

Original Articles

Reticulocyte Analysis by Coulter Gen S

Pornvaree Lamchiagdhase, Amphan Chaipun*,
Sirintorn Mahem**, and Kovit Pattanapanyasat***

Department of Clinical Microscopy, Faculty of Medical Technology, Mahidol University, Bangkok, *Kaunkanoon Hospital, Phatthalung, **Khon Kaen Hospital, Khon Kaen, ***Office for Research and Development, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok.

Abstract: The reticulocyte count is useful for measuring of erythropoietic activity, anemia diagnosis, monitoring of therapy or as a way of checking early regeneration after marrow or stem cell transplantation. We evaluated the counting of reticulocytes in peripheral blood with newly developed automated hematology analyzer, Coulter Gen S and compared the results with manual method. Venous blood was obtained from 45 healthy individuals with normal hematologic data and 70 anemic patients. Results showed that there was no statistical difference between both methods and a good correlation with $r = 0.900$ was observed. The automated method is precise and accurate and also provide other reticulocyte parameters including mean reticulocyte volume (MRV), immature reticulocyte fraction (IRF), percent and absolute count of high light scatter reticulocyte (HLR) which can be used for evaluation of reticulocyte maturity.

Key Words: ● Reticulocyte count ● Automation

Thai J Hematol Transf Med 1999;9:211-8.

A reliable reticulocyte count (RC) can offer valuable clinical information with regard to the erythropoietic status of the bone marrow. Most commonly, supravital dyes such as brilliant cr-

esyl blue or new methylene blue (NMB) used to stain RNA in reticulocytes and manually count based on their precipitation. Unfortunately, the manual method is time consuming, lacking of precision and having greater intra-observer variability¹⁻³ and is subjected to morphological definition of reticulocytes, especially for the more mature cells belonging to the fourth class according to Heilmeyer⁴⁻⁶.

Received September 17th, 1999. Accepted September, 1999.
Requests for reprints should be addressed to Ms. Pornvaree Lamchiagdhase, Department of Clinical Microscopy, Faculty of Medical Technology, Mahidol University, Bangkok. 10700. Thailand.

Recently, the Coulter Corporation has developed an automated method, utilizing the NMB to precipitate rRNA and then cleared of erythrocytic hemoglobin, leaving stained RNA. The sample is assayed by volume, conductivity and light scatter (VCS). The instrument separates reticulocytes from mature red blood cells (RBCs), white blood cells and platelets.

The aim of this study was to compare the automated RC function of the Coulter Gen S with the manual counting methods and also to relate other reticulocyte parameters with hematologic measurements.

Materials and Methods

Samples and Subjects

Fourty-five apparently healthy adults (21 males, 24 females) who have normal hematologic data on the basis of their automated CBC and have no medication. They are the 3rd and 4th year students of Faculty of Medical Technology, Mahidol University, Bangkok. Their ages are 19-32 years (21 ± 3). Anemic patients studied were from the Hematology Clinic of the Department of Medicine, Siriraj Hospital, Mahidol University, which their ages are 19-49 years (34 ± 12). Blood samples were collected into dipotassium EDTA anticoagulant from subjects and were analyzed for CBC, blood smear and RC within 4 hours.

Automated reticulocyte counting⁷

The performance characteristics of the Coulter Gen S reticulocyte measurement is in RETIC mode. Briefly, 34 μ L of whole blood

was automatically aspirated and incubated with 166 μ L of NMB to precipitate the basophilic RNA in reticulocytes. Hemoglobin and unbound stain were removed by adding a clearing reagent, leaving clear spherical mature RBCs and darkly stained reticulocytes. Stained reticulocytes were differentiated from mature RBCs and other cell populations by direct current measurements for volume, conductivity and light scatter (VCS). The 32,000 cells were counted to measure the following parameters:-

RET% = The number of reticulocytes per 100 RBCs. RET% is directly measured and reported as a percentage of RBCs.

RET# = The absolute number of reticulocytes. It is calculated from RET% and RBC number. Expressed and reported as 10^6 cells/ μ L or 10^9 cells/L.

IRF* = Immature reticulocyte fraction. The fraction is calculated from the ratio of HLR and total reticulocyte.

HLR%* = High light scatter reticulocyte. This number is directly measured and reported as a percentage of RBCs.

HLR#* = The absolute number of HLR. HLR# is calculated from HLR% and RBC number, and expressed as 10^6 cells/ μ L or 10^9 cells/L.

MRV* = Mean reticulocyte volume. This is the average volume of individual reticulocytes and expressed as femtolitre (fL).

MSCV* = Mean sphered cell volume. This is the average of total RBCs in the reticulocyte analysis as the effect of clearing solution and

expressed as fL.

* IRF, HLR, MRV and MSCV are the parameters for research use, not for use in diagnostic procedures.

Manual reticulocyte counting⁸

Supravital staining of unfixed RBCs with NMB, was performed by mixing 100 mL of whole blood with 100 μ L of 1% NMB solution. The number of reticulocytes per 1,000 RBCs was determined microscopically by each of two experient medical technologists on separate slides (2,000 cells total). The mean of two counts was used for comparison with automated method. If the number of the reticulocytes did not agree within 0.5%, a third medical technologist was recruited to count another 1,000 cells on the third slide, and the results were averaged. A reticulocyte was defined as a RBC containing at least two granules of reticulum. The absolute RC was calculated from the RBC count using Coulter Gen S.

Statistical analysis

For statistical analysis, SPSS/PC+ version 4.0 (Microsoft Corp., USA) was used to calculate descriptive statistics (mean, SD, CV and range). The reference ranges were estimated by percentile (P_5 - P_{95}) for all parameters. Difference between genders was calculated using Mann-Whitney U test. Comparison between automated and manual reticulocyte counting was performed with Wilcoxon Signed Ranks test, simple linear regression and the correlation coefficient (r) was also calculated. The threshold for statistical significance for all compari-

sons was chosen as $p = 0.05$.

Results

The hematologic parameters in 45 healthy Thai adults using Coulter Gen S are shown in Table 1. The mean of RBC count, hemoglobin concentration and hematocrit are difference between males and females. But there was no difference between the mean of white blood cell, blood indices, platelet count and its parameters.

The mean and reference ranges of RC by manual and automated method and other reticulocyte parameters are demonstrated in Table 2. There were no statistically significant difference between males and females except the average of absolute RC, IRF and absolute HLR ($p < 0.0001$). The mean of MRV (114.1 ± 7.9 fL) was also significant higher than MCV (86.2 ± 3.4 fL).

For both percentage and absolute RC, there was no statistical difference between manual and RC using Coulter Gen S. Fig. 1 shows the comparison between the manual reference method and the Coulter Gen S. The linear regression statistics showed the following results: intercept = 0.430, slope = 0.900, $r = 0.900$. For absolute RC, intercept = 0.041, slope = 0.720, $r = 0.824$.

The RC as percentage and absolute value showed high correlation with HLR% ($r = 0.882$, 0.803 , $p = 0.001$) and HLR# ($r = 0.840$, 0.876 , $p = 0.001$), respectively. IRF also correlated with HLR% ($r = 0.712$, $p = 0.001$), HLR# ($r = 0.680$, p

Table 1 Mean and standard deviation of complete blood count using Coulter Gen S.

Parameters	Males (n = 21)	Females (n = 24)	Total (n = 45)
Age (years)	21±3	22±4	21±4
White blood cell			
WBC count (x 10 ⁹ /L)	7.2±1.6	7.2±1.2	7.2±1.4
Red blood cell			
RBC count (x 10 ¹² /L)	5.26±0.30	4.53±0.30	4.87±0.46
Hb (g/dL)	14.9±0.7	12.8±0.8	13.9±1.3
Hct (%)	45.0±2.3	39.2±2.6	41.9±3.6
MCV (fL)	85.8±2.9	86.6±4.1	86.2±3.8
MCH (pg)	28.5±1.1	28.5±1.7	28.5±1.5
MCHC (%)	33.3±0.4	33.7±0.4	33.5±0.4
RDW (%)	12.9±0.6	13.1±0.9	13.0±0.8
Platelet			
Platelet count (x 10 ⁹ /L)	228.6±50.6	242.3±45.4	235.0±48.5
Pct (%)	0.201±0.037	0.214±0.043	0.206±0.040
MPV (fL)	8.9±0.7	9.0±0.7	8.9±0.7
PDW (%)	15.5±0.4	15.3±0.4	15.4±0.4

Table 2 Mean and standard deviation of reticulocyte count and other parameters using manual and Coulter Gen S.

Parameters	Males (n = 21)		Females (n = 24)		Total (n = 45)	
	x (SD)	95% range	x (SD)	95% range	x (SD)	95% range
Reticulocyte count (%)						
Manual	1.1±0.3	(0.6-1.9)	1.1±0.3	(0.5-1.8)	1.1±0.4	(0.5-1.9)
Coulter Gen S	1.1±0.3	(0.6-1.7)	1.2±0.3	(0.7-1.7)	1.1±0.4	(0.6-1.7)
Abs. reticulocyte count (x 10⁹/L)						
Manual	56±16	(27-83)	49±16	(24-82)	52±16	(26-83)
Coulter Gen S	58±15	(35-84)	53±16	(24-86)	55±16	(24-90)
IRF	0.323±0.061	(0.214-0.439)	0.293±0.046	(0.234-0.423)	0.308±0.055	(0.243-0.438)
HLR% (%)	0.366±0.128	(0.154-0.616)	0.338±0.099	(0.168-0.510)	0.355±0.111	(0.168-0.513)
HLR# (x 10⁹/L)	0.019±0.007	(0.008-0.033)	0.015±0.005	(0.008-0.024)	0.017±0.006	(0.008-0.026)
MRV (fL)	115.4±6.5	(101.6-128.0)	113.1±8.7	(95.3-125.0)	114.1±7.9	(98.5-125.5)
MSCV (fL)	92.7±3.4	(82.5-98.2)	91.7±9.5	(79.9-97.4)	92.1±4.3	(882.9-97.8)

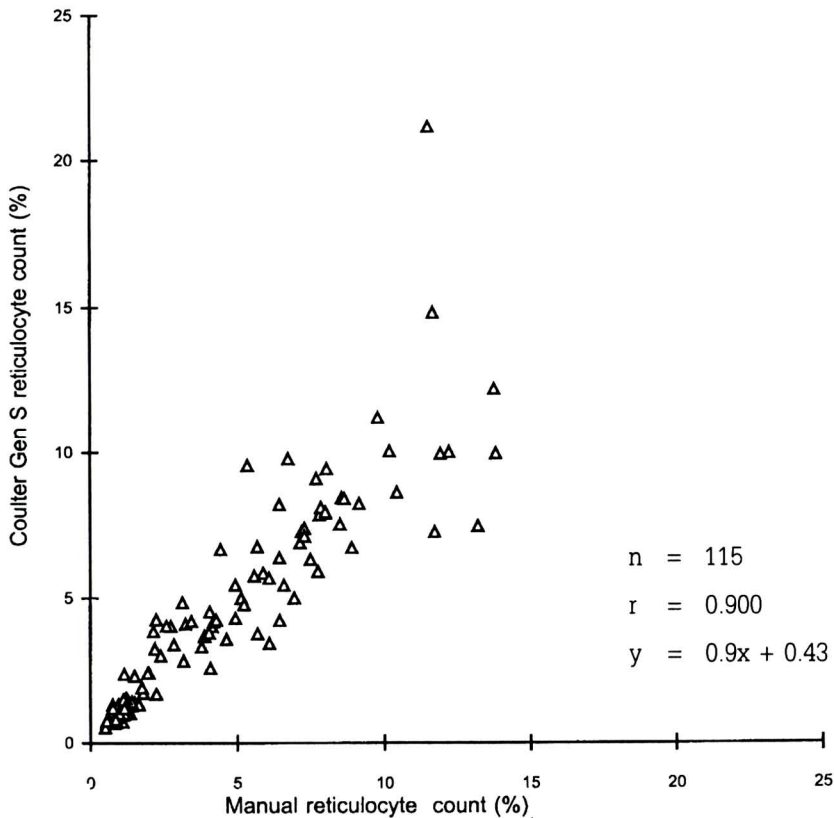


Fig. 1 Comparison of Coulter Gen S reticulocyte percentage count with manual reticulocyte count

= 0.001) and MRV ($r = 0.233$, $p = 0.01$) respectively. The MRV also significantly correlated with RC percentage with $r = 0.236$ ($p=0.01$). The correlation between the hematologic variables and RC and others parameters are summarized in Table 3, only RDW showed positive correlation.

Discussion

Counting of reticulocytes in the peripheral blood is a simple and outstanding method to assess erythroid bone marrow function. It provides not only useful clinical information, in the diagnosis and classification of anemia, but

also in monitoring therapeutic response. Unfortunately, the manual counting of reticulocytes presents a number of problems, especially time consuming and associated with imprecision¹⁻³. The automated analysis has significantly improved the precision and semiquantitative assessment of reticulocyte RNA levels. In this report, we have employed Coulter Gen S blood analyzer's for reticulocyte counting. This instrument is upgraded from semi-automated RC, Coulter STKS and MAXM, using supravital stain, NMB.

The mean of RC of healthy subjects for Coulter Gen S was compatible with the manual

Table 3 Correlation between reticulocyte count and other parameters obtained with Coulter Gen S.

	Rbc count (10 ¹² /L)	Hb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (%)	RDW (%)
Reticulocyte (%)							
Manual	-.4799*	-.5942*	-.4985*	-.1982	-.4481*	-.7201*	.5524*
Coulter Gen S	-.3265*	-.3068*	-.2861**	-.0227	-.1229	-.2419	.3271*
Abs. reticulocyte (10⁹/L)							
Manual	-.3006**	-.4695*	-.3610*	-.2046	-.4342*	-.7075*	.4225*
Coulter Gen S	-.2316**	-.5071*	-.3989*	-.3634*	-.5595*	-.7186*	.4590*

*p = 0.001 **p = 0.01

method. This is because both methods use the same supravital stain. The reference intervals of two methods agreed with those obtained by other automated studies in Thai population⁹⁻¹¹. The mean percentages of RC in this study showed no significant difference between males and females. Some earlier studies by both manual¹² and automated¹³ methods have described significantly higher reticulocyte percentages in females than males. However, other authors reported no appreciable difference by flow cytometry^{9,11,14-16}. Moreover, in one study of 265 adults, results showed a slightly lower mean percentage in females (0.92 %) than in males (1.07 %)¹⁷. In contrast, the mean of absolute RC in this study was higher in males than in females. These results agreed with other study using Technicon H-3⁹. For our data, the IRF and absolute HLR in males are significantly higher. IRF and HLR were the parameters which similar to IRF (MFR+HFR) of Sysmex R series. Takubo, et al¹⁸ and Tarallo, et al¹⁹ found that HFR and MFR counts among

males were higher than females. In contrast, the LFR counts of females were significantly higher than males. This may support that immature reticulocytes in males are higher than females and that the maturation of reticulocyte in females may be quicker than males. Another explanation may be that the RBC production in bone marrow is more active in males than females since male hormones accelerate red blood cell production²⁰.

The NMB method for counting reticulocyte on the Coulter Gen S provides an opportunity to improve the precision of reticulocyte enumeration. The manufacturer, Coulter electronics⁷ reported CV between 5.5-16.5% for RC 15-0%. In this study, although the within-run CVs of 2-17% over the range of reticulocyte values of 6.5-0.8% (data not shown) but the imprecision of the microscopic method has the CVs varied from 25-50%¹⁻³. This support the precision of automated technique over the manual method. Correlation analysis of Coulter Gen S and manual RC in this study produced an r

value of 0.900 which compatible with other reports²¹⁻²³.

Another advantages of the automated analyzers are the additional parameters of reticulocyte. For the Coulter Gen S, MRV of healthy subjects are significantly higher than MCV with mean 27.9% and the volume ratio (MRV/MCV) = 1.32. This confirms that maturation of the reticulocyte is associated with loss of size and increased in density²⁴. The value of MRV in this reports is slightly higher than those obtained by Technicon H-3 with a volume ratio (MCV of reticulocyte/MCV, 110.3/88.7) of approximately 1.249. Using the same instrument, d'Onofrio, et al²⁵ also found the same volume ratio in the Italian population and suggested that the measurement of reticulocyte and red blood cell indices seem to be more sensitive indicator of sudden erythropoietic changes than conventional MCV since a changes of MRV occurs much earlier than the MCV.

In conclusion, we have shown a good correlation between Coulter Gen S and manual method. The automated analyzer provides both efficient and reliable for assessing RC. Additional parameters i.e. HLR, MRV, are also obtained and should be further utilized in pathologic conditions to enhancement of the clinical utility in monitoring early recovery of erythropoietic activity of bone marrow.

References

1. Savage RA, Skoog DP, Rabinovitch A. Analytical inaccuracy and imprecision in reticulocyte counting. A preliminary report form the College of American

- Pathologists reticulocyte project. *Blood Cell* 1985;11:97-112.
2. Koepke JF, Koepke JA. Reticulocytes. *Clin Lab Haematol* 1986;8:169-79.
3. Lohmann RC, Crawford LN, Wood DE. Proficiency testing in reticulocyte counting. *Clin Lab Haematol* 1994;16:57-64.
4. Heilmeyer L. Blutfarbstoffwechselstudien. *Dtsch Arch Klin Med* 1931;171:123-53.
5. Peebles DA, Hochberg A, Clarke TD. Analysis of manual reticulocyte counting. *Am J Clin Pathol* 1981; 76:713-7.
6. NCCLS. Proposed standard H16-P. Method for reticulocyte counting. Villanova, PA: National Committee for Clinical Laboratory Standards, 1985.
7. Coulter Corp. Coulter Gen S System on line reference manual. Coulter Corp. PN 4237221, 1996.
8. Hall R, Malia RG. Basic haematological practice. In: Hall R, Malia RG, eds. *Medical laboratory Haematology*. 2nd ed. Oxford: Butterworth-Heinemann, 1991: 84-112.
9. Charuruks N, Limpanasithikul W, Voravud N, Virochpoka T, Nuchprayoon C. Reference ranges of reticulocytes in adults. *J Med Assoc Thai* 1998;81:357-64.
10. Khuapinant A, Bunyaratvej A, Tatsumi N, Pribwia M, Fucharoen S. Number and maturation of reticulocytes in various genotypes of thalassemia as assessed by flow cytometry. *Acta Haematol* 1994;91:119-25.
11. Lamchiagdhase P, Pattanapunyasat K, Tangtiyaphan W, Kulrisombat K. Flow cytometric reticulocyte analysis using thiazole orange. *J Med Tech Assoc Thai* 1996;24:157-66.
12. Disse A, Kurth D. Circulating reticulocytes in normal adults as determined by the new methylene blue method. *Am J Clin Pathol* 1970;53:481-4.
13. Carter JM, McSweeney PA, Wakem PJ, Nemet AM. Counting reticulocytes by flow cytometry: use of thiazole orange. *Clin Lab Haematol* 1989;11:267-71.
14. Ferguson DJ, Lee S, Gordon PA. Evaluation of reticulocyte counts by flow cytometry in a routine laboratory. *Am J Clin Hematol* 1990;33:13-7.
15. Bowen D, Bentley N, Hoy T, Cavill I. Comparison of a

- modified thiazole orange technique with a fully automated analyser for reticulocyte counting. *J Clin Pathol* 1991;44:130-3.
16. Lofness KG, Kohnke ML, Geier NA. Evaluation of automated reticulocyte counts and their reliability in the presence of Howell-Jolly bodies. *Am J Clin Pathol* 1994;101:85-90.
 17. Tatsumi N, Tsuda I, Kojima K, Nari M, Setoguchi K. An automated reticulocyte counting method: preliminary observations. *Med Lab Sci* 1989;46:157-60.
 18. Takubo T, Kitano K, Ohto Y, Ikemoto T, Kikuchi T, Inai S. The usefulness of the R-1000 automated reticulocyte counter in a clinical hospital laboratory. *Eur J Haematol* 1989;43:88-9.
 19. Tarallo P, Humbert JC, Mahassen P, Fournier B, Henny J. Reticulocytes: Biological variations and reference limits. *Eur J Haematol* 1994;53:11-5.
 20. Gardner FH, Nathan DG, Piomelli S, Cummins JF. The erythrocythaemic effects of androgen. *Br J Haematol* 1968;14:611-5.
 21. Buttarello M, Bulian P, De Pra M, Barbera P, Rizzotti P. Reticulocyte quantification by Coulter MAXM VCS (volume, conductivity, light scatter) technology. *Clin Chem* 1996;42:1930-7.
 22. Rudensky B. Comparison of a semi-automated new Coulter methylene blue method with fluorescence flow cytometry in reticulocyte counting. *Scand J Clin Lab Invest* 1997;57:291-6.
 23. Davies JI, Smyth MS, Martin JHJ. Automated reticulocyte counting :evaluation of the CoulterR STKS haematology analyser reticulocyte counting function. *Clin Lab Haematol* 1997;19:89-92.
 24. Lowenstein LM. The mammalian reticulocyte. *Int Rev Cytol* 1959;8:135-74.
 25. d'Onofrio G, Chirillo R, Zini G, et al. Simultaneous measurement of reticulocyte and red blood cell indices in healthy subjects and patients with microcytic and macrocytic anemia. *Blood* 1995;85:818-23.

การวิเคราะห์เรติคูลอไซท์ด้วยเครื่อง Coulter Gen S

พรวิทย์ ลำเจียกเทศ, อำพรธน์ ไชยพันธ์*, ศิรินทร มาเหิม** และ โกวิท พัฒนาปัญญาสัตย์***

ภาควิชาจุลทรรศน์ศาสตร์คลินิก คณะเทคนิคการแพทย์ มหาวิทยาลัยมหิดล, *โรงพยาบาลขอนแก่น จังหวัดพัทลุง, **โรงพยาบาลขอนแก่น จังหวัดขอนแก่น, ***สถานส่งเสริมการวิจัย คณะแพทยศาสตร์ศิริราชพยาบาล, มหาวิทยาลัยมหิดล, กรุงเทพมหานคร

บทคัดย่อ: จำนวนเรติคูลอไซท์ที่มีประโยชน์ในการวัดความสามารถในการสร้างเม็ดเลือดแดงของไขกระดูก ใช้ประกอบการวินิจฉัยภาวะโลหิตจาง ติดตามการรักษาหรือเป็นวิธีการตรวจสอบการเกิดใหม่ของเม็ดเลือดแดงหลังการปลูกถ่ายไขกระดูก หรือ stem cell รายงานนี้ได้ประเมินการนับจำนวนเรติคูลอไซท์โดยเครื่องอัตโนมัติ Coulter Gen S เปรียบเทียบกับวิธี manual โดยใช้เลือดจากหลอดเลือดดำของผู้มีสุขภาพดี 45 รายที่มีค่าทางโลหิตวิทยาปกติ และผู้ป่วยโลหิตจาง 70 ราย ผลการศึกษาพบว่า ไม่มีความแตกต่างทางสถิติระหว่างทั้ง 2 วิธี และมีความสัมพันธ์กันดี โดยค่า $r = 0.900$ วิธีใช้เครื่องอัตโนมัติเป็นวิธีที่มีความแม่นยำและความถูกต้อง และยังสามารถรายงานค่าพารามิเตอร์อื่นๆ ที่มีประโยชน์ในการประเมินการแก่ตัวของเรติคูลอไซท์ ได้แก่ mean reticulocyte volume (MRV), immature reticulocyte fraction (IRF), ร้อยละและค่าสัมบูรณ์ของ high light scatter reticulocyte (HLR)

Key Words: ● Reticulocyte count ● Automation

วารสารโลหิตวิทยาและเวชศาสตร์บริการโลหิต 2542;9:211-8.