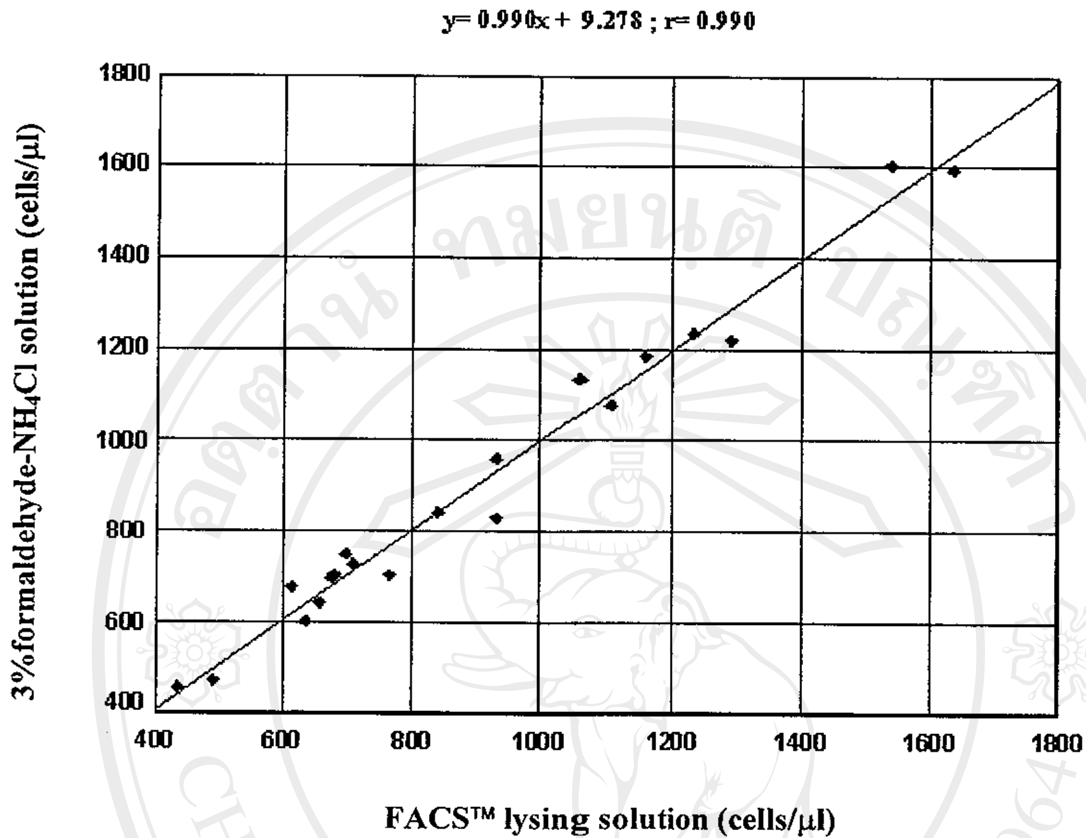

Figure 3.17 Correlation plot of absolute number of CD4 lymphocytes from 20 blood samples. Blood samples were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl lysing solutions.

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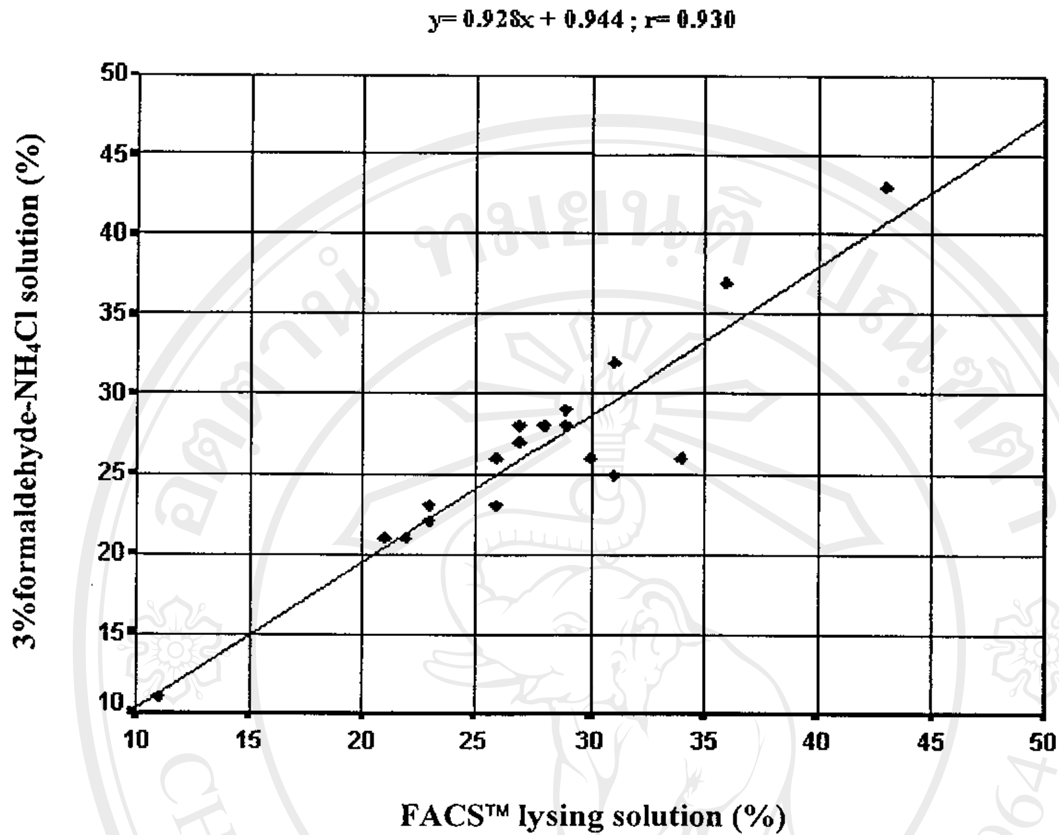

Figure 3.18 Correlation plot of percentage of CD8 lymphocytes from 20 blood samples. Blood samples were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl lysing solutions.

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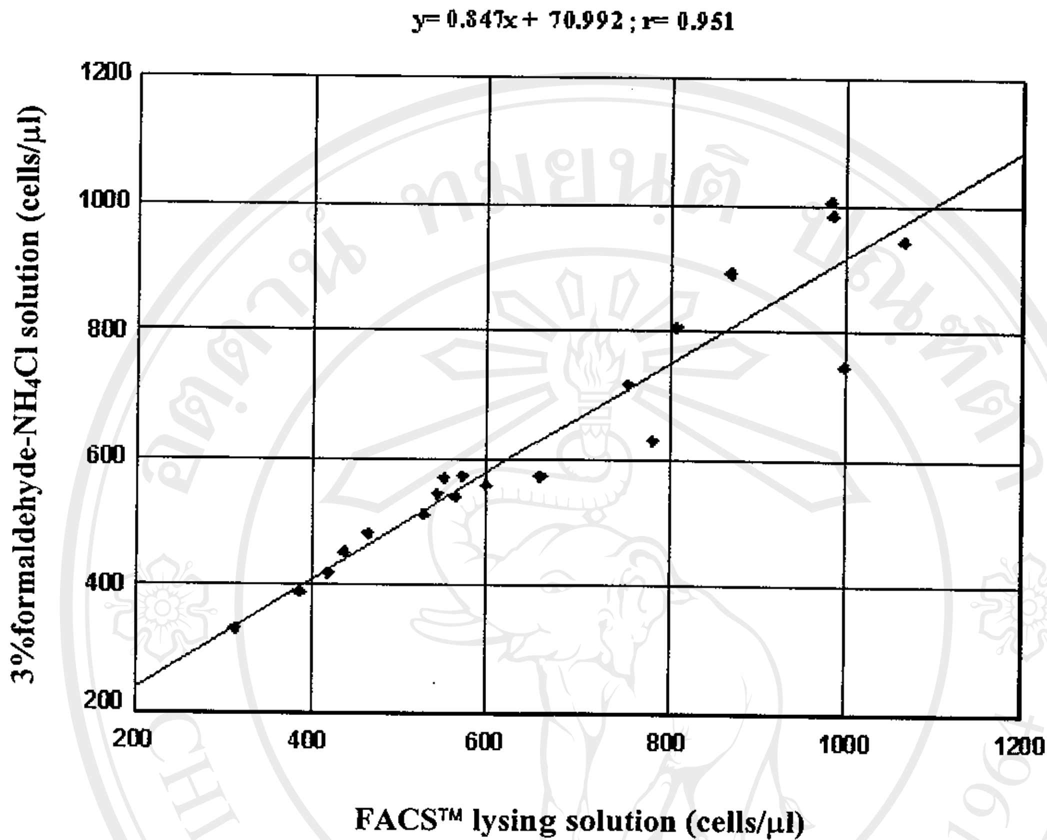

Figure 3.19 Correlation plot of absolute number of CD8 lymphocytes from 20 blood samples. Blood samples were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl lysing solutions.

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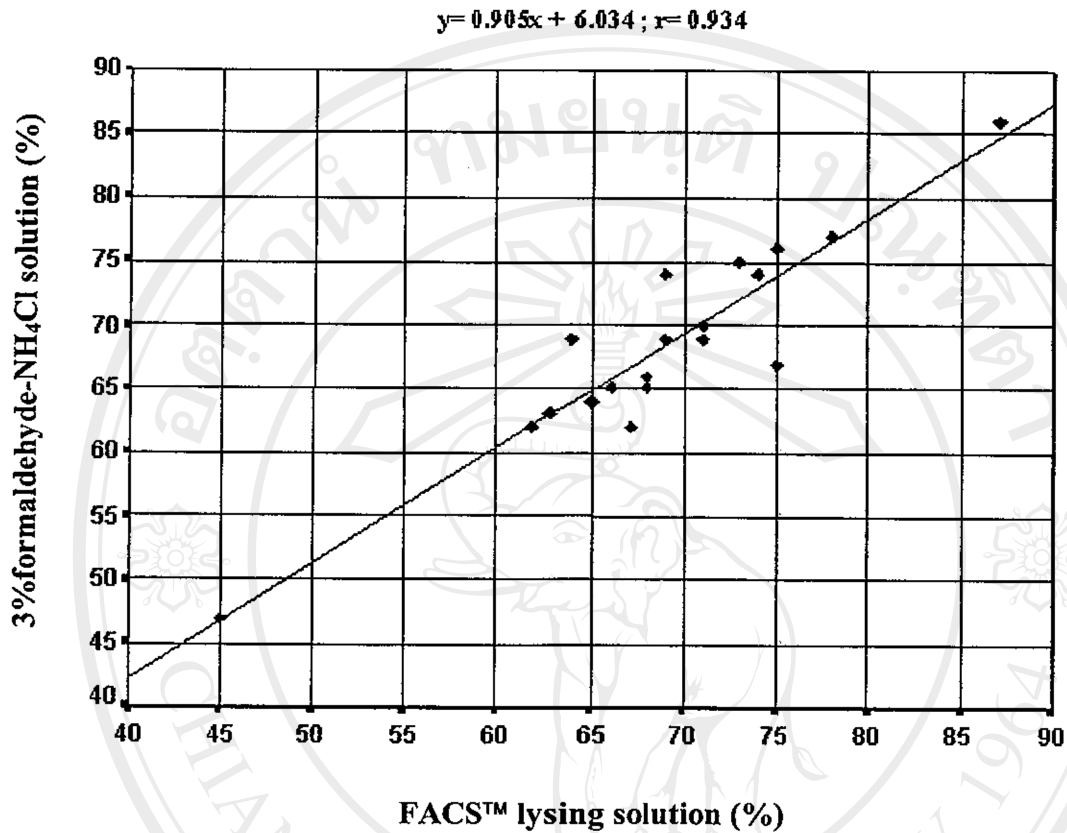

Figure 3.20 Correlation plot of percentage of CD3 lymphocytes from 20 blood samples. Blood samples were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl lysing solutions.

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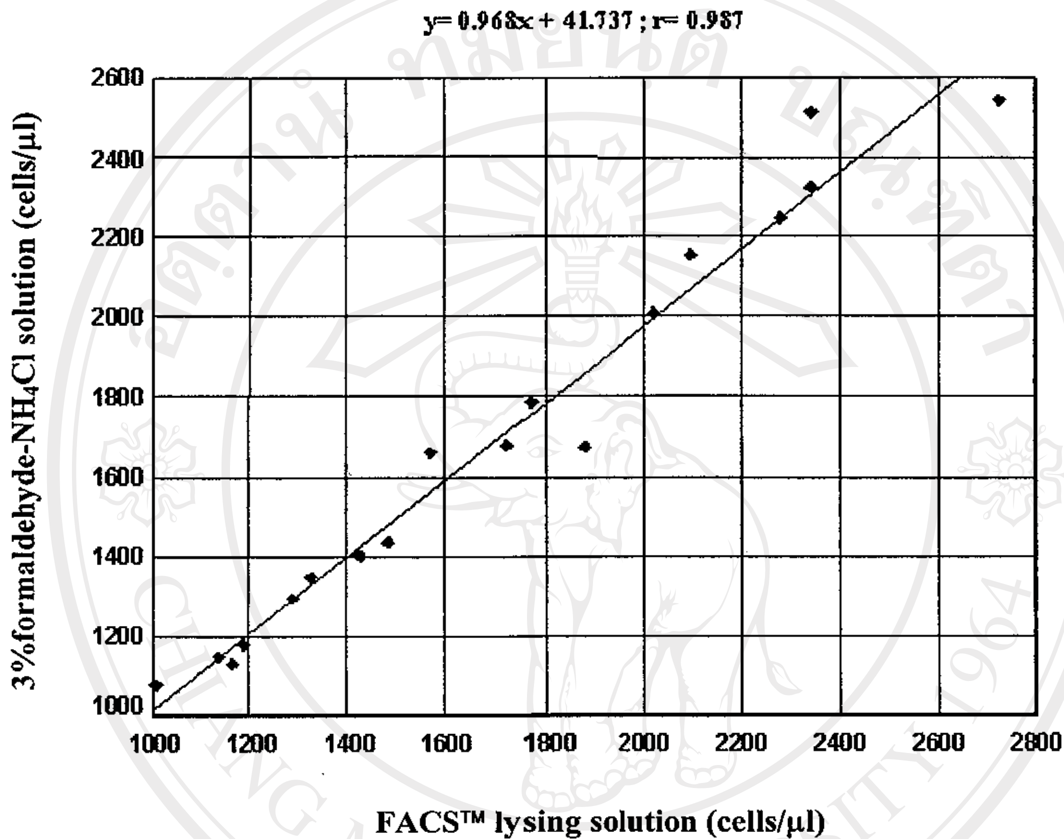

Figure 3.21 Correlation plot of absolute number of CD3 lymphocytes from 20 blood samples. Blood samples were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl lysing solutions.

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3.6.2 Evaluation of 3%formaldehyde-NH_4Cl-PBS lysing solution

Lymphocyte subset as CD4, CD8 and CD8 lymphocytes of 10 healthy donors and 10 HIV infected persons were determined using 3%formaldehyde-NH_4Cl-PBS lysing solution. Percentage and absolute number of CD4, CD8 and CD3 lymphocytes were compared with the results obtained from standard Simultest™ reagent kit. As shown in Table 3.12 and 3.13, both percentage and absolute number of CD4, CD8 and CD3 lymphocytes obtained from the prepared and commercial lysing solutions were similar with no statistically significant difference. A correlation plots comparing the percentage and absolute number of CD4, CD8 and CD3 lymphocytes obtained from both solutions were shown in Figure 3.22-3.27 respectively. Linear regression analysis resulted in a slope of 1.016, 1.051 and 0.998, an intercept of –0.479, -2.504 and 0.154 when percentage of CD4, CD8 and CD3 lymphocytes from the two lysing solution were compared respectively. The correlation coefficients were 0.995, 0.981 and 0.983 (Figure 3.22, 3.24 and 3.26). Linear regression analysis resulted in a slope of 1.010, 1.027 and 1.006, an intercept of –3.872, -32.524 and –7.026 when absolute number of CD4, CD8 and CD3 lymphocytes from the two lysing solution were compared respectively. The correlation coefficients were 0.997, 0.985 and 0.998 (Figure 3.23, 3.25 and 3.27). These results indicated that the home made 3%formaldehyde-NH_4Cl-PBS lysing solution can be used to enumerate CD4, CD8 and CD3 lymphocytes in blood samples equivalent to those given by the commercial FACS™ lysing solution.

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Table 3.12 Percentage of CD4, CD8 and CD3 lymphocytes determined by FACS™ and 3% formaldehyde-NH_4Cl-PBS lysing solutions from 10 healthy donors and 10 HIV infected persons.

Donor no.	FACS™ lysing solution			3%formaldehyde-NH_4Cl-PBS		
	%CD4	%CD8	%CD3	%CD4	%CD8	%CD3
H1	2	59	61	2	59	63
H2	11	47	63	11	47	63
H3	4	42	47	4	45	49
H4	26	55	89	25	57	91
H5	2	55	71	1	53	70
H6	11	45	61	8	42	59
H7	1	41	49	1	44	53
H8	5	29	34	3	27	32
H9	18	39	63	20	34	59
H10	15	52	69	17	56	73
N1	42	34	78	42	29	76
N2	31	30	68	30	25	66
N3	39	23	66	40	23	65
N4	45	23	71	45	22	70
N5	38	27	71	34	27	70
N6	38	26	65	38	26	65
N7	40	26	67	40	25	66
N8	27	29	63	27	27	65
N9	27	27	62	29	27	61
N10	42	23	72	45	24	74

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Whole blood specimens were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl-PBS lysing solutions. The stained cells were analyzed by Simulset software.

Table 3.13 Absolute number of CD4, CD8 and CD3 lymphocytes (cells/μl) determined by FACS™ and 3%formaldehyde-NH_4Cl-PBS lysing solutions from 10 healthy donors and 10 HIV infected persons.

Donor no.	FACS™ lysing solution			3%formaldehyde-NH_4Cl-PBS		
	CD4	CD8	CD3	CD4	CD8	CD3
H1	18	531	549	18	531	562
H2	218	931	1238	218	931	1237
H3	53	557	623	53	596	642
H4	674	1426	2294	648	1477	2345
H5	15	399	512	7	385	508
H6	133	543	736	96	507	705
H7	11	436	521	11	468	558
H8	48	281	329	29	262	310
H9	376	815	1306	418	711	1223
H10	400	1386	1826	453	1493	1946
N1	1235	1000	2279	1235	853	2234
N2	683	661	1487	661	551	1443
N3	710	419	1192	728	419	1183
N4	1103	564	1728	1103	539	1703
N5	765	544	1429	684	544	1399
N6	839	574	1424	839	574	1424
N7	1638	1065	2723	1638	1024	2703
N8	492	529	1140	492	492	1176
N9	437	437	996	470	437	988
N10	1200	657	2042	1285	685	2113

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Whole blood specimens were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl-PBS lysing solutions. The stained cells were analyzed by Simulset software.

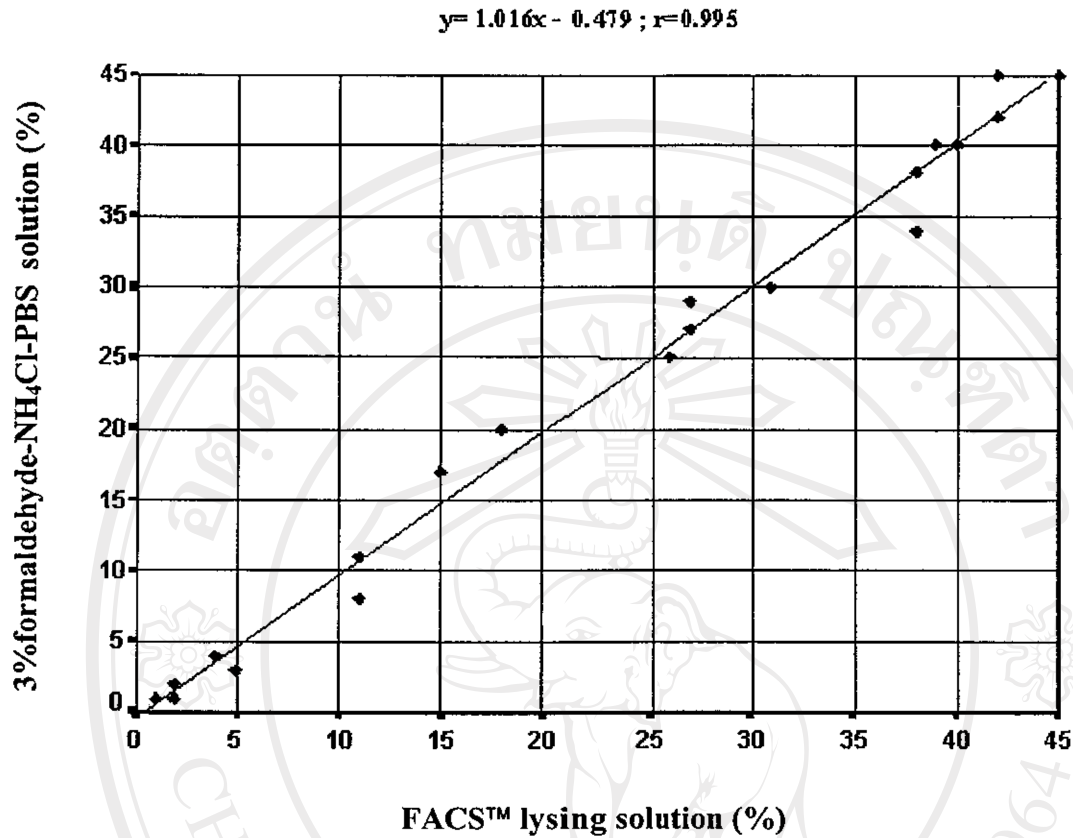

Figure 3.22 Correlation plot of percentage of CD4 lymphocytes from 20 blood samples. Blood samples were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl-PBS lysing solutions.

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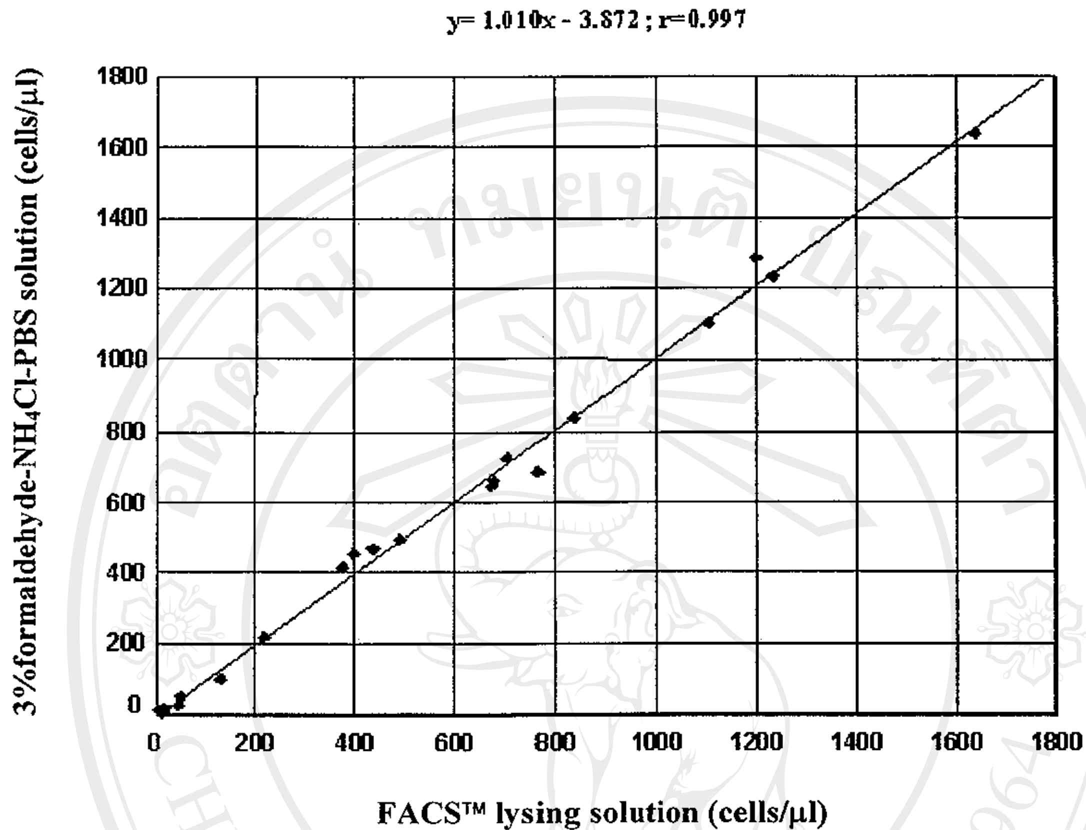

Figure 3.23 Correlation plot of absolute number of CD4 lymphocytes from 20 blood samples. Blood samples were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl-PBS lysing solutions.

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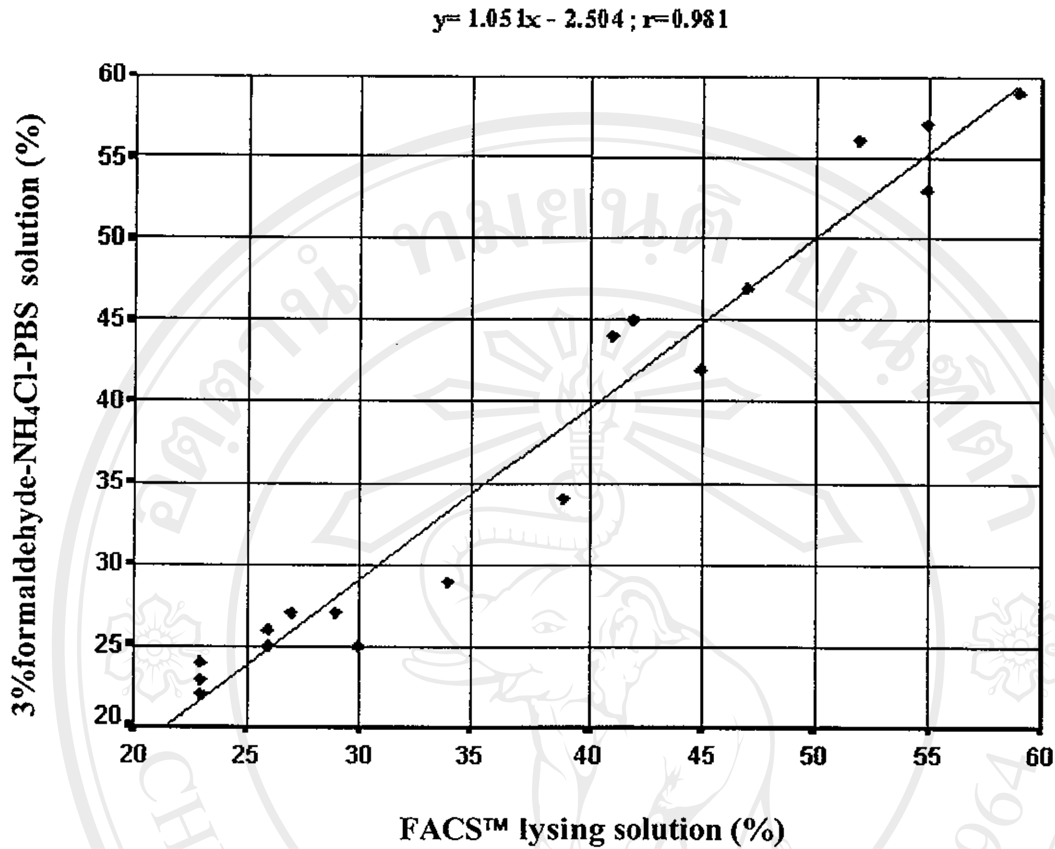

Figure 3.24 Correlation plot of percentage of CD8 lymphocytes from 20 blood samples. Blood samples were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl-PBS lysing solutions.

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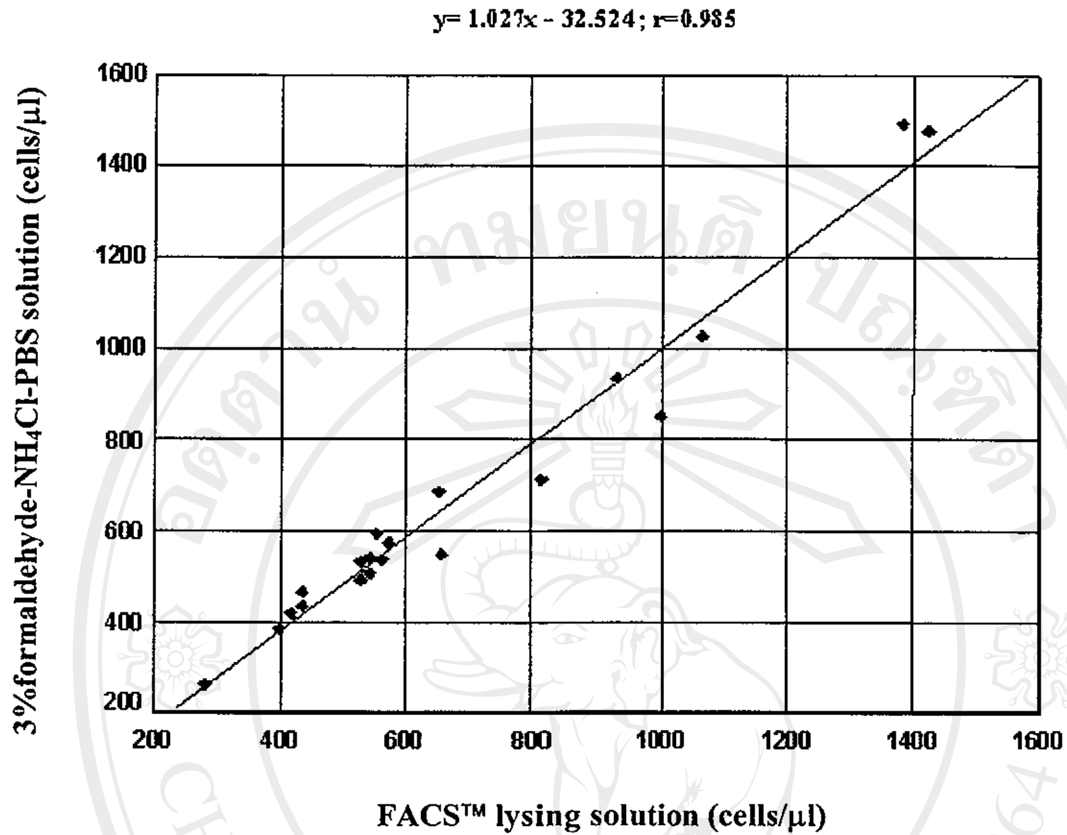

Figure 3.25 Correlation plot of absolute number of CD8 lymphocytes from 20 blood samples. Blood samples were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl-PBS lysing solutions.

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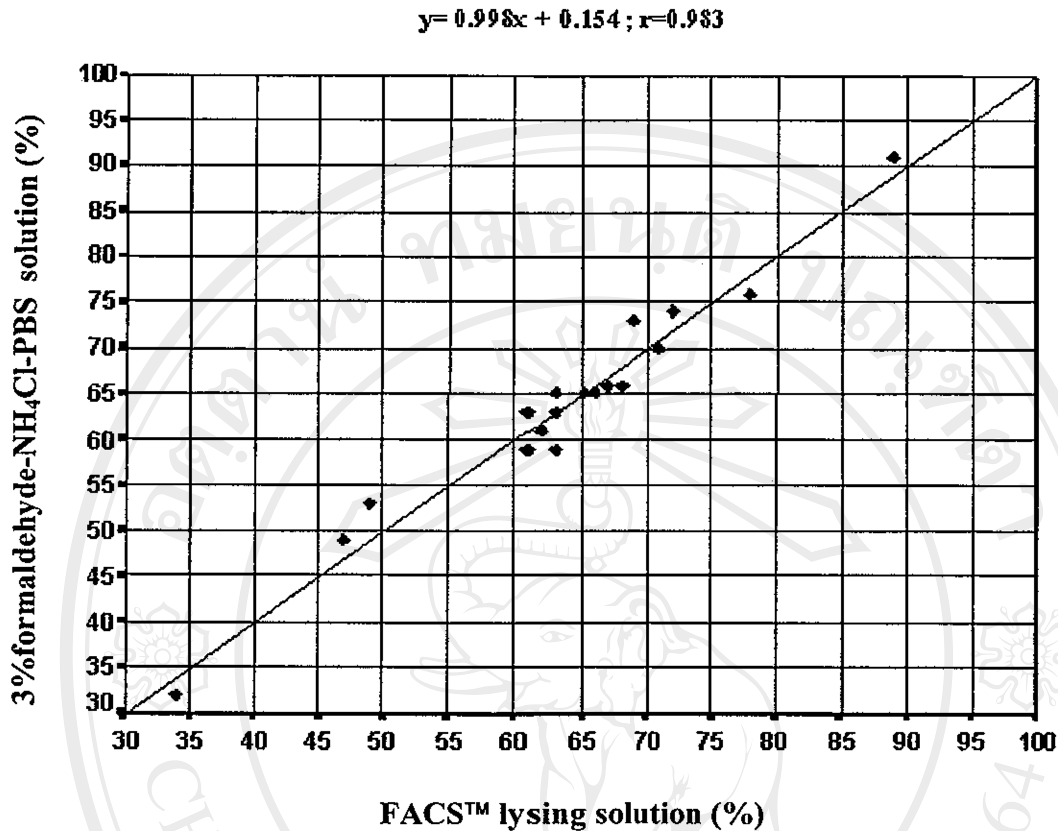

Figure 3.26 Correlation plot of percentage of CD3 lymphocytes from 20 blood samples. Blood samples were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl-PBS lysing solutions.

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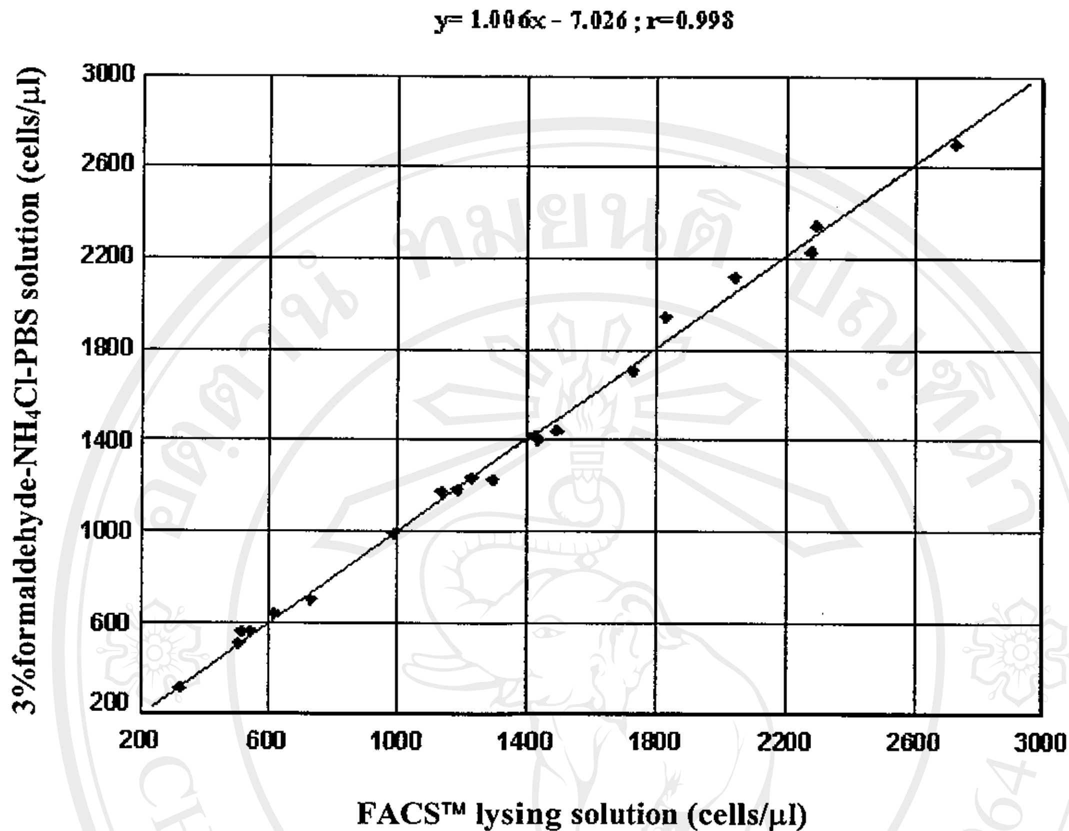

Figure 3.27 Correlation plot of absolute number of CD3 lymphocytes from 20 blood samples. Blood samples were stained two sets with Simultest™ reagent kit then lysed with FACS™ and 3%formaldehyde-NH_4Cl-PBS lysing solutions.

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