

Stability of New Erythrocyte and Reticulocyte Parameters in Testing for Anemia on the Sysmex XN 9000

Elise Schapkaitz, MD*

Laboratory Medicine 49:3:219-225

DOI: 10.1093/labmed/lmx095

ABSTRACT

Background: New erythrocyte and reticulocyte parameters provide improved classification of anemia and monitoring of erythropoietic activity. Parameters available on the Sysmex XN analyzers include the percentage of microcytic red blood cells (%Micro-R), percentage of macrocytic red blood cells (%Macro-R), percentage of hypochromic red blood cells (%Hypo-He), percentage of hyperchromic red blood cells (%Hyper-He), reticulocyte hemoglobin content (Ret-He), and immature reticulocyte fraction (IRF). However, use of these parameters is limited by specimen stability.

Methods: To verify the stability of the new erythrocyte and reticulocyte parameters during prolonged storage, 20 blood specimens were analyzed on the Sysmex XN-9000 hematology analyzer. The specimens included healthy patients (60%) and patients with anemia (40%). The specimens were stored at room temperature (RT) and at 4° to 8°C. Analysis was performed after 12, 24, 48, and 72 hours of storage.

Results: Reticulocyte parameters IRF and Ret-He were precise and stable for at least 72 hours after collection when stored at RT and 4° to 8°C. The volume-dependent parameters, %Macro-R and %Micro-R, were stable for less than 12 hours after collection at RT (mean [SD%], 6.55 [3.19%] and -20.70 [10.37%], respectively). Storage at 4° to 8°C showed a reduction in osmotic swelling. However, %Macro-R and %Micro-R were stable for less than 12 hours after collection (mean [SD%], 4.89 [2.02%] and -17.17 [8.38%], respectively). Similarly, %Hypo-He showed a mean (SD%) increase of 0.73 (4.05%) and %Hyper-He showed a decrease of -0.70 (9.72%) at less than 12 hours after storage at 4° to 8°C.

Conclusion: New reticulocyte parameters stored at RT and 4° to 8°C are suitable for testing on the Sysmex XN analyzer.

Keywords: reticulocyte hemoglobin content, percentage hypochromic cells, storage time, storage temperature

Anemia constitutes a significant disease burden in the developing world, particularly in Southern Africa, where tuberculosis and human immunodeficiency virus (HIV) are endemic. New extended reticulocyte and erythrocyte

parameters offer several advantages for the assessment of anemia and monitoring of erythropoietic activity.

Abbreviations

HIV, human immunodeficiency virus; %Micro-R, percentage of microcytic red blood cells; %Macro-R, percentage of macrocytic red blood cells; %Hypo-He, percentage of hypochromic red blood cells; %Hyper-He, percentage of hyperchromic red blood cells; Ret-He, reticulocyte hemoglobin content; IRF, immature reticulocyte fraction; RBCs, red blood cells; RT, room temperature; CMJAH, Charlotte Maxeke Johannesburg Academic Hospital; CLSI, Clinical and Laboratory Standards Institute; K₂, dipotassium; EDTA, ethylenediaminetetraacetic acid; %CV, percentage coefficient of variation; ANOVAs, analyses of variance; FBC, full blood count; MCV, mean cell volume

Parameters available on the latest Sysmex automated hematology analyzer, the XN 9000 (Sysmex Corporation), include the percentage of microcytic red blood cells (%Micro-R), percentage of macrocytic red blood cells (%Macro-R), percentage of hypochromic red blood cells (%Hypo-He), percentage of hyperchromic red blood cells (%Hyper-He), reticulocyte hemoglobin content (Ret-He), and immature reticulocyte fraction (IRF). Recent studies¹⁻⁴ have demonstrated the diagnostic usefulness of these parameters in different population groups. In combination, these parameters provide information at a cellular level regarding iron availability in red blood cells (RBCs) and reticulocytes. The most widely studied is the Ret-He, the hemoglobin content of the reticulocytes, which allows for early detection of iron deficiency.² Also, the Sysmex analyzer reports the %Hypo-He, or the mature RBC subpopulations with reduced hemoglobin content,

Department of Molecular Medicine and Hematology, University of Witwatersrand Medical School, Johannesburg, South Africa

*To whom correspondence should be addressed.
elise.schapkaitz@nhls.ac.za

which reflects the iron status of the RBC during the circulating lifespan. In thalassemia, a greater proportion of RBC is microcytic, compared with iron deficiency. The % microcytic/% hypochromic ratio index, based on new RBC parameters, has been shown to be a more sensitive screening tool, compared with alternative discriminant formulae indices, for discriminating iron deficiency and thalassemia.⁵

However, a limitation of these new extended parameters is that they are affected by storage time and temperature. Osmotic swelling of RBC during storage at RT affects volume-dependent variables and the measurement of the cellular hemoglobin concentration.

It is recommended that the new reticulocyte parameters be analyzed 24 hours after specimen collection when stored at room temperature (RT).^{6,7} However, erythrocyte parameters useful for diagnosis and monitoring of anemia are unreliable after 6 hours.^{6,7} On a practical level, this fact makes it difficult to refer specimens from remote locations to a centralized laboratory for anemia work-up. These studies, however, are small and specific to the hematology analyzers investigated. Further, studies on stability beyond 48 hours are limited. Large academic laboratories, such as the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) Laboratory in Johannesburg, South Africa, are commonly faced with the situation in which a specimen collected on Friday is not received for analysis until Monday morning. In accordance with the Clinical and Laboratory Standards Institute (CLSI) recommendation that laboratories should evaluate stability in their specific settings, a study was performed to investigate the stability of the new erythrocyte and reticulocyte parameters on the Sysmex XN 9000 during storage at RT and 4° and 8°C.⁸ The objective of this study was to determine laboratory criteria for storage time and temperature for specimens referred for the work-up of anemia.

Materials and Methods

Study Design and Population

This study was conducted at the Main Hematology Laboratory at CMJAH National Health Laboratory Service Complex in Johannesburg, South Africa. Blood specimens were selected from 20 adult patients (mean [SD] age of 46 [13] years, with a male:female ratio of 1:1) from the hematology workload

representative of the patient population. Of these, 60% were normal specimens and 40% were from patients with anemia, with median hemoglobin of 87.5 g/L (range, 41–125 g/L). The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (M090688).

Blood Specimen Collection

Ten milliliters of venous blood were collected in dipotassium (K₂) ethylenediaminetetraacetic acid (EDTA) vials (1.5–2.2 mg of dipotassium EDTA per milliliter of blood) (Becton, Dickinson and Company) left over after routine testing of each patient. K₂EDTA is the anticoagulant of choice for automated hematology analyzers. The specimens were analyzed within 2 hours of collection (time 0) at RT. Because of limited specimen volume, specimens were not analyzed in duplicate. Aliquots of the selected specimens were stored at RT (18°–24°C) and 4°–8°C. Analysis was performed after 12, 24, 48, and 72 hours of storage to assess changes with time.

We performed between-run precision analysis with normal and abnormal high- and low-quality control material (Sysmex Corporation) once a day during a 10-day period. The state-of-the-art acceptance criteria for the obtained percentage coefficient of variation (%CV) was less than 10% for reticulocyte parameters and less than 1.1% for erythrocyte parameters.⁹

Analytical Methods

The following tests were performed on the Sysmex XN 9000 analyzer in the CMJAH laboratory: %Micro-R, %Macro-R, %Hypo-He, %Hyper-He, Ret-He, and IRF. On the XN 9000, RBCs are counted in the RBC/platelet channel using the sheath flow direct current detection method with hydrodynamic focusing. The RBC parameters, %Micro-R and %Macro-R, indicate the percentage of RBC with a volume less than 60 fl and greater than 120 fl, respectively. The %Hypo-He and %Hyper-He are determined from high-angle forward-scattered light, which is directly proportional to RBC-He. %Hypo-He and %Hyper-He refer to the percentage of RBC with a hemoglobin content of less than 17 pg and greater than 49 pg, respectively.

In the reticulocyte channel, cells are stained with a nucleic-acid dye and analyzed by fluorescent flow cytometry. Forward-scattered light versus fluorescence is represented as a scattergram showing mature RBC and reticulocytes. The Ret-He is a log transformation of Ret-Y that is derived from the forward-scattered light of the reticulocyte. Reticulocytes are separated from mature RBC according to

Table 1. Stability Analysis (Mean Percentage Difference) at Room Temperature (18°–24°C) and 4–8°C

Analyte	Room-Temperature Stability Analysis, Mean (SD)				Stable Until (h)
	n = 20				
	12 h (%)	24 h (%)	48 h (%)	72 h (%)	
Ret-He (pg)	–1.20 (1.37)	–1.31 (2.20)	–3.49 (1.75)	–4.16 (2.33)	>72
%Micro-R (%)	–20.70 (10.37)	–23.95 (19.11)	–57.57 (11.43)	–63.88 (10.46)	<12
%Macro-R (%)	6.55 (3.19)	7.29 (3.89)	13.40 (6.35)	16.58 (7.89)	<12
%Hypo-He (%)	3.23 (6.71)	2.22 (4.94)	–9.14 (16.73)	–13.89 (36.71)	<12
%Hyper-He (%)	–8.33 (13.61)	–10.00 (17.48)	–15.83 (21.68)	–25.33 (25.55)	<12
IRF (%)	0.77 (11.55)	–0.26 (15.68)	–6.13 (9.75)	–5.05 (12.78)	>72
4°–8°C Stability Analysis Mean (SD)					
Ret-He (pg)	0.30 (0.90)	–1.04 (1.78)	0.99 (1.98)	0.91 (2.35)	>72
%Micro-R (%)	–17.17 (8.38)	–23.47 (23.72)	–27.85 (26.00)	–34.15 (32.46)	<12
%Macro-R (%)	4.89 (2.02)	5.39 (1.85)	5.25 (2.13)	5.19 (2.04)	<12
%Hypo-He (%)	0.73 (4.05)	2.59 (9.54)	3.18 (9.71)	10.71 (27.30)	<12
%Hyper-He (%)	–0.70 (9.72)	–10.18 (16.97)	–4.73 (18.90)	–4.42 (20.18)	<12
IRF (%)	–0.72 (7.44)	0.55 (10.32)	1.25 (10.02)	–1.5 (12.26)	>72

Ret-He, reticulocyte hemoglobin content; %Micro-R, percentage of microcytic red cells; %Macro-R, percentage of macrocytic red cells; %Hypo-He, percentage of hypochromic red cells; %Hyper-He, percentage of hyperchromic red cells; IRF, immature reticulocyte fraction.

their fluorescence intensity, which is directly proportional to RNA content. The IRF is the sum of the MFR (medium-fluorescence reticulocytes) and HFR (high-fluorescence reticulocytes).

Statistical Analysis

The mean percentage difference from the value at time 0 was calculated. The *stability of a parameter* was defined in accordance with the CLSI definition, namely, when the difference between the means exceeds the SD of the first mean at 95% confidence intervals.⁸ Data were analyzed using repeated measures analyses of variance (ANOVAs). Statistical significance was set at a *P* value of 0.05 or less.

Results

Stability Analysis

Table 1 presents the mean percentage difference (SD) of specimens stored at RT and 4° to 8°C, respectively. Reticulocyte parameters IRF and Ret-He were stable for at least 72 hours after collection when stored at RT and 4° to 8°C. After RT storage for 24 hours, we observed a gradual decrease in the Ret-He and IRF. Storage at 4° to 8°C showed improved stability for at least 72 hours (**Figures 1**

and **2**). The volume-dependent parameters % Hypo-He, %Hyper-He, %Micro-R, and %Macro-R were stable for less than 12 hours when stored at RT and 4° to 8°C. Storage at 4° to 8°C showed a reduction in the degree of osmotic swelling during a 72-hour period.

During storage at RT, %Macro-R showed a mean% (SD) increase of 6.55% (3.19) at 12 hours, 7.29% (3.89) at 24 hours, 13.40% (6.35) at 48 hours, and 16.58% (7.89) at 72 hours. In contrast, during storage at 4° to 8°C %Macro-R showed a mean (SD) increase of 4.89% (2.02) at 12 hours, 5.39% (1.85) at 24 hours, 5.25% (2.13) at 48 hours, and 5.19% (2.04) at 72 hours. Similarly, %Hypo-He showed an increase of 3.23% (6.71), and %Hyper-He showed a mean% (SD) decrease of –8.33% (13.61) at less than 12 hours after collection at RT. A decrease in the % microcytic/% hypochromic ratio was also observed, of –17.93% (16.99%) at 12 hours, –11.07 (19.50%) at 24 hours, –55.76% (15.52%) at 48 hours, and –59.98% (21.81%) at 72 hours during storage at RT. Smaller differences were observed during storage at 4° to 8°C; however, %Hypo-He, %Hyper-He, and % microcytic/% hypochromic ratio were unstable beyond 12 hours.

Precision Analysis

Precision data were collected during a 10-day period (**Table 2**). The volume-dependent erythrocyte parameters, namely, %Hypo-He, %Hyper-He, %Micro-R, and

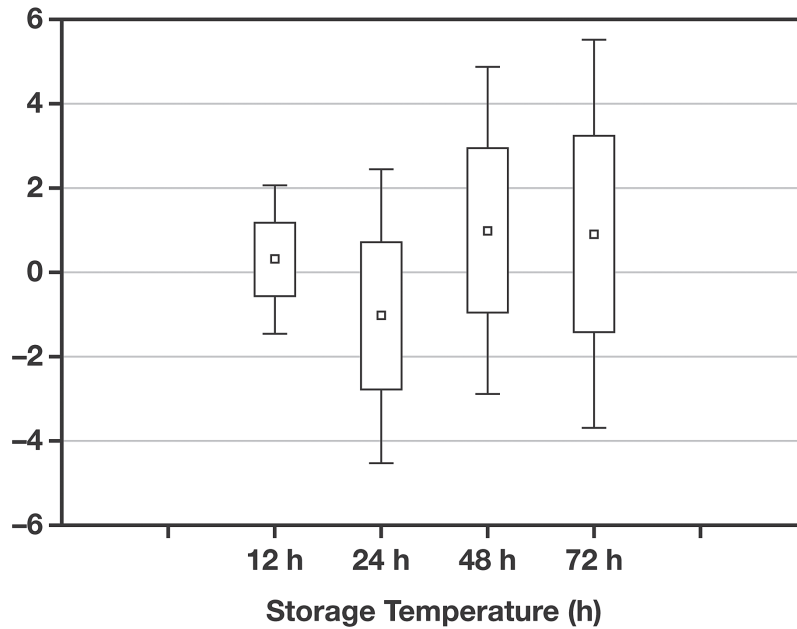


Figure 1

Boxplot of the mean percentage difference during storage at 4°–8°C for the reticulocyte hemoglobin content (Ret-He).

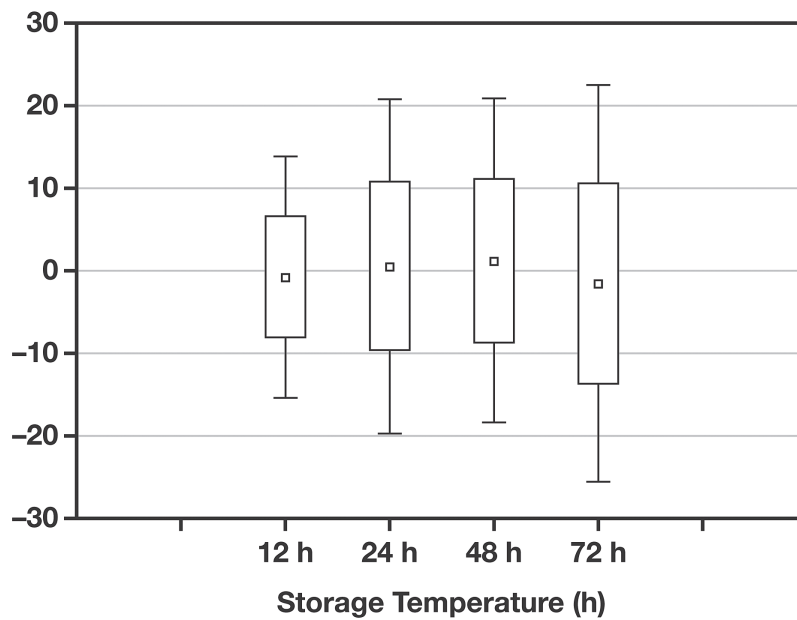


Figure 2

Boxplot of the mean percentage difference during storage at 4°–8°C for the immature reticulocyte fraction (IRF).

Table 2. Between-Run Precision Analysis During a 10-Day Period

Normal Control Specimens							
Value	Ret-He	RBC-He	%Hypo-He	%Hyper-He	%Micro-R	%Macro-R	IRF
SD	0.15	0.13	0.05	0.03	0.50	0.07	1.71
%CV	0.6	0.46	11.74	6.20	10.10	1.65	7.21
Control Specimens with Abnormally Low Values							
SD	0.12	0.11	0.04	0	1.48	0.06	1.35
%CV	0.52	0.43	19.17	0	9.23	2.46	4.81
Control Specimens with Abnormally High Values							
SD	0.25	0.17	0.15	0	0.17	0.10	2.33
%CV	0.94	0.55	8.91	0	10.20	2.07	7.09

Ret-He, reticulocyte hemoglobin content; RBC-He, hyperchromic red blood cells; %Hypo-He, percentage of hypochromic red blood cells; %Hyper-He, percentage of hyperchromic red blood cells; %Micro-R, percentage of microcytic red blood cells; %Macro-R, percentage of macrocytic red blood cells; IRF, immature reticulocyte fraction; %CV, percentage coefficient of variation.

%Macro-R, when reanalyzed daily, revealed a %CV of 11.74%, 6.20%, 10.10%, and 1.65%, respectively, for the normal quality-control specimen.

Nevertheless, storage at 4° to 8°C showed minimal changes during a 72-hour period.

Discussion

New generation automated hematology analyzers provide information regarding individual cell characteristics by measuring RBC and reticulocyte subpopulations with inadequate hemoglobin content and volume. On the Sysmex hematology analyzer, these new reticulocyte and erythrocyte parameters offer a simple and cost-effective alternative to traditional laboratory tests, particularly in areas where resources are limited. The main disadvantage is that these parameters are affected by storage time and temperature. In centralized laboratories where referred aged specimens make up a significant proportion of the workload, the storage time and temperature must be taken into consideration.

This study confirms that reticulocyte parameters, including IRF and Ret-He, were precise and least affected by storage temperature and time. These parameters can be analyzed 72 hours after specimen collection when stored at RT. Previous studies^{3,6} have also demonstrated that the Ret-He is highly stable during a 48-hour period, with minimal changes at RT and 4° to 8°C storage. With prolonged RT storage, we observed a decrease in the Ret-He mean (%) difference (SD) of -3.49% (1.75%) at 48 hours and -4.16% (2.33%) at 72 hours. Similarly, we observed a decrease in the IRF of -6.13% (9.75%) at 48 hours and -5.05% (12.78%) at 72 hours. However, this finding is unlikely to have an effect on clinical diagnoses and management.

The finding that reticulocyte parameters (Ret-He and IRF) are stable for up to 72 hours adds to the available evidence that specimen stability varies according to storage time and temperature. Reticulocyte parameters are frequently referred from peripheral pediatric clinics, primary healthcare clinics, or dialysis units to centralized laboratories as part of the diagnostic work-up for anemia. Thus, it is important for laboratory staff to be aware of the changes that occur during storage, so they can decide whether to accept or reject aged specimens.

In addition, the Ret-He test has several advantages for routine use. It offers a simple and cost-effective alternative to traditional biochemical tests, particularly where resources are limited. Unlike biochemical tests, these parameters are not influenced by infection or inflammation and can be performed on the same specimen used for full blood count (FBC) analysis. This test can be performed on 1.0 to 1.5 mL of blood in a single K₂EDTA tube; in children, a finger prick would produce an adequate specimen, thus eliminating the need for additional tubes of blood to assay for the biochemical parameters.

In this study, as expected, the RBC volume-dependent measurements (%Hypo-He, %Hyper-He, %Micro-R, and %Macro-R) were stable for less than 12 hours when stored at RT and at 4° to 8°C. A moderate degree of imprecision was also observed during a 10-day period. This finding indicates drift over time that also contributed to the variability in the absence of storage time and temperature-related changes. On the Sysmex analyzer, the %Hypo-He reflects the percentage of mature RBC subpopulations with a

reduced hemoglobin content of less than 17 pg, whereas on the ADVIA 120 (Siemens Healthcare Diagnostics), the Hypo% is based on the intracellular hemoglobin concentration.

Although the analytical methods differ, the percentage of hypochromic red cells (%HPO) on the Sysmex showed a progressive increase at less than 12 hours after collection during storage at RT. Similarly, the %Hypo-He on the CELL-DYN Sapphire analyzer (Abbott Laboratories Inc.) was stable for up to 6 hours at RT.^{6,10} Sellors et al⁷ observed a decrease in the Hypo% on the ADVIA (equivalent of the % Hypo-He) during storage at 4°C. In contrast, we observed an increase in the mean (SD) %Hypo-He of 0.73% (4.05%) at 12 hours, 2.59% (9.54%) at 24 hours, 3.18% (9.71%) at 48 hours, and 10.71% (27.30%) at 72 hours with storage at 4° to 8°C. A possible explanation is that Sellors et al included only specimens from iron-replete healthy volunteers, whereas we selected specimens of which 60% had normal amounts of iron and 40% indicated anemia, with median hemoglobin of 87.5 g per L (range, 41–125 g/L), which is representative of our patient population.

Another useful parameter that we evaluated is the %Hyper-He, an indicator of the presence of spherocytes in the assessment of hereditary spherocytosis (HS) and autoimmune hemolysis.^{11,12} As expected, storage at RT showed a subsequent mean (SD) decrease in %Hyper-He of –8.33% (13.61%) at 12 hours, –10.00% (17.48%) at 24 hours, –15.83% (21.68%) at 48 hours, and –25.33% (25.55%) at 72 hours of storage. However, no cases of HS were included. The hyperchromic RBC parameter (%HPR) has been evaluated on the CELL-DYN Sapphire analyzer in a population of patients with HS. Similarly, this study also showed a significant decrease in the hyperchromic RBC after 6 hours of storage.¹²

A sensitive screening tool based on new erythrocyte parameters for discriminating iron deficiency and thalassemia is the % microcytic/% hypochromic ratio index.⁵ In thalassemia, a greater proportion of RBCs are microcytic, compared with those having iron deficiency. We observed a decrease in the % microcytic/% hypochromic ratio owing to osmotic RBC swelling at less than 12 hours during storage at RT. Alternative indices to differentiate iron deficiency and thalassemia use traditional RBC parameters, such as mean cell volume (MCV), mean cell hemoglobin, RBC count, RBC distribution width, and hemoglobin.

It is well established that the stability of the RBC and hemoglobin is acceptable after 24 hours.¹³ Stability studies on new-generation automated analyzers^{14,15} have also demonstrated longer stability—as long as 24 hours—for the MCV and, consequently, the hematocrit level at RT, regardless of the analyzer principle. Further, storage of specimens at 4° to 8°C increases the stability of the MCV with a predictable increase in RBC swelling.¹³ Thus, such indices are more suitable than the % microcytic/% hypochromic ratio in referred specimens owing to storage degradation.

A limitation of this study is that stability was evaluated under optimal conditions on specimens that were received in the laboratory within 2 hours of collection. Specimens referred for testing, however, are often subject to variation in temperature during collection and transportation. Further, the specimen size and the proportion of specimens indicating anemia was small.

In conclusion, this study recommends that measurement of the Ret-He and IRF can be performed on K₂EDTA specimens stored, preferably, at 4° to 8°C for as long as 72 hours. This practice, however, should be the exception, owing to additional preanalytical variables that may interfere with the accuracy of the results. However, storage at 4° to 8°C is not a solution for specimens referred from remote laboratories for erythrocyte parameters, namely, %Hypo-He, %Hyper-He, %Macro-R, and %Micro-R, owing to progressive RBC swelling during storage. Although the results of this study are comparable to those from other analyzers, the findings of this study are specific to the Sysmex hematology analyzers. **LM**

Acknowledgements

We thank Shilla Raburabu, BTech, Phoyisile Nkosi, BTech, and Bongiwe Moyake, BTech, for their technical assistance.

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