CHAPTER III

RESULTS

3.1 Production and purification of anti-CD4 mAb clone MT4

A Balb/C mouse was intraperitoneally immunized with hybridoma clone MT4 for large scale production of anti-CD4 mAb. Eight ml of acitic fluid was obtained from the immunized mouse. MT4 mAb was purified from the induced ascitic fluid by affinity chromatography using HiTrap column. After purification process, the concentration of purified mAb was determined by measuring at the absorbance of 280 nm and protein concentration was calculated in accordance with 1 mg/ml solution of IgM has extinction coefficient as 1.18. In this study, approximately of 3 mg of MT4 mAb were obtained from 1.2 ml of ascitic fluid. Therefore, total approximately 24 mg of purified MT4 mAb could be produced.

3.2 Determination the activity and specificity of purified MT4 mAb

3.2.1 Determination of purity of the purified MT4 mAb

To verify the purity of the purified MT4 mAb obtained from affinity chromatography. Three fractions of purified proteins obtained from the affinity column were determined by SDS-PAGE under 12.5% gel. As shown in figure 3.1-3, under reducing condition, two protein bands representative of heavy chain and light chain were emerged. No other protein could be observed. Under non-reducing condition, no protein band could be observed. These results indicated that the purity of purified MT4 mAb was qualified for further study (Fig. 3.1-3).

3.2.2 Determination of specificity of the purified MT4 mAb

Indirect immunofluorescence staining and flow cytometry were used to determine the activity and specificity of the purified MT4 mAb. In this study, to examine the activity of the purified MT4 mAb, PBMC were incubated with two concentrations of the purified MT4 mAb, 20 μg/ml and 50 μg/ml. In addition, to inspect the specificity, a human T cell line Sup T1 of which expressing CD4 molecules on their surfaces was incubated with MT4 mAb at concentration of 20 μg/ml. The anti mouse immunoglobulin-FITC was used as conjugate. The MT4 mAb stained PBMC and Sup T1 cell line were analyzed by a FACScalibur

As shown in figure 3.1-1 and 3.1-2, PBMC and Sup T1 cells were positive with the purified MT4 mAb. The positive reactivity of lymphocyte sub-population and Sup T1 cells illustrated at FACS profiles indicated the reactivity and the specificity of purified MT4 mAb in binding of CD4 molecules expressed on the surface of helper T lymphocyte sub-population and Sup T1 cells.

3.3 Preliminary study: Preparation of MT4 bead reagent and development of method for enumeration of CD4+ T lymphocytes.

3.3.1 Preliminary study on MT4 bead reagent preparation

M450 Epoxy magnetic beads were coated with purified MT4 mAb as was described in materials and methods. Immunofluorescent staining was applied for proving the binding of MT4 mAb on the bead surface. Detection of fluorescent intensity was examined under both fluorescent microscopy and flow cytometer. By fluorescent microscope, under visible light field, brown circle of beads were observed (Fig. 3.2-1A). By scrutinizing under the fluorescent light, all uncoated bead had no fluorescence (Fig. 3.2-1B), while all MT4 mAb coated beads had homogenous positive green fluorescence (Fig. 3.2-1C). The results demonstrated the success of coating beads with MT4 mAb.

To confirm the observed results, the stained beads were analyzed by flow cytometer. As predicated, the uncoated beads were not fluorescence, whereas MT4 mAb coated beads were strongly fluorescence.(Fig. 3.2-2). The results indicated that all beads were coated with MT4 mAb. The coated beads, therefore, were capable to be used for further development of method for enumeration of CD4+ lymphocytes.

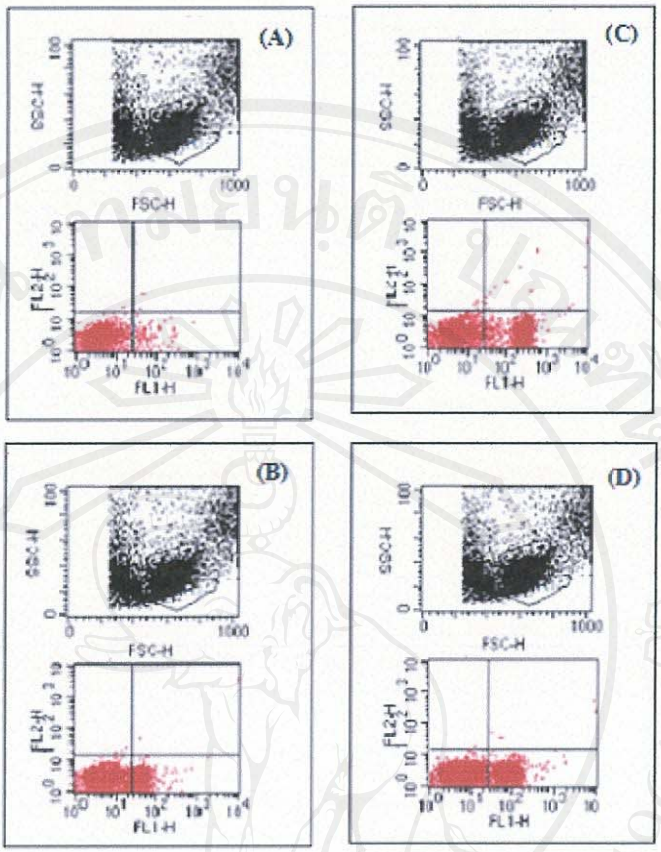


Figure 3.1-1: Flow cytometric analysis of the activity of purified MT4 mAb using PBMC. PBMC were stained with various concentrations of MT4 mAb by indirect immunofluorescent technique analyzed by flow cytometer. Lymphocytes were gated according to their size and granularity (upper panels) and the fluorescent intensities were determined (lower panels). (A) Conjugate background control, (B) Purified MT4 mAb 20 μg/ml, (C) Purified MT4 mAb 50 μg/ml; and (D) Positive control

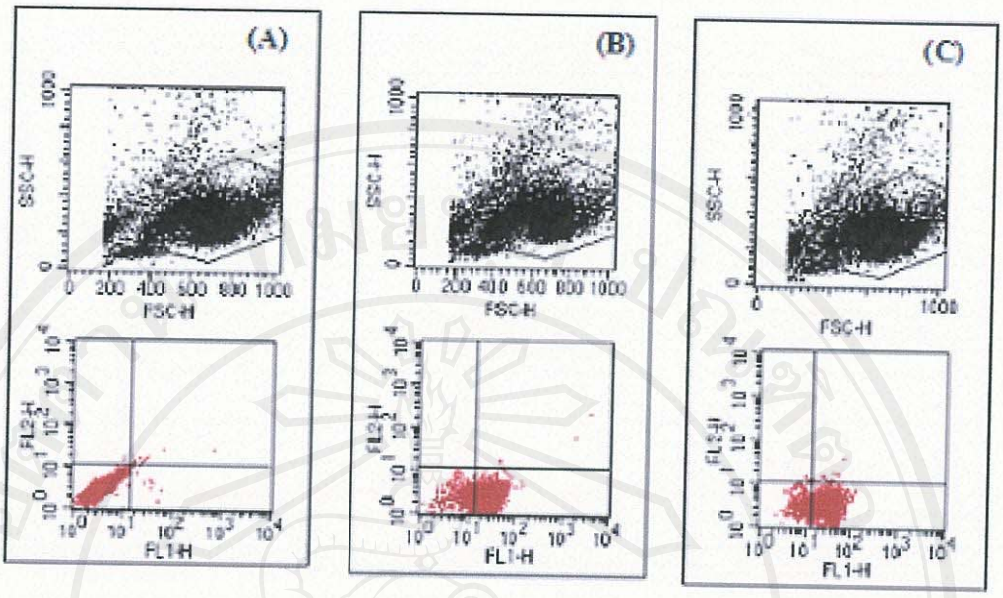
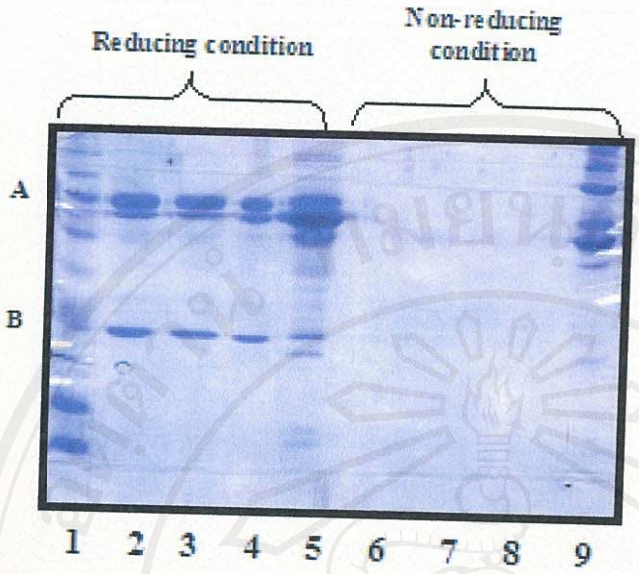


Figure 3.1-2: Flow cytometric analysis of the activity of purified MT4 mAb using Sup T1 T cell line. Sup T1 cell line was incubated with MT4 mAb 20 μg/ml by indirect immunofluorescence. The stained cells were analyzed by flow cytometer. (A) Conjugate background control (B) Positive control and (C)Purified MT4 mAb.



Lane 1: Standard protein markers
Lane 2-4: Fraction 1, 2, 3
Lane 5: Unbound proteins
Lane 6-8: Fraction 1, 2, 3
Lane 9: Unbound proteins

A: MW ~ 50 kDa (heavy chain)
B: MW ~ 25 kDa (light chain)

Figure 3.1-3: SDS-PAGE analysis of purified MT4 mAb. Electrophoresis was performed under reducing and non-reducing conditions. Molecular mass of heavy chain and light chain at 50 kDa and 25 kDa were indicated at A and B, respectively.

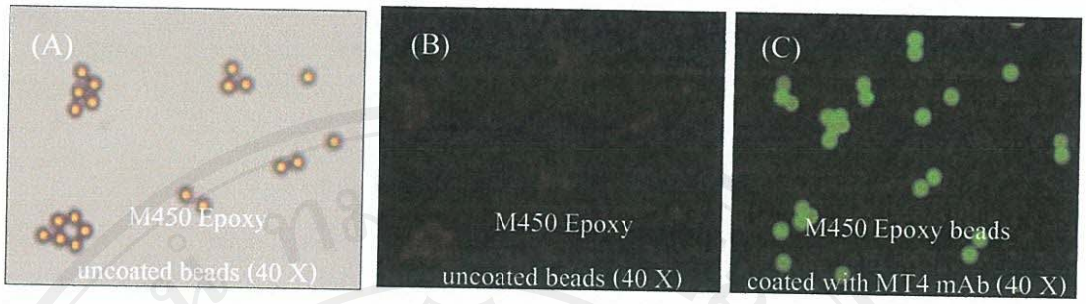


Figure 3.2-1: Determination of MT4 mAb coated beads by immunofluorescent staining using fluorescent microscope. (A) Observation under visible light, (B) Uncoated beads were observed under fluorescent light and (C) MT4 mAb coated beads were observed under fluorescent light.

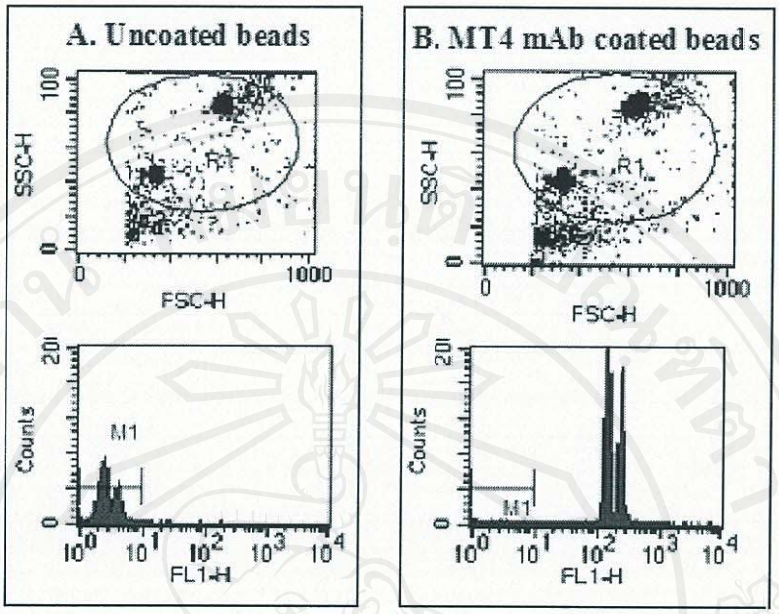


Figure 3.2-2: Determination of MT4 mAb coated beads by immunofluorescent staining using flow cytometric analysis. Beads were gated according to their size and granularity (upper panels). Fluorescent intensities (FL1) were shown in lower panels. (A) Uncoated beads, (B) MT4 mAb coated beads.

3.3.2 Preliminary study: Development of method for enumeration of CD4+ T lymphocytes in whole blood by non-flow cytometric method

To develop method for enumeration of CD4+ T lymphocytes (MT4 method) by using hematology analyzer, volume of blood and number of MT4 coated beads were firstly optimized. In this study, various volumes of blood were incubated with the various number of MT4 beads (test tube). Control tube was done simultaneously by mixing blood with the same volume of PBS. After that, both tubes were incubated for cell-bead binding with a constant rotation. Then, the test tube was placed on magnetic particle concentrator (MPC) for 3 minutes, and un-attachment cells were removed to a new tube leaving attached cells-beads-magnet undisturbed. In control tube, blood was transferred to a new tube. Both tubes were subjected for complete blood count using COULTERR-MAXM hematology analyzer. The expected CD4+ T lymphocytes were obtained by subtracting the absolute lymphocyte number from control tube with the test tube. The results were shown in table 3.1. When comparing the CD4+ T lymphocytes obtained by MT4 method and standard flow cytometry, at the optimal condition, the CD4 counts obtained from both methods were comparable (Table 3.1).

From these preliminary results, indicating the determination of CD4+ T lymphocytes in whole blood by using MT4 coated beads and hematology blood analyzer was possible.

3.3.3 Optimization amount of MT4 mAb for coating beads

After demonstrating that the MT4 method could be used for enumeration of CD4+ T lymphocytes, we then studied the optimal amount of MT4 mAb for coating beads. M450 Epoxy beads were coated with various amount of purified MT4 mAb. The bindings of anti-CD4 mAb on bead surface were verified by immunofluorescent staining. Fluorescent intensity was determined under flow cytometer. It was found that mean fluorescence intensities were varied from 17.42 to 98.65, respectively (Fig. 3.3).

The results demonstrated that the optimal amount of MT4 mAb coated bead should be at 10 and 20 $\mu g/10^7$ beads which given the higher fluorescent intensities compared to those lower amount of mAb coated beads. However, to ensure the capability of those various MT4 beads could be used to enumerate CD4+ T lymphocyte, CD4+ T lymphocytes in seven subjects were determined by MT4 method compared to flow cytometry (Table 3.2). It was found that at MT4 mAb 20 μg per 10^7 beads given the best comparable results. Therefore, coating beads using MT4 mAb 20 μg per 10^7 beads was selected for further study.

Table 3.1: Optimization of conditions for determination CD4+ T lymphocytes by a non-flow cytometric method (MT4 method)

Tube condition	100 μl of MT4 beads concentration	WB (μl)	PBS (μl)	Final volume (μl)	Final dilution	MT4 method CD4 cells (cells/μl)	Flow cytometric method (cells/μl)
1	$4x10^6$	100	300	500	1:5	500	
	$4x10^6$	100	800	1000	1:10	1000	880
2	$4x10^6$	200	100	400	1:2	600	
	$4x10^6$	100	300	500	1:5	500	
	$4x10^6$	100	800	1000	1:10	-	676
	$4x10^6$	100	1800	2000	1:20	2000	
3	$4x10^6$	200	100	400	1:2	700	831
4	10^7	200	100	400	1:2	1200	
	10^7	200	100	400	1:2	1000	1263
5	10^7	200	100	400	1:2	700	746
	10^7	200	100	400	1:2	600	483
6	10^7	200	100	400	1:2	550	592

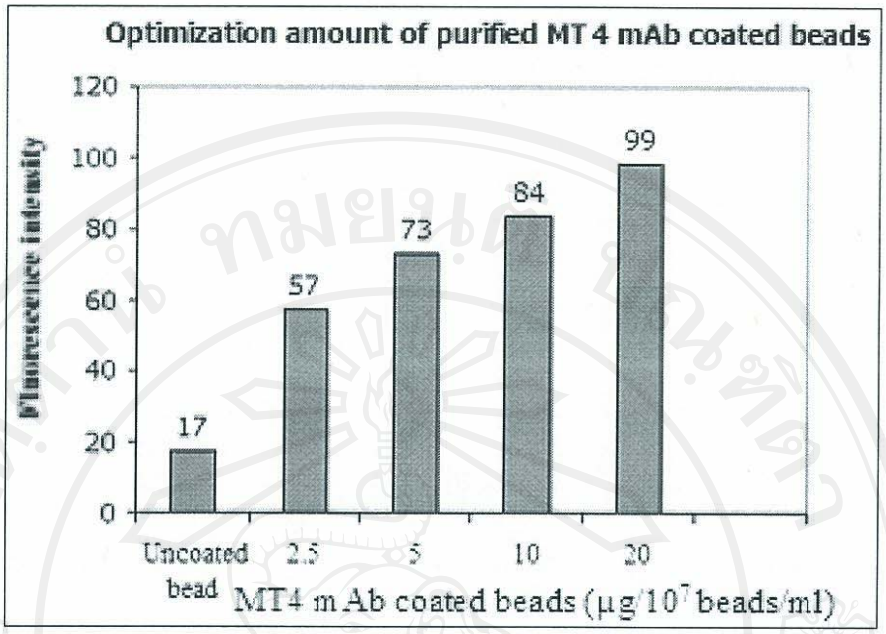


Figure 3.3: Mean fluorescence intensities from flow cytometry analysis of MT4 beads coated with different amount of MT4 mAb.

Table 3.2: Enumeration of CD4+ T lymphocyte by MT4 method comparing to flow cytometry

Subject	CD4 cells from MT4 method (cells/μl) Amount of MT4 mAb coated 10^7 beads				FCM CD4 cells (cells/μl)
	2.5 μg	5 μg	10 μg	20 μg	
1	200	400	600	600	670
2	-	0	-200	0	1
3	-	0	0	0	38
4	-	-	400	400	423
5	-	-	400	600	547
6	-	-	600	600	590

3.3.4 Optimization of incubation time for CD4+ T lymphocyte depletion

To optimize the suitable period for cell-beads binding, the incubation time between MT4 beads and blood at 5, 10, 20 and 30 minutes were investigated. Briefly, the MT4 beads was mixed with blood sample at various incubation times. Then, bead bound cells were depleted using magnetic particle concentrator. To inspect the depletion efficacy of CD4+ T lymphocytes, the remained non-adhered cells were determined for CD4+ T lymphocyte number by indirect immunofluorescence and flow cytometer. It was found that 30 minutes was the best incubating time for CD4+ T lymphocyte depletion (Fig. 3.4-1, Table 3.3 and Fig. 3.4-2). All this incubation time, 90% of CD4+ T lymphocytes were depleted.

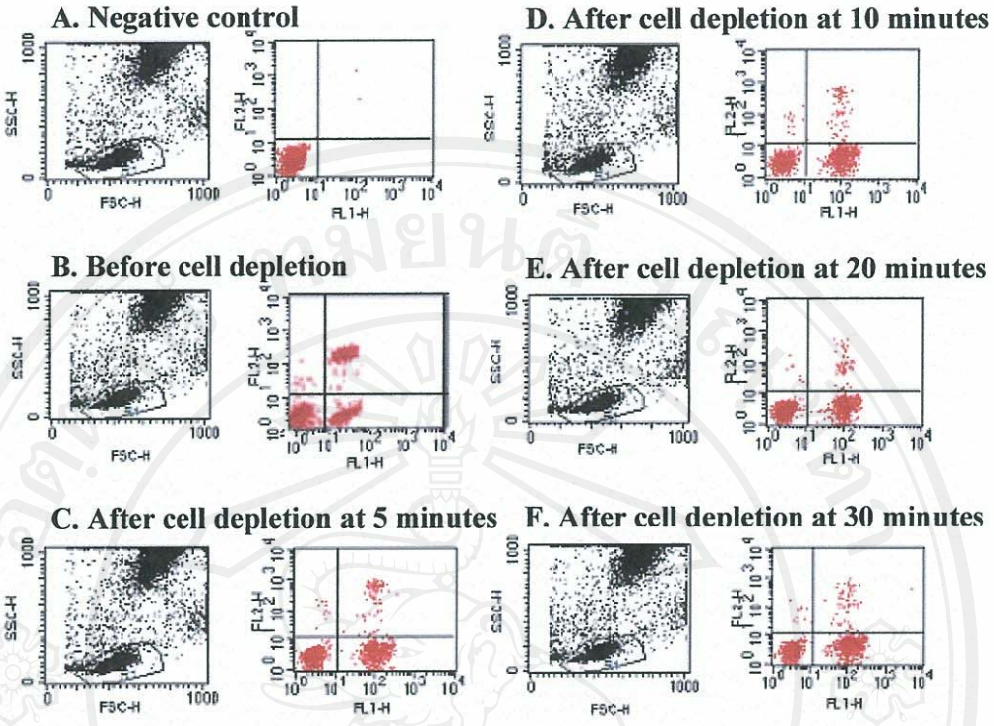


Figure 3.4-1: The flow cytometric analysis of the depletion efficacy of CD4+ T lymphocytes using MT4 beads. CD4+ T lymphocytes were depleted using MT4 beads. The remaining cells were stained with CD3-FITC/CD4-PE. (A) negative control, (B) Before cell depletion, (C, D, E, and F) After cell depletion at 5, 10, 20 and 30 minutes incubation times, respectively.

Table 3.3: CD4+ T lymphocyte depletion efficacy by MT4 beads method at different incubation times.

Sample	% CD4 before	% CD4 remained after depletion			% Depletion efficacy		
	cell depletion	5 min	15 min	30 min	5 min	15 min	30 min
1	7.78	4.3	2	1.25	44.73	74.73	83.93
2	27.06	6.12	4.42	3.37	77.38	83.67	87.55
3	32.79	-	3.63	4.14	-	88.93	87.37
4	10.73	-	1.39	0.91	-	87.05	91.52
5	24	-	-	1.91	-	-	92.04
6	12	-	-	0.67	-	-	94.42
7	19	-	-	1.47	-	-	92.26
8	10	-	-	0.78	-	-	92.2
9	37	-	-	2.27	-	-	93.86
10	7	-	-	1.33	-		81

Note: (-) not done

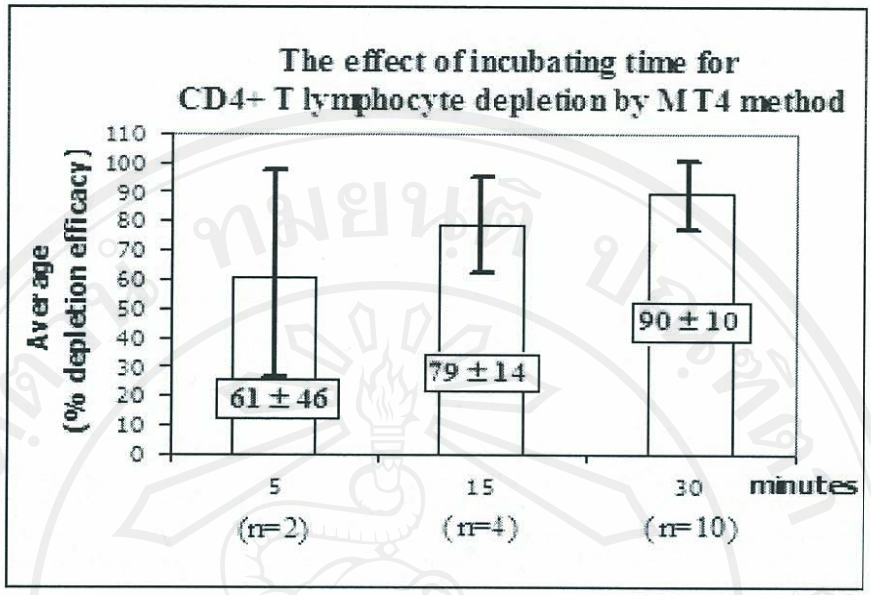


Figure 3.4-2: Depletion efficacy of CD4+ T lymphocyte by MT4 bead method at several of incubating times.

3.4 Validation of developed MT4 method by comparing with standard flow cytometry

To evaluate the developed reagent and method, CD4+ T lymphocytes of 100 blood samples, 32 normal and 68 HIV infected persons, were determined by the developed non-flow cytometric method (MT4 method) and compared to the standard flow cytometric method.

3.4.1 MT4 method for enumerating CD4+ T lymphocytes

From various optimizations, the method for CD4+ T lymphocytes count was fixed as follows: In test tube: 100 µl of MT4 bead at concentration of 10^7 beads/ml were mixed with 200 µl of whole blood, then 100 µl of 0.2% NaN_3 - PBS was added. In control tube: 200 µl of 0.2% NaN_3-PBS were mixed with 200 µl of blood. Then both tubes were incubated at room temperature with constant rotation for 30 minutes. After that, place the test tube on MPC for 3 minutes, then remove blood to a new tube leaving attached cells-beads-magnet binding undisturbed. In this step, CD4+ T lymphocytes were depleted. Meanwhile, transfer the diluted blood from control tube to a new tube. Both tubes, complete blood counts were analyzed using COULTER-MAXM hematology analyzer. The CD4+ T lymphocyte numbers were obtained by subtracting the absolute lymphocyte numbers from both tubes (non-CD4 depleted and CD4 depleted).

3.4.2 Flow cytometric method for enumerating CD4+ T lymphocytes

Same samples were simultaneous tested for CD4 count by flow cytometry. Whole blood was stained with Simultest reagents and then analyzed using Simulset software. Figure 3.5 represented the report form of flow cytometry analysis; Leukogate tube showed gating (R1) of lymphocyte population to reduce red blood cells debris, monocytes, and granulocytes (Fig. 3.5A), isotype control tube had nonspecific staining

(Fig. 3.5B). Lymphocyte sup-populations, CD4+ and CD8+ T lymphocytes, were determined at upper right quadrant (Fig. 3.5C and 3.5D).

3.4.3 Comparison of CD4+ T lymphocytes enumeration of the developed MT4 method with flow cytometric method

To validate the accuracy of the developed MT4 method, CD4+ T lymphocytes from 32 healthy donors and 68 HIV infected persons were determined by using the developed MT4 method and standard Simultest^{TM} reagent kit. It was found that the absolute number of CD4+ T lymphocytes obtained from both methods were very similar (Table 3.4-1).

To see discrepant results, the data were divided into three groups according to their absolute CD4+ T lymphocyte value as follow: less than 200, between 200-499 and greater than 500 cells/μl. The results found that only 1 in 39 (2.6%) discrepancy among CD4+ T lymphocyte number less than 200 cells/μl, 5 in 26 (19.2) when between 200-499 and 4 in 35 (2.7%) when CD4 number greater than 500 cells/μl. The results was indicating the few discrepancy of CD4 count in totally of 10 in 100 (10%) (Fig. 3.6-1).

3.4.3.1 Regression line analysis

The regression analysis between the two techniques was plotted and correlation coefficient (r) between the developed method and standard flow cytometry was 0.91 (Fig. 3.6-2). Linear regression analysis illustrated a slope of 0.9, and an intercept of 74. This finding indicated that both methods had good correlation.

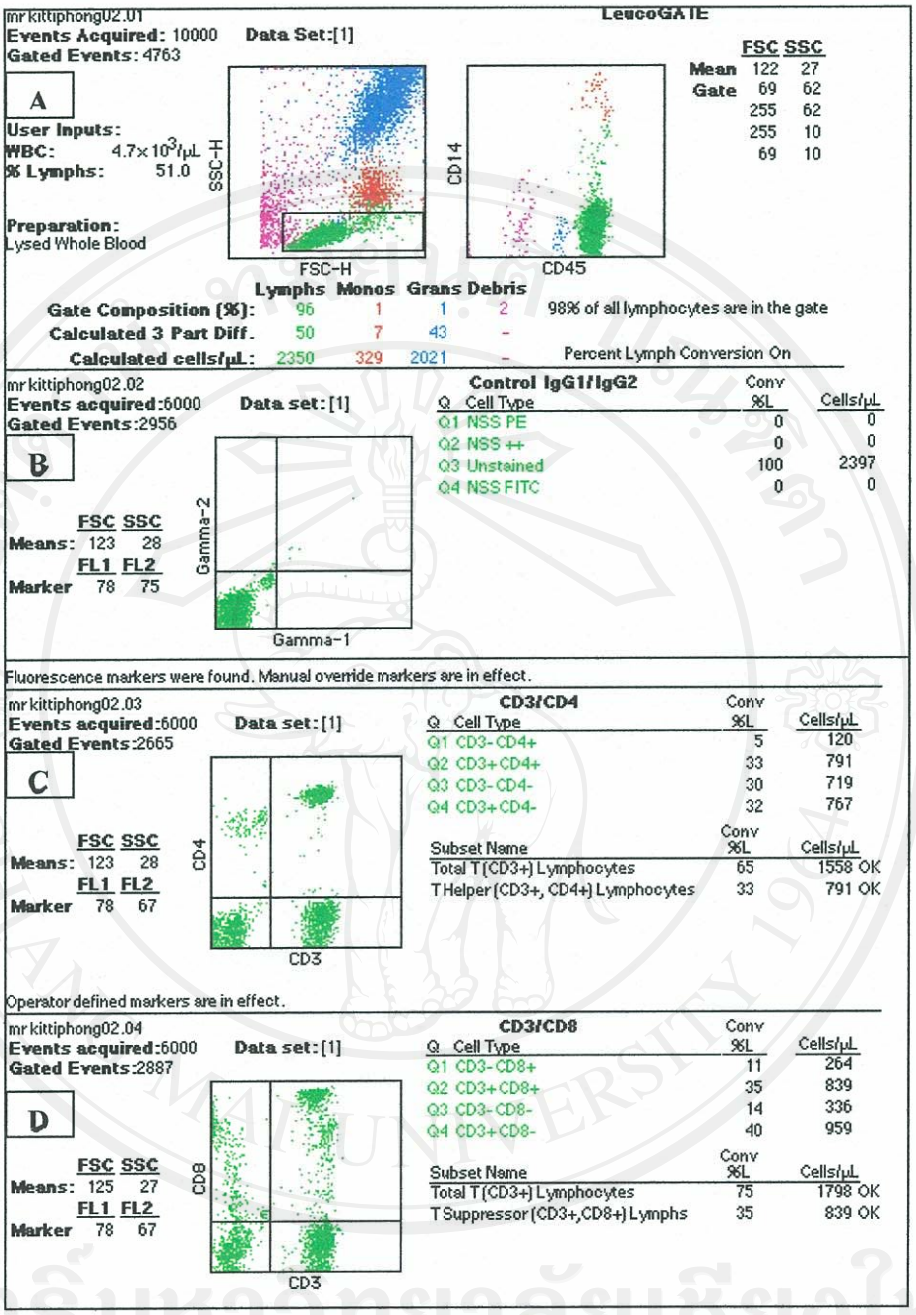


Figure 3.5: Flow cytometric analysis of whole blood stained by Simulset reagent. Simultest leukogate (A), Isotype control (B). The percentage of CD4+ T lymphocytes (C) and CD8+ T lymphocytes (D) were number of events in Q2 displayed in percent lymphocyte conversion automatically calculated with SimulSet software.

Table 3.4-1: Comparison of absolute CD4+ T lymphocyte numbers obtained from developed MT4 method and standard flow cytometric method

N:	MT4 method	FCM	N:	MT4 method	FCM	N:	MT4 method	FCM
1	610	788	35	538	747	69	0	56
2	879	959	36	936	956	70	43	69
3	336	410	37	1026	936	71	6	9
4	651	683	38	408	285	72	168	152
5	742	777	39	340	285	73	62	232
6	704	559	40	487	551	74	286	239
7	880	1036	41	1349	1127	75	1009	903
8	90	203	42	535	562	76	238	313
9	885	887	43	713	686	77	400	200
10	442	689	44	214	322	78	699	331
11	197	378	45	176	213	79	152	43
12	365	542	46	313	143	80	185	178
13	728	754	47	86	110	81	104	126
14	585	484	48	477	394	82	132	378
15	0	81	49	135	77	83	0	184
16	83	11	50	842	699	84	76	70
17	212	272	51	786	742	85	0	0
18	720	651	52	127	310	86	363	86
19	221	192	53	495	519	87	525	226
20	45	92	54	514	580	88	120	244
21	221	119	55	650	862	89	207	180
22	0	103	56	319	646	90	118	130
23	400	380	57	475	583	91	343	115
24	707	514	58	104	42	92	202	65
25	103	88	59	34	54	93	326	178
26	508	519	60	220	143	94	268	51
27	22	19	61	0	199	95	151	193
28	161	147	62	19	175	96	207	317
29	614	614	63	811	703	97	150	226
30	0	117	64	717	627	98	271	417
31	224	312	65	0	121	99	864	823
32	256	384	66	0	102	100	789	810
33	456	517	67	72	110			
34	584	837	68	151	211			

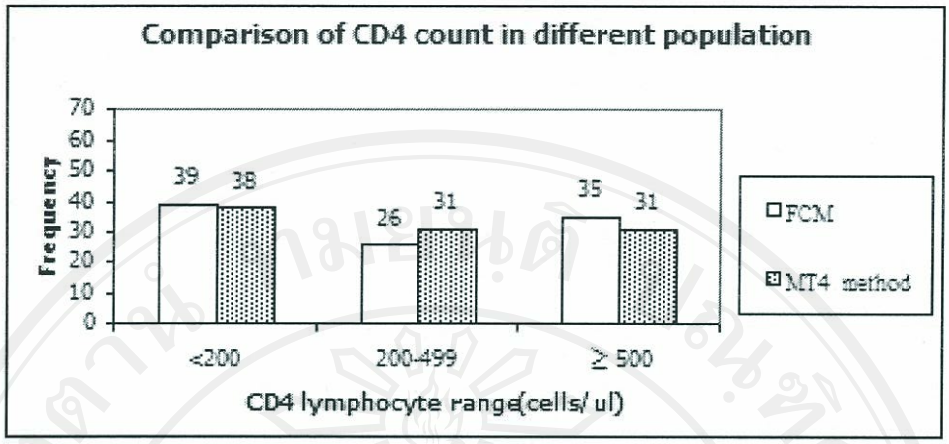


Figure 3.6-1: Frequency of the agreement of CD4 counts using MT4 method and flow cytometry. Discrepancy results of 1 in 39 (2.6%), 5 in 26 (19.2%) and 4 in 35 (11.4%) among different groups of CD4+ T lymphocyte number: less than 200, between 200-499 and greater than 500 cells/μl respectively

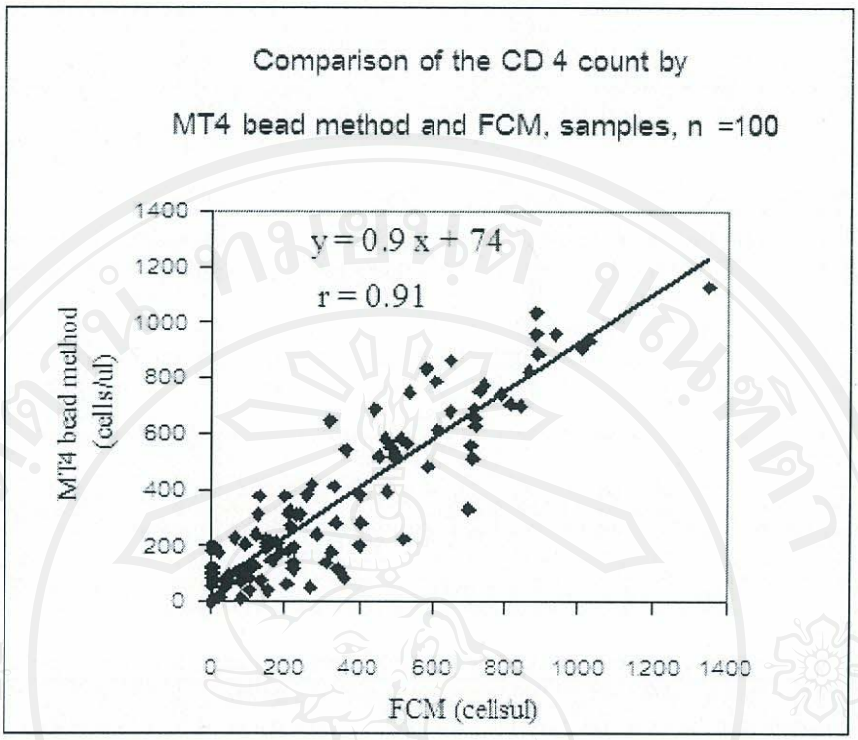


Figure 3.6-2: Regression line analysis of absolute CD4+ T lymphocyte counts between developed MT4 method and Flow cytometric method.

3.4.3.2 Testing proficiency of developed MT4 method

The number of CD4+ T cells from MT4 method were paralleling compared with results from flow cytometry. The agreement between the MT4 method and flow cytometry in classifying CD4+ T lymphocytes at the threshold of CD4 200 or 350 cells/μl was determined. The agreement of 80 in 100 (80%) when classifying threshold of 200 cells/μl and 94 in 100 (94%) at the threshold of 350 cells/μl were obtained (Table 3.4-2).

The agreement number was used to evaluate the testing proficiency by calculating the sensitivity, specificity, accuracy, negative predictive value (NPV), and positive predictive value (PPV). The finding results of sensitivity, specificity, and accuracy were 73%, 85%, and 80% respectively. 76% of PPV and 82% of NPV were also obtained in classifying patient at threshold of 200 cells/μl. At threshold of 350 cells/μl, the percentages of sensitivity, specificity, and accuracy were 96, 91, and 94, respectively and 93% of PPV and 95% of NPV were obtained (Table 3.4-3). These results indicating that the testing proficiency of MT4 method was sufficient to identify patient at different levels of CD4+ T lymphocyte.

Table: 3.4-2: Agreement between the two methods in classifying absolute CD4+ T lymphocyte numbers at the threshold of CD4 200 or 350 cells/μl from 100 samples.

		Flow cytometry		Total
		≤200/350	> 200/350	
MT4 method	**≤200/350**	29/53	9/4	38/57
	> 200/350	11/2	51/41	62/43
Total		40/55	60/45	100/100

Table: 3.4-3: Proficiency testing of developed MT4 method

Parameter	**≤ 200 cells/μl**	**≤ 350 cells/μl**
Sensitivity	73% (29/34)	96% (53/55)
Specificity	85% (51/61)	91% (41/45)
Accuracy *	80% (80/100)	94% (94/100)
Positive predictive value (PPV) **	76% (29/38)	93% (53/57)
Negative predictive value (NPV) ***	82% (51/62)	95% (41/43)

* Efficacy of the test

** Efficacy of the test when count less then threshold

*** Efficacy of the test when count greater than threshold

3.4.1.3 Bland-Altman statistics analysis

Bland-Altman statistics analysis was used to demonstrate how much bias of the developed MT4 method different from flow cytometry. The CD4 values from flow cytometry were plotted against the difference values obtained from subtracting result of MT4 method and flow cytometry. Bland-Altman analysis in Figure 3.6-3 showed very low bias with average mean different of 13 cells/μl. Within one hundred samples analysis, it was found that 96% of the samples were accepted within 2SD, and 70% was within 1SD.

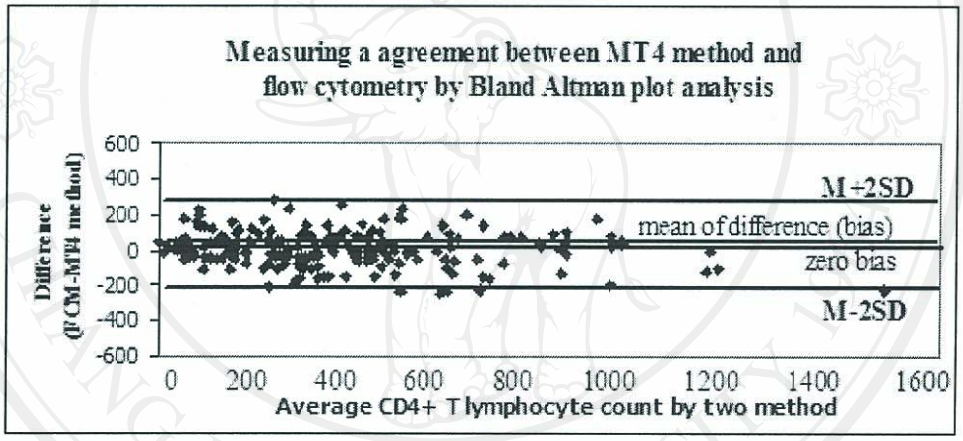


Figure 3.6-3: Bland-Altman statistics analysis to measure the agreement between the developed MT4 method to flow cytomery. The absolute number of CD4+ lymphocytes from flow cytometry presented at X-axis and the different value of both methods at Y-axis.

3.5 Improvement of MT4 method

The above results suggested that MT4 method is an effective non-flow cytmetric method for enumeration of CD4+ lymphocytes. The developed MT4 method was then modified in order to obtain more practically method. Some materials and condition were changed (Table 3.5). The modified method were as follows: In test tube, 200 μl of blood was mixed with 200 μl of MT4 beads at concentration of 10^7 beads/ml. In control tube, 200 μl of blood was mixed with 200 μl of PBS. Both tubes were incubated at room temperature for 30 minutes with constant rotation. After that, the test tube was placed on magnetic particle concentrator (MPC) for 5 minutes. Blood were transfered into a new microtube with cap. Complete cell count (CBC) was analyzed using hematology analyzer, COULTER LH 750 for the both tubes. The absolute CD4+ T lymphocyte numbers were obtained by subtracting the absolute lymphocyte number of control tube from the test tube.

Table 3.5: Procedure of the improved MT4 method

Materials	The old MT4 method	Improved MT4 method
- Blood volume	200 μl	200 μl
- MT4 beads (10^7 beads/ml)	100 μl (10^6 beads)	200 μl ($2x10^6$ beads)
- 0.2% NaN_3–PBS	100 μl	None
- Final dilution	1:2	1:2
- Tube	FACS tube (5 ml)	Micro tube (1.8 ml)
- Ag-Ab incubation time	30 minutes	30 minutes
- Depletion time	3 minutes	5 minutes

3.5.1 Validation of the improved MT4 method comparing to flow cytometry

To validate the accuracy of the developed method, CD4+ T lymphocytes from 17 healthy blood donors and 178 HIV infected persons were determined for absolute number by using the improved MT4 method and standard flow cytometry using Simultest^{TM} reagent kit. It was found that absolute numbers of CD4+ T lymphocytes obtained from the two methods were comparable (Table 3.6-1).

To see discrepant results, the data were divided into three groups according to their absolute CD4+ T lymphocyte value as follow: less than 200, between 200-499 and greater than 500 cells/μl. The results found that 8 in 63 (12.7%) discrepancy among CD4+ T lymphocyte number less than 200 cells/μl, 9 in 97 (9.2%) when between 200-499 and only 1 in 35 (2.8%) when CD4 number greater than 500 cells/μl. The results was indicating the few discrepancy of CD4 count in totally of 18 in 195 (9.2%) (Fig. 3.7-1).

3.5.1.1 Regression line analysis

Correlation plots comparing absolute number of CD4+ lymphocytes obtained from both methods were shown in Figure 3.7-2. Linear regression analysis resulted in a slope of 0.98, an intercept of –0.09. The correlation coefficient was 0.907.

These results indicated that the developed MT4 method can be used to enumerate CD4 lymphocytes in whole blood samples equivalent to flow cytometry.

Table 3.6-1: Absolute CD4+T lymphocyte numbers determined by the improved MT4 method and standard flow cytometry.

N:	MT4 method	FCM	N:	MT4 method	FCM	N:	MT4 method	FCM
1	809	847	**47**	3	29	**93**	275	383
2	566	465	**48**	464	392	**94**	-43	183
3	322	225	**49**	299	92	**95**	130	224
4	36	152	**50**	386	403	**96**	286	437
5	684	463	**51**	-11	145	**97**	8	140
6	499	496	**52**	460	310	**98**	590	462
7	674	512	**53**	437	381	**99**	461	450
8	1412	1185	**54**	399	366	**100**	129	136
9	787	655	**55**	261	184	**101**	450	648
10	1052	949	**56**	374	423	**102**	141	254
11	771	848	**57**	676	696	**103**	134	90
12	1039	916	**58**	680	784	**104**	454	433
13	562	495	**59**	381	362	**105**	340	522
14	457	450	**60**	-18	12	**106**	162	106
15	189	121	**61**	312	200	**107**	138	188
16	124	146	**62**	611	684	**108**	288	209
17	418	417	**63**	399	433	**109**	177	219
18	292	259	**64**	636	397	**110**	181	216
19	75	25	**65**	228	308	**111**	112	352
20	23	30	**66**	325	433	**112**	-230	9
21	398	339	**67**	36	33	**113**	313	489
22	346	444	**68**	169	143	**114**	209	186
23	402	350	**69**	70	52	**115**	376	231
24	655	505	**70**	40	65	**116**	594	668
25	740	719	**71**	81	202	**117**	260	160
26	799	819	**72**	268	227	**118**	20	78
27	984	982	**73**	340	408	**119**	213	293
28	385	326	**74**	281	280	**120**	300	310
29	178	223	**75**	159	109	**121**	333	170
30	548	317	**76**	135	114	**122**	190	245
31	311	391	**77**	288	344	**123**	473	564
32	237	134	**78**	356	336	**124**	258	299
33	-78	12	**79**	145	186	**125**	145	118
34	243	206	**80**	128	127	**126**	107	224
35	652	581	**81**	580	653	**127**	166	228
36	693	871	**82**	231	159	**128**	491	434
37	185	237	**83**	392	353	**129**	284	253
38	316	305	**84**	193	335	**130**	364	382
39	6	53	**85**	226	233	**131**	101	271
40	93	180	**86**	126	120	**132**	331	523
41	517	531	**87**	224	203	**133**	327	239
42	-21	19	**88**	243	213	**134**	269	321
43	231	370	**89**	182	68	**135**	361	346
44	14	4	**90**	282	212	**136**	717	721
45	904	709	**91**	540	487	**137**	296	220
46	16	67	**92**	75	85	**138**	658	754

Table 3.6-1: Absolute CD4+ T lymphocyte number determined by the improved MT4 method and stansdard flow cytometry (Continue).

N:	MT4 method	FCM	N:	MT4 method	FCM	N:	MT4 method	FCM
139	115	146	**158**	135	26	**177**	512	648
140	409	256	**159**	405	393	**178**	-44	134
141	266	348	**160**	323	347	**179**	71	22
142	192	215	**161**	72	149	**180**	-29	168
143	83	134	**162**	407	355	**181**	593	544
144	113	70	**163**	60	39	**182**	385	394
145	87	197	**164**	224	192	**183**	803	855
146	490	351	**165**	97	148	**184**	246	204
147	363	203	**166**	228	314	**185**	238	256
148	135	26	**167**	133	77	**186**	443	514
149	369	216	**168**	210	309	**187**	703	758
150	48	41	**169**	277	372	**188**	282	242
151	160	151	**170**	277	233	**189**	316	552
152	81	46	**171**	69	113	**190**	76	125
153	300	270	**172**	505	565	**191**	104	62
154	336	147	**173**	197	448	**192**	285	355
155	654	701	**174**	52	206	**193**	442	442
156	445	351	**175**	434	578	**194**	627	380
157	363	203	**176**	17	146	**195**	65	346

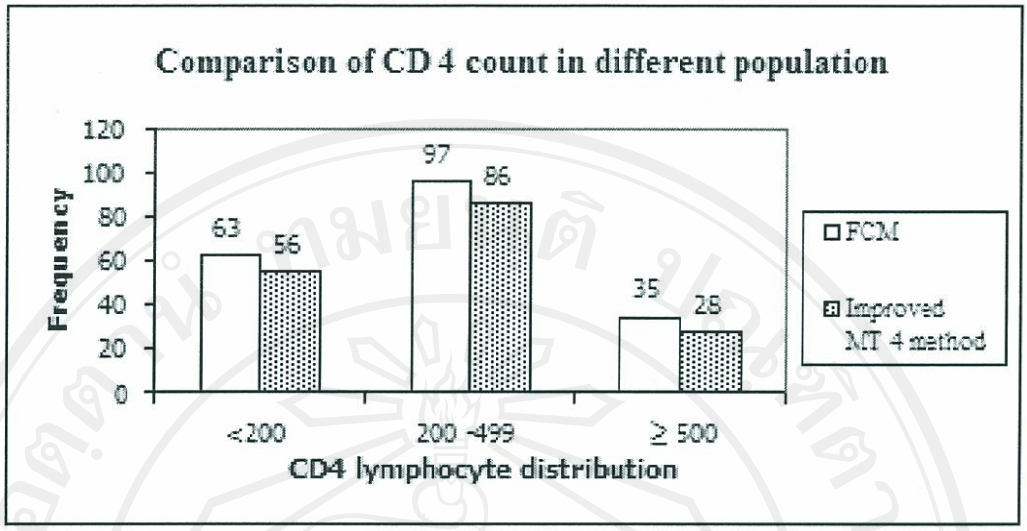


Figure 3.7-1: Frequency of the agreement in enumeration of CD4+ T lymphocyte using MT4 method and flow cytometry. Discrepancy results of 8 in 63 (12.7%), 9 in 97 (9.2%) and 1 in 35 (2.8%) among different groups of CD4+ T lymphocyte number: less than 200, between 200-499 and greater than 500 cells/μl respectively

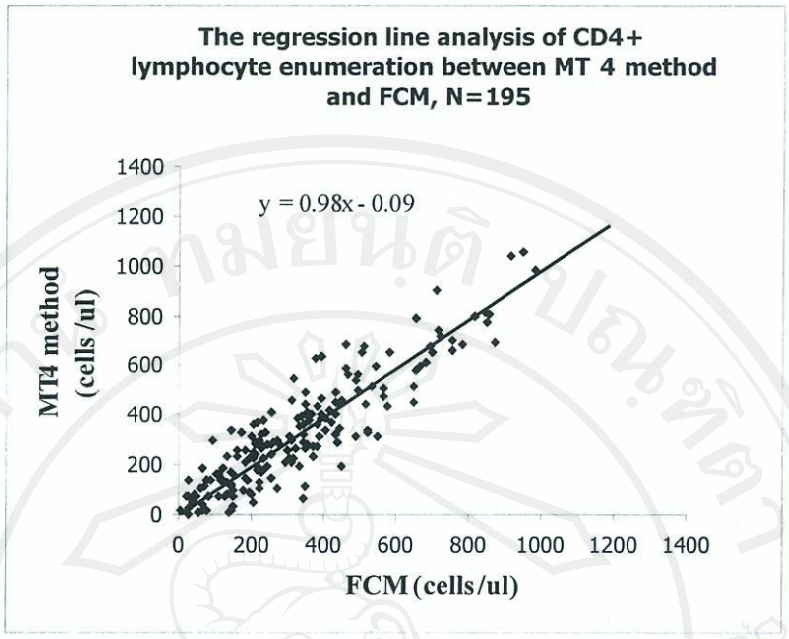


Figure 3.7-2: Regression line analysis of CD4+ T lymphocyte counts between the improved MT4 method and flow cytometry.

3.5.1.2 Testing proficiency of developed MT4 method

The number of CD4+ T lymphocytes from MT4 method were compared with the results from flow cytometry. The agreement results between the MT4 method and flow cytometry in classifying CD4+ T lymphocyte at the threshold of CD4 200 or 350 cells/μl was studied. The agreement of 167 in 195 (85.6%) when classifying at threshold of 200 cells/μl and 173 in 195 (88.7%) at the threshold of 350 cells/μl were obtained (Table 3.6-2). The agreement number was used to evaluate the testing proficiency by calculating the sensitivity, specificity, accuracy, negative predictive value (NPV), and positive predictive value (PPV) (Table 3.6-3). At threshold of 200 cells/μl, the sensitivity, specificity, and accuracy were 86%, 85%, and 86%, respectively. PPV and NPV were 74% and 93%, respectively. At threshold of 350 cells/μl, the percentages of sensitivity, specificity, accuracy, PPV and NPV were 93, 83, 89, 90% and 87%, respectively.

Table: 3.6-2: Agreement between improved MT4 method and flow cytometric method in classifying patients at the threshold of CD4 200 or 350 cells/μl

		Flow cytometry		Total
		≤200/350	> 200/350	
MT4 method	≤ 200/350	55/111	19/13	74/124
	> 200/350	9/9	112/62	121/71
Total		64/120	131/75	195/195

Table: 3.6-3: Testing proficiency of improved MT4 method

Parameter	**≤ 200 cells/μl**	**≤ 350 cells/μl**
Sensitivity	86% (55/64)	93% (111/120)
Specificity	85% (121/131)	83% (62/75)
Accuracy *	86% (167/195)	89% (173/195)
Positive predictive value (PPV) **	74% (55/74)	90% (111/124)
Negative predictive value (NPV) ***	93% (112/121)	87% (62/71)

* Efficacy of the test

** Efficacy of the test when count less then threshold

*** Efficacy of the test when count greater than threshold

3.5.1.3 Bland-Altman statistics analysis

Bland-Altman statistics analysis was then used to demonstrate how much bias of the developed MT4 method different from flow cytometry. In Bland-Altman analysis of 195 samples (Fig. 3.7-3), the CD4 values from standard flow cytometry were plotted against the difference in values between both methods. The result showed very low bias with average mean different of 7 cells/μl, 95% of population was accepted within 2 SD.

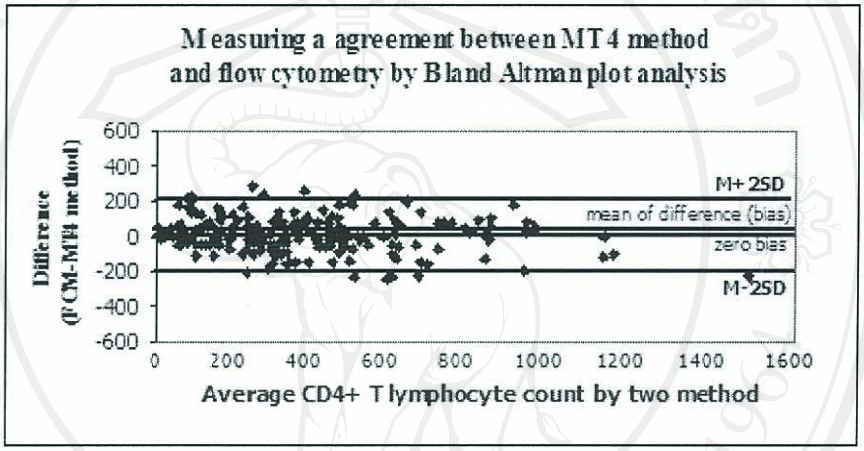


Figure 3.7-3: Bland-Altman statistics analysis to measure the agreement between the MT4 method and flow cytometry. The absolute number of CD4+T lymphocytes from flow cytometry presented at X-axis and the different value of both methods at Y-axis.