Application of Flow Cytometric Beads for Simultaneous CD4 and CD8 Determinations in HIV-1 Infected Thalassemia Patients

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Background: *CD4 and CD8 are determined by either a dual step assay using a calculation of absolute lymphocytes obtained from routine CBC or by a single step assay by including cytometric beads and fluorescent microspheres in the sample so that an absolute cell count can be made, simultaneously. Since thalassemia is common in Thailand, and a number of nucleated red blood cells (NRBC) are observed in peripheral blood of thalassemia patients with severe anemia, we speculated that NRBC in HIV-1 infected thalassemia patient with severe anemia might cause an error in CD4 and CD8 determinations in the dual step assay.*
Objective: *Comparing cytometric beads in three-color-lyse-no-wash in the single step assay in CD4 and CD8 with dual step assay by calculation using absolute lymphocytes obtained from routine CBC in HIV-1 infected thalassemia patients with severe anemia.*
Material and Method: *Simultaneous screening of alpha (SEA type)- and beta thalassemia using a multiplex PCR was done. In the thalassemia patients with severe anemia having 13-1,392 NRBC/100 WBC, it found significant differences ($p < 0.001$-0.002, paired-t-test) of the means of cytometric bead CD4 and CD8 and the means of NRBC corrected CD4 and CD8 as compared to the means of NRBC uncorrected CD4 and CD8 in dual step determinations. In the thalassemia patients with lesser severe anemia, having less than 10 NRBC/100 WBC, there were no significant differences ($p > 0.05$, paired-t-test) of the means of cytometric bead CD4 and CD8 and the means of NRBC corrected $CD4^+$ and $CD8^+$ as compared to the means of NRBC uncorrected CD4 and CD8 in dual step assay. In comparison of CD4 and CD8 determinations in HIV-1 infected thalassemia patients with severe anemia having more than 10 NRBC/100 WBC, there were significant differences ($p < 0.002$, paired-t-test) of the means of cytometric bead CD4 and CD8 and the means of NRBC corrected CD4 and CD8 as compared to the means of NRBC uncorrected CD4 and CD8 in dual step assay.*
Conclusion: *Results indicated that the NRBC in HIV-1 infected or uninfected thalassemia with severe anemia having more than 10 NRBC/100WBC do cause an error in CD4 and CD8 determinations in dual step in routine assay. Therefore, either cytometric beads application in the single step or the conventional calculation using NRBC corrected absolute lymphocytes in three-color-lyse-no-wash assay is essentially needed in the flow cytometric assay for CD4 and CD8 determinations.*

Keywords: *CD4-positive T-lymphocytes, CD4 lymphocyte count, CD4-CD8 ratio, CD8-positive T-lymphocytes, Erythroblasts, Flow cytometry, HIV-1, Thalassemia*

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Thalassemia is an inherited disorder of hemoglobin synthesis. It is common in Mediterranean countries, Chinese, and Southeast Asians where its prevalence can reach up to 30-40% in some populations of Thailand. The disorder can be asymptomatic up to life threatening severity depending upon whether the individual is heterozygote, homozygote, or concomitant with other hematological disorders. In the severe cases, a number of nucleated red blood cells (NRBC) are observed in peripheral blood. These NRBC may be included in the cluster of lymphocytes and therefore cause an error in the lymphocyte enumeration. To overcome this problem Borowitz et al have introduced CD45, a common leukocyte antigen marker for a three-color flow cytometric assay to exclude the NRBC from the lymphocyte cluster[1].

Even though, the NRBC were excluded from CD4 and CD8 lymphocyte determinations in the HIV-1 infected patients. However, an absolute CD4 count is normally calculated using an absolute lymphocyte count from a routine complete blood cell (CBC) determination, and this is subjected to an error due to the inclusion of NRBC in the absolute lymphocytes in CBC determination.

Recently, cytometric beads, fluorescent microspheres were included in the flow cytometric assay so that an absolute cell count (cell/μL) of the positive cells in the peripheral blood sample can be simultaneously determined, thus can be free of an error from the CBC determination[2,3]. Janossy et al revealed the practical advantages of volumetric two-color flow cytometry with CD45/CD4 and CD45/CD8 analysis by adding known numbers of fluorospheres[4]. The authors have used cytometric beads in the CD4 and CD8 determinations in the HIV-1 infected thalassemia patients using three-color lyse no wash assay. This single step CD4 and CD8 determinations is free from the error caused by NRBC that normally encounters an error in the conventional dual step using a calculation from absolute lymphocytes obtained from a routine CBC.

Material and Method

Multiplex PCR for thalassemia

A multiplex polymerase chain reactions (PCR) was used to determine thalassemia in EDTA-blood samples[5]. Briefly, 0.2 ml of EDTA-whole blood was lysed in 2.0 ml of BD-FACS lysis solution (Becton Dickinson) at room temperature for 15 min, and white blood cells (WBC) were obtained after two washings in phosphate buffer saline (PBS). DNA extracts were obtained by lysis the WBC pellets in 0.1 ml of proteinase K lysis buffer. A multiplex PCR was carried out using following primers; alpha thalassemia: Pr-A1B: 5'-GGTTCCCTGAGCCCCGACACG-3' and Pr-A7: 5'-CTCTGTGTTCTCAGTATTGGAG-3' for a normal alpha globulin; and Pr-A9: 5'-ATATATGGGT CTGGAAGTGTATC-3' for Southeast Asian type alpha thalassemia[6]. For beta thalassemia; using Pr-B17-N: 5'-ACTTCATCCACGTTCA CCTT-3' for normal and Pr-B17-M: 5'-ACTTCATCCACGTTCACCTA-3' for A-T codon 17; and Pr-B-IVS-N: 5'-CTGTCTTGTAAC CTTGATAC-3' for normal and Pr-B-IVS-M: 5'-CTGT CTTGTAACCTTGATAG-3' for G-C IVS; using Pr-B-41/42-N: 5'-GAGTGGACAGATCCCCAAAG GACT CAAAGA-3' for normal and Pr-B-41/42-M: 5'-GAGTGGACAGATCCCCAAAGGACTCAACCT-3' for 4 bp codons 41/42; using Pr-B-E-N: 5'-CGTGGA TGAAGTTGGTGGTG-3' for normal and Pr-B-E-M: 5'-CGTGGATGAAGTTGGTGGTA-3' for G-A codon 26, HbE; and using Pr-B-A53: 5'-TCCCATAGACTCA CCCTGAA-3' and Pr-B-N-S1: 5'-TGTCATCACTTA GACCTCAC-3' for the internal control of normal beta globulin[7]. The Multiplex PCR was done by a DNA Thermal Cycler-480 (Perkins Elmer) for 40 cycles as described previously[8].

Flow cytometric beads for CD4 and CD8 determinations

BD-TruCount tube was used in simultaneous-determining absolute count of CD4 and CD8 in EDTA-whole blood samples by three color-lyse no wash-flow cytometric assay. The TruCount tube was containing a freeze-dried pellet of fluorescent beads in a single-use tube. Briefly, 15 μL of a mixture of CD4-FITC, CD8-PE-, and CD45-PerCP was added into the TruCount tube followed by adding of 50 μL of EDTA blood in to the tube. The reaction mixture was incubated for 30 min in the dark at room temperature. After which 450 μL of FACS lysing solution (BD) was added into the tube, it was mixed by vortex gently. After incubation for 15 min in the dark at room temperature, the sample was analyzed by FACSCalibur (BD) using BD-CellQuest program by setting the bead counts according to the reagent package insert.

The data obtained were absolute CD4 and CD8 cell counts. The results were compared to the absolute cell counts obtained from the calculation from the percentages of CD4 and CD8 and an absolute lymphocyte counts from a routine CBC with or without NRBC correction.

Results

Screening of thalassemia

The authors have used a multiplex PCR in determination of EDTA-whole blood samples of either HIV-1 seropositive and HIV-1 seronegative anemia patients. The assay designed was able to simultaneously detect beta thalassemia as well as alpha thalassemia-1 (SEA-type) as previously reported[5]. The authors have found that the peripheral blood mononuclear cells (PBMC) purified by a density gradient Histopaque (Sigma) was normally contaminated by NRBC, especially in thalassemia patients with high NRBC. In addition, those contaminated NRBC interfere with the PCR amplification due to their hemoglobin content. The authors have, therefore, used the red blood cell lysis reagent (BD FACS lysing solution), normally used in CD4 and CD8 determinations by flow cytometer. The authors have found this is very effective and more convenient in preparation of WBC pellet for DNA-cell lysis since the PCR assay could be carried out along with the flow cytometric assay.

As shown in Fig. 1, examples of five patients were screened by the multiplex PCR showed internal PCR markers of an alpha globulin- and a beta globulin gene of approximate 314 bp and 660 bp, respectively. Patients A, B, C showed hemoglobin-E amplification of approximate 400 bp whereas patients D and E were normal hemoglobin. Interestingly, patient B showed a band of 243 bp corresponding to A-T 17 codon[5].

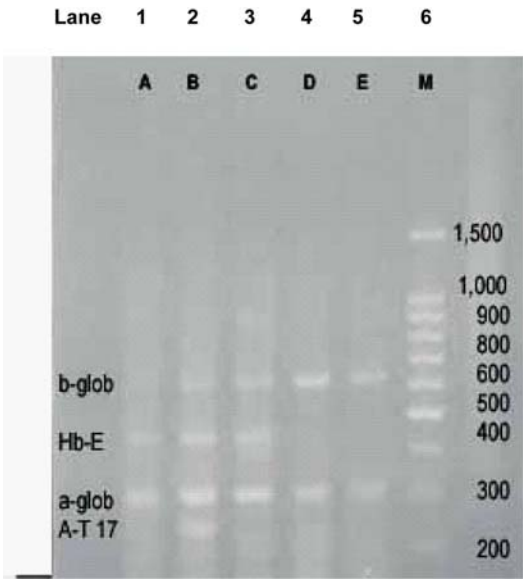

Fig. 1 A representative agarose gel electrophoresis of multiplex polymerase chain reactions for simultaneous detection of alpha (SEA type)- and beta thalassemia Internal PCR markers of 314 bp and 660 bp for alpha- and beta-thalassemia, respectively were seen in all patients. Patient A, B, and C showed Hb E amplification products of 400 bp. In addition, patient B showed a band of 243 bp of A-T 17 codon mutant. Lane M was 100 bp markers

Genotyping of beta thalassemia

Patients A, B, and C were genotyping with normal and mutant primers for beta thalassemia. As shown in Fig. 2, Panel 1; lanes 1-3 were beta thalassemia patients genotyped by normal beta thalassemia primer set. Whereas lanes 4-6 were amplification products by a mutant beta thalassemia primer set. Lane 7 was 100 bp markers. There were approximately, 400 bp seen in normal beta thalassemia primer set as well as in mutant beta-thalassemia primer set. These indicated patients A, B, and C were heterozygotes or a genotype of HbEA. This hemoglobin E trait is quite common in Thailand and is the most common beta thalassemia phenotype in the world

Genotyping of AT-17 codon thalassemia

In Fig. 2, panel 2, patient B shows a band of approximate 243 bp for AT-17 codon observed in both AT-17 normal (lane 8) and AT-17 mutant assays (lane 9).

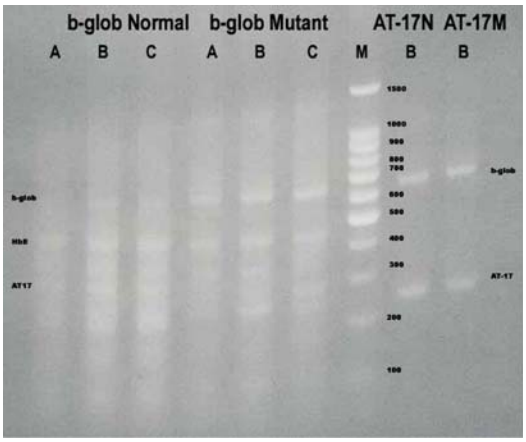

Fig. 2 Genotyping of beta thalassemia
Panel 1: Lane 1, 2, and 3 were genotyped with normal beta globulin primer set for patients A, B, and C, respectively. Lane 4, 5, and 6 were genotyped with mutant of beta globulin primer set for patients A, B, and C respectively. Lane 7 was 100 bp markers
Panel 2: Lane 8 was genotyping with normal AT-17 codon primers. Lane 9 was genotyping with mutant AT-17 codon primers for patient B

Therefore, it is indicated that the patient B was a double heterzygote for Hb AT-17 in addition to HbEA.

Effect of NRBC on flow cytometric plot

In the flow cytometric determination, the forward scatter (FSC) determining the size of the cells and the side scatter (SSC) determining the granularity in the cells were plotted, and populations of lymphocytes, monocytes, and granulocytes were easily separated. Therefore, the number of $CD4^+$ and $CD8^+$ cells can be determined within the lymphocyte-gating. However, the two color-flow cytometric assay by an immunological staining with fluorochrome conjugated CD4 and CD8 can be interfered by the contaminated nucleated RBC in the lymphocyte-gating because of the incomplete lysis of NRBC by FACS lysis solution.

To overcome NRBC interfering, a three-color flow cytometric assay was introduced by using CD45, a common leukocyte antigen, and a plot was made by CD45-PerCP intensity and the SSC[1,10]. Fig. 3 shows that the lymphocyte-gating was not interfered by

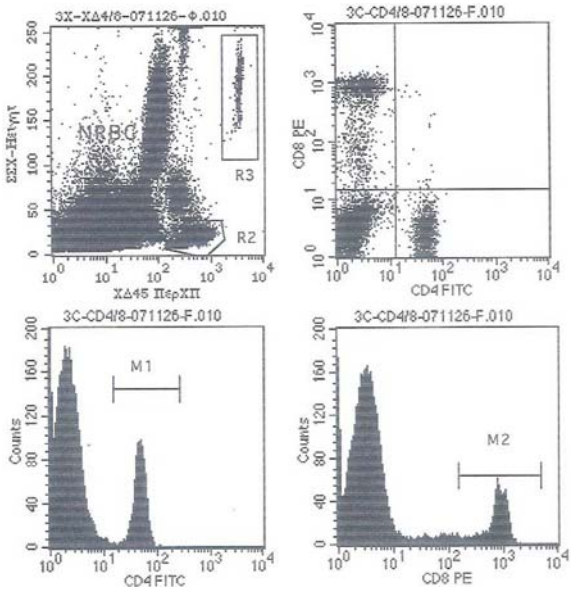

Fig 3. Flow cytometric plots of cytometric bead -3-color lyse-no-wash assay
The upper left showed separated clusters of NRBC, lymphocyte (R2), and cytometric beads (R3). The upper right showed $CD4^+$ counts in quadrant 4, and $CD8^+$ counts in quadrant 1 of lymphocytes obtained from gate R2
Absolute numbers of CD 4^+ and $CD8^+$ were obtained from regions M1 and M2, respectively

NRBC. The authors, therefore, used CD4-FITC, CD8-PE, and CD45-PerCP conjugated monoclonal antibodies for a three-color flow cytometric assay in CD4 and CD8 determinations.

Comparison of CD4 and CD8 counts, NRBC-corrected CD4 and CD8 counts, and Cytobeads CD4 and CD8 counts in thalassemia patients with NRBC more than 10/100 WBC

As shown in Table 1, 50 thalassemia patients having 13-1,392 NRBC/100 WBC were determined for CD4 and CD8. NRBC corrected CD4 cell count was mean of 1,655 $\pm$ 180 cells, and similar to the mean of Cytobead CD4 cell count of 1,764 $\pm$ 154 cells. There were significant differences ($p < 0.001$) compared to the mean of 5,109 $\pm$ 729 cells for CD4 cell count without NRBC correction. Similarly, means of 1,597 $\pm$ 194 cells and 1,360 $\pm$ 141 cells for NRBC corrected CD8 cell count and Cytobead CD8 cell count had significant differences ($p < 0.002$) compared to the mean cell count of 4,966 $\pm$ 818 cells of the absolute CD8 cells based on the calculation using absolute lymphocytes obtained from routine CBC determination.

Comparison of CD4 and CD8 cell counts, NRBC-corrected CD4 and CD8 cell counts, Cytobead-CD4 and CD8 cell counts in thalassemia patients with NRBC less than 10.

As shown previously, CD4 and CD8 determinations were elevated, probably due to the NRBC in the thalassemia patients. The authors, therefore, determined CD4 and CD8 cell counts in the less severe patients with NRBC less than 10 NRBC/ 100 WBC. From results shown in Table 1, there were no significant differences ($p > 0.05$) with the mean of 1,186 $\pm$ 151 cells for NRBC-corrected CD4 cell count and the mean of 1,302 $\pm$ 181 cells for the Cytobead CD4 cell count compared to the mean of 1,244 $\pm$ 158 cells for CD4 cell count from the calculation using absolute lymphocytes obtained from routine CBC determination.

Similarly, there were no significant differences ($p > 0.05$) of the mean cell count of 1,280 $\pm$ 143 cells for NRBC-corrected CD8 cell count and the mean cell count of 1,115 $\pm$ 157 cells of the Cytobead CD8 cell count compared to the mean cell count of 1,343 $\pm$ 151cells for CD8 cell count from the calculation using absolute lymphocytes obtained from routine CBC determination.

Comparison of CD4 and CD8 cell counts, NRBC-corrected CD4 and CD8 cell counts, and Cytobead-CD4 and CD8 cell counts in HIV-1 infected-thalassemia patients with NRBC more than 10.

As seen in Table 2, means of 401 $\pm$ 72 and 355 $\pm$ 69 cells for NRBC corrected CD4 cell count and

Table 1. Comparison of CD4/CD8, NRBC corrected CD4/CD8, Cytobead CD4/ CD8 in thalassemia-patients

Cell count	NRBC > 10/100WBC		NRBC < 10/100WBC	
	Mean ± SEM (n = 50)	p-value	Mean ± SEM (n = 48)	p-value
CD4 cell count	5,109 ± 729		1,244 ± 158	
NRBC-corrected CD4 cell count	1,655 ± 180	<0.001*	1,186 ± 151	>0.05***
Cytobead CD4 cell count	1,764 ± 154	<0.001*	1,302 ± 181	>0.05***
CD8 cell count	4,966 ± 818		1,343 ± 151	
NRBC-corrected CD8 cell count	1,597 ± 194	<0.002**	1,280 ± 143	>0.05****
Cytobead CD8 cell count	1,360 ± 141	<0.002**	1,115 ± 157	>0.05****

* Compared to CD4 cell count of thalassemia patients with NRBC > 10/100WBC
** Compared to CD8 cell count of thalassemia patients with NRBC > 10/100WBC
*** Compared to CD4 cell count of thalassemia patients with NRBC < 10/100WBC
**** Compared to CD8 cell count of thalassemia patients with NRBC < 10/100WBC

Table 2. Comparison of CD4/CD8, NRBC corrected CD4/CD8, Cytobead CD4/ CD8 in HIV + thalassemia-patients with NRBC >10/100WBC

Cell count	Mean ± SEM	n	p-value
CD4 cell count	621 ± 98	22	
NRBC-corrected CD4 cell count	401 ± 72	22	<0.002*
Cytobead CD4 cell count	355 ± 69	22	<0.002*
CD8 cell count	2,306 ± 235	22	
NRBC-corrected CD8 cell count	1,511 ± 147	22	<0.002**
Cytobead CD8 cell count	1,601 ± 151	22	<0.002**

* Compared to CD4 cell count of HIV + thalassemia patients withNRBC > 10/100WBC
** Compared to CD8 cell count of HIV + thalassemia patients withNRBC > 10/100WBC

Cytobead CD4 cell count in HIV-1 infected thalassemia patients had significant differences ($p < 0.002$) from the mean of absolute CD4 count (621 ± 98) obtained from the calculation using absolute lymphocytes from routine CBC determination.

Similarly, means of 1,511 ± 147 and 1,601 ± 151 cells for NRBC corrected CD8 cell count and Cytobead CD8 cell count, respectively were significant differences ($p < 0.002$) compared to CD8 cell count (2,306 ± 235 cells).

Discussion

The authors have successfully modified WBC preparation by using BD-FACS lysing solution to lyse the red blood cells in EDTA blood samples. The cell pellet is used in DNA preparation and further amplified in a multiplex PCR for a simultaneous alpha- and beta-thalassemia detection in routine screening. The modified method is rapid and convenient in a flow cytometric laboratory.

It is known that anti-retroviral agents such as Zidovudine cause the development of anemia in HIV-1-seropositive patients in HAART (highly active anti-retroviral therapy)[9]. Since thalassemia is a common anemia in Thailand, therefore, the authors speculated that NRBC in the thalassemia with severe anemia of the HIV-1 infected patients might interfere with the counts of CD4 and CD8 determinations.

It is known that NRBC interfere with the lymphocyte getting in the two-color flow cytometric assay and cause an error in CD4 and CD8 cell counts. This interference can be overcome by using the three-color flow cytometric assay.

The absolute lymphocyte count is not normally corrected for NRBC in routine CBC determination. Even the absolute cell count is manually corrected for NRBC, it is still subjected up to 20% of human error, and this may contribute to 20% of error of CD4 and CD8 cell count using a calculation from the absolute lymphocyte count. To avoid the error, one

must use either cytometric beads, fluorescent microspheres for a simultaneous direct cell count, or NRBC corrected absolute lymphocytes in the calculation. In the thalassemia patients with more than 10 NRBC/100WBC, the NRBC corrected CD4 cell count and Cytobead CD4 cell count are significant differences than that of CD4 cell count obtained from the calculation using the absolute lymphocytes from a routine CBC without correction for NRBC ($p < 0.001$). These are also in the cases of NRBC corrected CD8 cell count and Cytobead CD8 cell count as compared to the calculated CD8 cell count without NRBC correction ($p < 0.002$). However, in the thalassemia patients with less than 10 NRBC/100 WBC, there are no significant differences for NRBC corrected CD4 and CD8 cell counts and for Cytobead CD4 and CD8 cell counts compared to the calculated CD4 and CD8 cell counts without correction for NRBC ($p > 0.05$).

In the HIV-1 infected thalassemia patients with severe anemia having more than 10 NRBC/100 WBC, there are significant differences for NRBC corrected CD4 and CD8 cell count as well as cytobead CD4 and CD8 cell counts compared to CD4 and CD8 cell counts without correction for NRBC ($p < 0.002$).

In conclusion, the NRBC interfere with CD4 and CD8 determinations in dual step routine assay in the thalassemia patients with severe anemia having more than 10NRBC /100 WBC. Therefore, in the case of HIV-1 infected-thalassemia with severe anemia patients either Cytobead cell count for a single step-simultaneous CD4 and CD8 cell determinations by adding fluorescent references beads of known concentration must be used to define the volume of blood sample analyzed. Another method is to use the NRBC corrected absolute lymphocytes for a conventional calculation in dual-step routine assay as it is essentially needed in the flow cytometric assay.

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การใช้โฟลวไซโตเมตริกบีดในการตรวจซีดี 4 และซีดี 8 พร้อมกันในผู้ป่วยติดเชื้อเอชไอวีที่เป็นโรคธาลัสซีเมีย

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CD4 และ CD8 ตรวจโดยใช้แบบสองขั้นตอน (dual step) โดยคำนวณจากจำนวน absolute lymphocytes ที่ได้จากการตรวจ CBC หรือโดยใช้แบบขั้นตอนเดียว (single step) โดยการผสม cytometric beads ซึ่งเป็นเม็ดพลาสติกเคลือบด้วยสารฟลูออเรสซีนที่ทราบจำนวนเม็ดต่อปริมาตรทำให้ทราบผลเป็นจำนวน absolute CD4 และ CD8 ทันที เนื่องจากธาลัสซีเมียเป็นโรคที่พบทั่วไปในประชากรไทยและปกติจะพบตัวอ่อนของเม็ดเลือดแดง (NRBC) จำนวนมากในเลือดของผู้ป่วยที่มีภาวะโลหิตจางรุนแรง คาดว่าจำนวน NRBC ในผู้ป่วยที่ติดเชื้อเอชไอวีที่เป็นธาลัสซีเมียที่มีภาวะโลหิตจางรุนแรงจะทำให้ผลการตรวจ CD4 และ CD8 ผิดพลาดโดยใช้แบบ dual step ดังนั้นในการศึกษาครั้งนี้จึงใช้ cytometric beads ผสมในเลือดของผู้ป่วยดังกล่าวในการตรวจ CD4 และ CD8 โดยการย้อมสามสีใช้แบบ single step แล้วเปรียบเทียบผลที่ได้กับการตรวจแบบ dual step การตรวจกรองธาลัสซีเมียโดยวิธีมัลติเพล็กพีซีอาร์ทำให้สามารถตรวจแอลฟาธาลัสซีเมีย (ชนิด เอสอีเอ) และเบต้าธาลัสซีเมียในเวลาเดียวกัน ผลการตรวจผู้ป่วยธาลัสซีเมียที่มีภาวะโลหิตจางรุนแรงโดยมี NRBC ระหว่าง 13-1,392 เซลล์ต่อเม็ดเลือดขาว 100 เซลล์พบจำนวน CD4 และ CD8 โดยวิธี single step ใช้ cytometric beads และวิธี dual step โดยคำนวณจาก absolute lymphocytes จาก CBC ที่ปรับลดจำนวนตามจำนวน NRBC มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($p < 0.001$-0.002) เมื่อเปรียบเทียบกับผลที่ได้จากการตรวจใช้วิธี dual step โดยคำนวณจาก absolute lymphocytes จาก CBC ที่ไม่ได้ปรับลดจำนวนตามจำนวน NRBC ในขณะที่ผลการตรวจผู้ป่วยธาลัสซีเมียที่มีภาวะโลหิตจางที่มีอาการรุนแรงน้อยกว่าโดยมี NRBC น้อยกว่า 10 เซลล์ต่อเม็ดเลือดขาว 100 เซลล์พบจำนวน CD4 และ CD8 โดยวิธี single step ใช้ cytometric beads และวิธี dual step โดยคำนวณจาก absolute lymphocytes จาก CBC ที่ปรับลดจำนวนตามจำนวน NRBC ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($p > 0.05$) เมื่อเปรียบเทียบกับผลที่ได้จากการตรวจใช้วิธี dual step โดยคำนวณจาก absolute lymphocytes จาก CBC ที่ไม่ได้ปรับลดจำนวนตามจำนวน NRBC ผลการตรวจผู้ป่วยที่ติดเชื้อเอชไอวีที่เป็นโรคธาลัสซีเมียโดยมีภาวะโลหิตจางที่มีอาการรุนแรงโดยมี NRBC มากกว่า 10 เซลล์ต่อเม็ดเลือดขาว 100 เซลล์พบจำนวน CD4 และ CD8 โดยวิธี single step ใช้ cytometric beads และวิธี dual step โดยคำนวณจาก absolute lymphocytes จาก CBC ที่ปรับลดจำนวนตามจำนวน NRBC มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($p < 0.002$) เมื่อเปรียบเทียบกับผลที่ได้จากการตรวจใช้วิธี dual step โดยคำนวณจาก absolute lymphocytes จาก CBC ที่ไม่ได้ปรับลดจำนวนตามจำนวน NRBC (NRBC) ผลจากการวิจัยแสดงว่าจำนวน NRBC ในผู้ป่วยธาลัสซีเมียที่มีภาวะโลหิตจางรุนแรงมากกว่า 10 NRBC ต่อ 100 WBC ทั้งที่ติดเชื้อ และไม่ติดเชื้อเอชไอวีจะทำให้ผลการตรวจ CD4 และ CD8 ผิดพลาดโดยใช้แบบ dual stepโดยคำนวณจาก absolute lymphocytes จาก CBC ที่ไม่ได้ปรับลดจำนวนตามจำนวน NRBC สรุปว่าวิธี single step ใช้ cytometric beads และวิธี dual step โดยคำนวนจาก absolute lymphocytes จาก CBC ที่ปรับลดจำนวนตามจำนวน NRBC มีความจำเป็นในการตรวจ CD4 และ CD8 โดยใช้การย้อมสามสีแล้วทำให้เม็ดเลือดแดงแตกและไม่ปั่นล้าง โดยการตรวจโดยวิธีโฟลวไซโตเมตรี