Original Article

Evaluation of Nucleated Red Blood Cells in Thalassemia by the Coulter LH 750 Hematology Analyzer

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Abstract : *The complete blood count and white blood cells (WBC) differential count provide important data in hematological diagnosis and monitoring of therapy. Most of the laboratories use automated analyzers to evaluate these data. The Coulter LH 750 is a new hematology analyzer which incorporates the features of nucleated red blood cells (NRBC) and reticulocyte including the immature reticulocyte fraction (IRF) parameters. In this study, we evaluated the NRBC parameter from thalassemic blood samples by using Coulter LH 750 and comparing the results with microscopic examination. The whole blood of 160 β-thalassemia/HbE patients were obtained from leftover samples, which submitted to routine examination. The findings of automated NRBC% values of <49 /100WBC (n=44) have a good correlation with microscopic method. However, automated NRBC% values of > 50 /100WBC (n=35) have a poor correlation. In conclusion, the Coulter LH 750 could decrease workload on blood smear examination in normal differential WBC count without flags and need no reagent for NRBC enumeration. However, the review of blood smear is still recommended in case of the automated abnormal flags and NRBC for accuracy.*

Key Words : ● *Nucleated red blood cell* ● *Coulter LH 750* ● *Automated hematology analyzer* ● *β-thalassemia/HbE*

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Nucleated red blood cells (NRBC) or normoblasts can usually be found in the peripheral blood films of almost any patient with severe anemia, after splenectomy, and in the presence of extramedullary erythropoiesis. Small numbers are found in the cord blood of normal infants, whereas quite large numbers are found in that of premature infants or in hemolytic disease of the newborn.[1] Therefore, NRBC should be identified correctly, even at low numbers, because their presence may indicate significant underlying disease. NRBC are variably and unpredictability included within the lymphocyte count, according to their heterogeneous size and sensitivity to lysis. If there are significant numbers of NRBC or non-lysis red blood cells, the falsely high white blood cell (WBC) count and inaccurate differential WBC count may occur in automated cell counting.[2] So, it is necessary to correct the total nucleated cell count for the number of NRBC by counting their percentage on the blood smear. Traditionally, they have been counted as part of the 100-cell manual differential white blood cells (WBC) count and reported as number of NRBC per 100 WBC. Unfortunately, this method is time-consuming, lack of sensitivity, and statistically imprecise due to a small number of cell counted and heterogeneity of cell distribution in manual spreader-slide blood smear and

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also subjectiveness, depending on the technical skill of the examiners.[3-7] Recent hematology analyzers have improved performance on differential WBC as they can detect and report NRBC count in peripheral blood as a separate population out of WBC count. The Coulter LH 750 hematology analyzer (Beckman Coulter, Miami, FL, USA) is automated system that provides a complete blood cell count, 5-part differential WBC mode, and NRBC counts. The 5-part differential including percentage and absolute number of total neutrophils, lymphocytes, monocytes, eosinophils, and basophils, is generated by volumetric (V) impedance using direct current, conductivity (C) using high frequency electromagnetic energy, and laser light scatter (S). Particles greater than 35 fL after red blood cells lysis are counted as WBC. The NRBC percentage (NRBC%) is derived from both the WBC histogram and volume conductivity scatter (VCS) information and represents the number of NRBC per 100 WBC, since the NRBC population will lie under the lymphocytes population in VCS scatter plot. The total number of NRBC is calculated from the NRBC% and the total WBC count. The WBC count is automatically corrected via a WBC interference algorithm for adjusting for interference by the particles greater than 35 fL.[8]

Thalassemia (Thal) is a genetic disorder caused by a partial or complete deficiency of α- or β-globin chain synthesis.[9] α- and β-thalassemia and abnormal hemoglobin (Hb) E are common in Thailand.[10-12] The pathophysiology of diseases related to the degree of anemia is caused by both intramedullary hemolysis and RBC destruction in peripheral blood. Because of the heterogeneity of the clinical syndrome, variation in the results of laboratory and clinical features are found. The RBC morphology of β-thal/HbE shows some degree of microcytic and hypochromic with slight-to-severe poikilocytosis, including target cells, fragmented RBC, elliptocytes, polychromasia, basophilic stippling, and NRBC. In splenectomized β-thal/HbE patients, a lot of NRBC will be seen in peripheral blood smear. This study aimed to compare the NRBC counts performance of β-thal/HbE between Coulter LH 750 and microscopic NRBC counts.

Matherials and Methods

Blood samples

The blood samples of 160 β-thal/HbE patients were obtained from leftover samples, which submitted to routine examination from the Out-patient Laboratory of the Division of Hematology, Department of Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University. All samples were collected in evacuated K_3-EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA).

Automated WBC differential counts

The Coulter LH 750 measures the WBC, RBC, HGB, MCV, MPV, RDW, and platelet count and calculates HCT, MCH, MCHC.[8] Three hundred microliters of blood samples were analyzed in the Coulter LH 750 in the cap-piercing mode. For WBC differential and NRBC count, about 8,000 WBC are analyzed by VCS technology and reported both in percentage and in absolute number. The calibrators and quality control materials recommended by the manufacturer were used in this study.

Manual WBC differential counts

In order to evaluate the manual NRBC counts, three smears were prepared by the manual wedge technique and stained with Wright-Giemsa by using Hematek 1000 (Miles Laboratories, Elkhart, IN, USA). According to NCCLS H20-A protocol[13], two manual 200-cell WBC differential counts were randomly performed with different slides by two medical technologists. The NRBC were counted microscopically to determine the NRBC percentage (referred to 100 WBC) according to the routine method of the laboratory. The average value of NRBC percentage obtained by the 400 WBC was used to compare with the results obtained by the Coulter LH 750.

Statistical analysis

All statistical calculations (means, standard deviations, paired t-test, coefficient of correlations) were performed with the SPSS version 10.0 statistical software (SPSS, Chicago, IL, USA)

Results

From a total of 160 β-thal/HbE samples, only 125 (78.1%) samples yielded the automated WBC differential reports. As shown in Table 1, NRBC were not detected in 14 samples by both methods and it was found that 32 samples had no NRBC (false negative) by the automated method, but the NRBC, 0.5 to 10.8 cells/100 WBC, were seen on the blood smear. The Coulter LH 750 reported 2.9 NRBC% (false positive) on one sample where no NRBC was seen on the smear. A total of 113 samples (70.6%) from 160 β-thal/HbE contained NRBC either by the Coulter LH 750 or by visual counting. Of these samples, 46 (40.7%) have no information of WBC differential by the automated, since there were many flags such as dimorphic RBC, giant platelet and cellular interference. To determine whether the degree of correlation varies with the degree of normoblasthemia, the correlation data were divided into two groups based on the range of manual NRBC% values, representing a fair number of samples, as indicated in Table 1. The manual NRBC% values in the range of 1 to 49 made up the first group of 44 samples. The second group of 35 samples represented the manual NRBC% values in the range of 50 to 1,186. In group I, there was no significant difference detected between the automated and manual NRBC counts. However, the mean of automated NRBC count was significantly lower than manual count (mean 248.7±80.8 versus 566.3±342.1, respectively, $p < 0.001$; range 53.7-410.2, 50.0-1186.0) in group II. Linear regression analysis performed on each group of data yielded correlation coefficients (r) of 0.784 (Figure 1a) and 0.426 (Figure 1b) for groups I and II, respectively. All data revealed good correlation (r=0.848) (Figure 1c), though the mean of automated derived NRBC counts was lower than the manual number. There were 9 samples which have big difference between the manual and the automated NRBC% results (Table 2). All of them had low RBC count, hemoglobin, and hematocrit. In group I, all of them showed spuriously high WBC count, normal MCV, and high platelet count and RDW, respectively. Similar findings were observed in three cases of group II, in which the automated NRBC counts were very lower than manual counts. These cases had the LH 750 flagged for abnormalities of variant lymphocytes (Variant LY) and cellular interference. Another two cases, in which the automated NRBC counts were higher than manual counts, the WBC count and platelet count were with in normal range but the instrument showed abnormal flagged of lymphocyte blasts (LY blast) and variant lymphocytes and cellular interference, respectively. The complete blood count obtained from the Coulter LH 750 was shown in Table 3. The mean values of WBC count, MCV, RDW, and platelet count of the β-thal/HbE patients which NRBC% > 50 /100 WBC were higher than the others group.

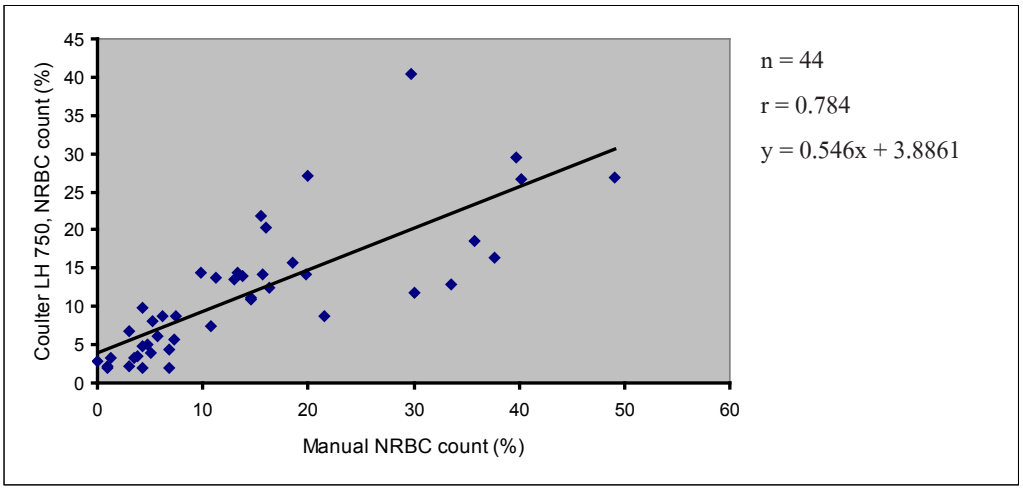
Table 1 Distribution of the manual and automated NRBC% values of β-thal/HbE samples.

	NRBC% counts (/100 WBC)				Correlation (r)
	Coulter LH 750		Manual[a]		
	mean (SD)	range	mean (SD)	range	
No NRBC% (n=46)					
both methods (n=14)	0.0	-	0.0	-	-
automated method (n=32)	0.0	-	3.9 (2.3)	0.5 – 10.8	-
Group I : NRBC% < 49 (n=44)	11.6[+] (8.7)	2.0 – 40.5	14.2[+] (12.5)	1.0 – 49.0	0.784*
Group II : NRBC% > 50% (n=35)	248.7[#] (80.8)	53.7 – 410.2	566.3[#] (342.1)	50.0 – 1186.0	0.426**
Total (n=125)	73.7[#] (117.7)	0.0 – 410.2	164.6[#] (308.9)	0.0 – 1186.0	0.848*

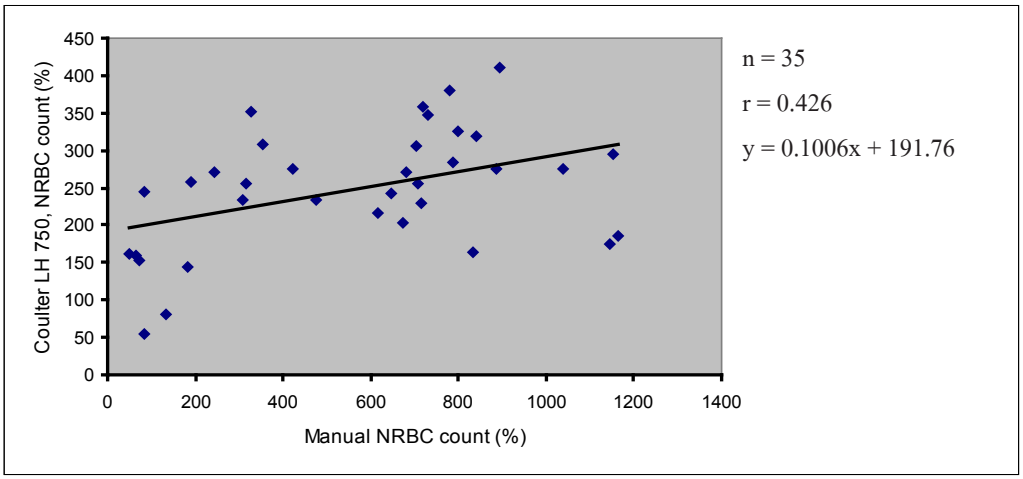
[a] average NRBC% values obtained by performing a-400 WBC differential;

[+] non statistically significant difference; [#] $p < 0.001$; * $p < 0.001$; ** $p = 0.011$

(a)



(b)



(c)

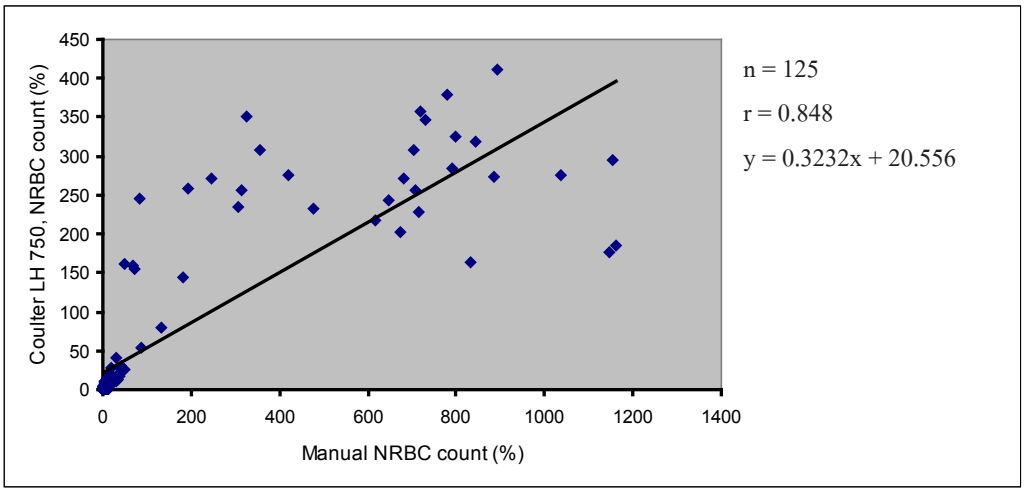


Figure 1 Correlation analysis from automated Coulter LH 750 NRBC% versus manual NRBC% per 100 WBC. Range (a) 1-49; (b) 50-1186; (c) 0-1186 cells/100 WBC.

Table 2 NRBC counts and some hematologic parameters from 9 samples that have different results between Coulter LH 750 and manual method.

Sample No.	NRBC% counts(/100WBC)		WBC	UWBC	RBC	HGB	HCT	MCV	RDW	PLT
	Coulter LH750	Manual	(x 10^9/L)	(x 10^9/L)	(x 10^{12}/L)	(g/dL)	(%)	(fL)	(%)	(x 10^9/L)
Group I : NRBC < 49/100 WBC										
1[1]	20.2	982.2	347.6	364.3	2.41	5.4	22.9	95.3	34.9	494.0
2[1]	16.3	923.0	338.8	374.0	2.60	6.1	25.6	98.6	32.4	541.0
6[2]	43.1	719.5	123.0	159.8	3.08	6.7	26.1	84.6	30.5	750.0
66[3]	39.2	693.7	100.0	135.7	2.46	5.5	20.5	83.4	36.9	992.0
Group II : NRBC% > 50/100 WBC										
202[4]	175.8	1118.7	133.2	196.2	3.24	6.7	28.3	87.5	35.0	926.0
649[5]	184.9	1163.8	174.1	174.1	2.52	5.5	22.8	90.6	34.8	599.0
658[5]	294.4	1186.0	89.0	124.2	2.34	5.3	20.8	88.5	32.2	528.0
3[6]	161.4	50.0	11.5	16.0	3.37	8.6	27.7	82.2	20.6	570.0
33[7]	245.4	82.0	6.0	9.5	2.73	5.9	20.0	73.5	30.6	188.0

UWBC = uncorrected white blood cell count; RDW = red blood cell distribution width

[1] *LH 750 flagged for abnormalities : Verify Diff; Cellular interference; Dimorphic RBCs; H&H check failed; Giant platelets*

[2] *LH 750 flagged for abnormalities : Verify Diff; Imm. NE; Cellular interference; Dimorphic RBCs; H&H check failed; Giant platelets*

[3] *LH 750 flagged for abnormalities : LY blasts; Imm. NE; Cellular interference; Dimorphic RBCs; H&H check failed; Giant platelets*

[4] *LH 750 flagged for abnormalities : Variant LY; Verify Diff; Cellular interference; Dimorphic RBCs; H&H check failed; Giant platelets*

[5] *LH 750 flagged for abnormalities : Variant LY; Imm. NE; Verify Diff; Cellular interference; Dimorphic RBCs; H&H check failed; Giant platelets*

[6] *LH 750 flagged for abnormalities : Variant LY; Cellular interference*

[7] *LH 750 flagged for abnormalities : Verify Diff; Cellular interference; Dimorphic RBCs*

Table 3. Complete blood count of 125 β-thal/HbE samples determined by the Coulter LH 750.

Parameters	Group of patients			
	No NRBC (n=14)	**NRBC (0-10.8 /100WBC)** (n=32)	**NRBC (< 49 /100WBC)** (n=44)	**NRBC (> 50 /100WBC)** (n=35)
WBC (x 10^9/L)	8.35 ± 2.40	7.79 ± 2.95	8.48 ± 5.35	47.24 ± 38.06
RBC (x 10^{12}/L)	3.97 ± 0.82	3.45 ± 0.45	3.32 ± 0.87	2.90 ± 0.42
HGB (g/dL)	9.32 ± 1.99	7.97 ± 1.15	7.28 ± 1.72	6.28 ± 1.11
HCT (%)	28.85 ± 5.89	24.72 ± 3.37	23.05 ± 5.40	22.80 ± 3.36
MCV (fL)	73.44 ± 10.71	72.38 ± 7.14	70.20 ± 8.77	78.93 ± 7.84
MCH (pg)	23.82 ± 4.29	23.41 ± 2.60	22.19 ± 3.22	21.74 ± 2.73
MCHC (%)	32.28 ± 1.44	32.31 ± 0.78	31.55 ± 1.26	27.55 ± 2.26
RDW (%)	23.20 ± 7.12	29.02 ± 6.20	30.11 ± 5.72	31.61 ± 6.10
PLT (x 10^9/L)	347.10 ± 192.40	255.60 ± 213.7	248.40 ± 179.80	725.20 ± 199.60

NRBC: average NRBC% values obtained by performing a-400 WBC differential

Discussion

Identification and enumeration of NRBC has clinical importance because hematology analyzers include NRBC in the WBC counts. Thus, high NRBC in peripheral blood cause falsely elevated WBC counts and require the correction. We performed the evaluation of NRBC parameter of the Coulter LH 750 from β-thal/HbE samples (NRBC range, 0-1186 /100WBC) by comparing with manual reference method. There were 35 samples that the automated could not report WBC differential and had flagged as partial clog. This meant that there were noises or too many particles or incomplete red blood cell lysis passed through the flow cell. In some cases (data not shown), there were only the reported of uncorrected WBC (UWBC), NRBC% and "Cellular interference" flag, which means that the sample contain other particles (such as giant platelets, NRBC, etc.). According to Coulter principle, any particles 35 fL or greater are considered to be a WBC and is included in WBC count reported by the instrument.[8] If the size of NRBCs were small (<35 fL) they were not included in the WBC count. Zandecki, et al.[14] proposed that after NRBC were in contact with lysis agents that destroyed their membrane, leaving nuclei free (< 40-50 fL), then they would disturb WBC counts. The blood samples of thalassemia in this study (in some cases, especially the splenectomized patients) contained many platelets and NRBC, including polychromatic normoblast. Moreover, their RBC morphology showed marked anisocytosis and poikilocytosis. It is possible that the immature NRBC with large nuclei may not cause a flag if all of the cells fall above the 35 fL threshold. The different NRBC% values between the Coulter LH 750 and the manual method might be the different in NRBC size which some of NRBC were included in WBC count and could be counted as variant lymphocytes, lymphoblasts, or immature neutrophils (Table 3). Normal MCV and high RDW represent the different size of RBC population. Moreover, high platelet count and giant platelets could affect the WBC count and also might be the cause of falsely elevated results. In some cases, there were falsely high platelet counts because of the present of many fragmented RBC. Then, the estimation of NRBC and WBC differential from blood smear examination still need as a reference method. However, it has been shown that the presence of NRBC in low numbers can be missed reported by the automated, and also the manual method, especially the 100-cell eye count differential method.[4] In contrast, it is difficult to assess the relevance of numeric difference between manual differential and 8,000-cell automated differential WBC.

The precise method for evaluating NRBC can be done with the aid of antibodies (CD71, CD 45 etc.) in combination with flow cytometry.[15-17] Igout, et al.[18] had reported a good correlation of NRBC percentage given by the Coulter LH 750 with flow cytometry (r=0.977, n=60). However, the flow cytometric determination was expensive and not suitable for routine laboratory. Zini, et al.[19] had evaluated the NRBC counting performance by Coulter LH 750 as compared with reference manual method and found good correlation between two methods (n= 71; r^2=0.89; NRBC range, 2-42/100WBC). Our results in group I agreed with them. Aulesa, et al.[20] had studied the correlation of Coulter LH 750 results and some interferences with those of manual reference method. They studied 46 samples with NRBC range of 1 to 110/100WBC and found that the Coulter LH 750 reported NRBC count only in 39 samples (85%) with the mean value of 13.69/100WBC (range, 0–94.6 /100WBC) but manual method obtained NRBC count of 19.35/100WBC (range, 1-110/100WBC). Therefore, Coulter LH 750 under estimates the NRBC count, but that is not significantly different, according to Passing-Bablock regression analysis. Overall correlation between the automated and manual results in the present study was good, although the mean NRBC of manual was higher than the automated method. Recently, Kang, et al.[21] evaluated the clinical usefulness of the four hematology analyzers : Cell-Dyn Sapphire (Abbott Diagnostics, Santa Clara, CA, USA), Advia 120 (Bayer Diagnostics, Tarrytown,

NY, USA), Sysmex XE-2100 (TOA Medical Electronics, Kobe, Japan), and Coulter LH 750, and demonstrated that Coulter LH 750 had highest sensitivity for NRBC flag (75%). However, the four analyzers are comparable in overall performance.

In summary, the Coulter LH 750 could decrease workload on blood smear examination in normal differential WBC count without flags and need no reagent for NRBC enumeration. However, the review of blood smear is still recommended in cases with the automated abnormal flags and NRBC for accuracy.

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การประเมินจำนวนเม็ดเลือดแดงที่มีนิวเคลียสของธาลัสซีเมีย โดยเครื่องวิเคราะห์ทางโลหิตวิทยา Coulter LH 750

พรวรีย์ ลำเจียกเทศ, ยูริ คุโรซากิ และ อริยาพร เอี่ยมทิม

ภาควิชาจุลทรรศนศาสตร์คลินิก คณะเทคนิคการแพทย์ มหาวิทยาลัยมหิดล วิทยาเขตโรงพยาบาลศิริราช

บทคัดย่อ : *การตรวจ complete blood count และการนับแยกชนิดเม็ดเลือดขาวเป็นการทดสอบที่ให้ข้อมูลที่มีความสำคัญในการช่วยประกอบการวินิจฉัยโรคทางโลหิตวิทยาและช่วยติดตามผลการรักษา ห้องปฏิบัติการส่วนใหญ่นิยมใช้เครื่องอัตโนมัติในการตรวจ Coulter LH 750 เป็นเครื่องอัตโนมัติทางโลหิตวิทยาที่เพิ่มความสามารถการรายงานค่าเม็ดเลือดแดงที่มีนิวเคลียส และเรติคูโลซัยท์ รวมถึงพารามิเตอร์ immature reticulocyte fraction (IRF) ในการศึกษานี้ได้ประเมินค่าพารามิเตอร์เม็ดเลือดแดงที่มีนิวเคลียสของเครื่องอัตโนมัติ Coulter LH 750 เปรียบเทียบกับวิธีนับจำนวนด้วยกล้องจุลทรรศน์ โดยใช้ตัวอย่างเลือดที่เหลือจากการตรวจในงานประจำวันของผู้ป่วย β-thalassemia/HbE 160 ราย ผลการศึกษา พบว่าค่าการนับจำนวนเม็ดเลือดแดงที่มีนิวเคลียสระหว่างทั้งสองวิธีมีความสัมพันธ์กันดีในกลุ่มที่เครื่องอัตโนมัติรายงานเม็ดเลือดแดงที่มีนิวเคลียส <49 /100 WBC (n=44) ส่วนกลุ่มที่เครื่องอัตโนมัติรายงานเม็ดเลือดแดงที่มีนิวเคลียส >50 /100 WBC (n=35) มีความสัมพันธ์กันต่ำ โดยสรุปการใช้เครื่องอัตโนมัติ Coulter LH 750 สามารถลดการดูสเมียร์เลือดในกรณีที่การนับแยกชนิดเม็ดเลือดขาวมีค่าปกติและไม่มี flags และไม่จำเป็นต้องใช้น้ำยาเพิ่มในการนับจำนวนเม็ดเลือดแดงที่มีนิวเคลียส อย่างไรก็ตาม การตรวจดูสเมียร์เลือดยังคงมีความจำเป็นในกรณีที่เครื่องอัตโนมัติรายงาน flags และมีเม็ดเลือดแดงที่มีนิวเคลียสเพื่อความถูกต้อง*

Key Words : ● *Nucleated red blood cell* ● *Coulter LH 750* ● *Automated hematology analyzer* ● *β-thalassemia/HbE*

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