Identification and Enumeration of Nucleated Red Blood Cells in Peripheral Blood

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Erythropoiesis is an exquisitely regulated process that results in the production of red blood cells. In humans, nucleated red blood cells (NRBC) do not appear in the peripheral blood except in the fetal and neonatal periods. The presence of NRBC in the peripheral blood in adults and children and elevated counts in neonates is encountered in many disease states and associated with a poor prognosis. Traditional methods for identification and enumeration of NRBC in the peripheral blood by manual morphology is time consuming and has low precision. The Sysmex XE-2100 automated hematology analyzer generates an automated NRBC count using a combination of technologies. The automated NRBC count has a high degree of precision, a wide range of linearity and compares favorably with the manual morphology method and with a proposed flow cytometric reference method. When the instrument is run in a discreet complete blood count (CBC)/ differential mode, the "NRBC?" suspect flag is efficient for screening for the presence of NRBC in peripheral blood. The XE-2100 NRBC count is robust, precise and accurate and represents an important advance in the analytic capabilities of the clinical hematology laboratory.

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INTRODUCTION

Erythropoiesis

The production and destruction of red blood cells (RBC) are finely regulated processes which effectively maintains the body's homeostasis and requirements for oxygen transport. In the adult, erythropoiesis occurs only in the bone marrow and involves a complex interaction of progenitor and precursor cells, cytokines and growth factors, and the stromal microenvironment. The pluripotential hemopoietic stem cell (PHSC) is present predominately in the bone marrow, but also in small amounts in the peripheral blood. The dormant PPSC is induced to cycle by cytokines including interleukins (IL) -1, -3, -4, -6, -11, -12, colony-stimulating-factor-granulocyte and kit ligand. The proliferation of PPSC is supported by cytokines, such as IL-3, -4 and granulocyte-macrophage-colony-stimulating-factor (GMCSF)[1]. PPSC are capable of generating committed progenitor cells for multiple hematopoietic cell lineages. It is hypothesized that generation of a specific committed progenitor cell is dependant on the random expression of differentiation genes corresponding to the different lineages[2] and the availability of lineage specific cytokines.

The first committed erythroid progenitor cells are the burst forming unit-erythroid (BFU-E). These cells are also present in the bone marrow and in minute amounts in the peripheral blood. The proliferation and maturation of BFU-E is dependant on cytokines including IL-3, GMCSF, Kit Ligand and erythropoietin[3, 4]. The BFU-E gives rise to a more mature progenitor cell, the colony forming unit erythrocyte (CFU-E).

This cell is exquisitely responsive to erythropoietin and dependant on this cytokine for its survival, proliferation and maturation[5]. The BFU-E and CFU-E cannot be identified morphologically. However, the CFU-E gives rise to the erythroid precursor cells, which are a series of morphologically recognizable cells[6, 7]. The proerythroblast is a large cell, with a large round nucleus, finely stippled chromatin, deeply basophilic cytoplasm and one or more nucleoli.

Further maturation of the erythroid precursor cells entails a progressive decrease in size along with nuclear and cytoplasmic changes. The basophilic erythroblast has basophilic cytoplasm, coarse nuclear chromatin and lacks nucleoli. The polychromatophilic eythroblast demonstrates a pink tinge to the cytoplasm and moderate heterochromatin. The late (orthochromic) erythroblast has pink cytoplasm and clumped chromatin. Following this

stage, the nucleus is extruded from the cell to produce a reticulocyte. The reticulocyte is released into the peripheral blood where it matures to a RBC within 24-48 hours.

NRBC in peripheral blood in normal and pathological conditions

NRBCs are not seen in the peripheral blood of normal adult humans[8]. In the fetal and neonatal period, NRBCs are routinely present in the peripheral blood and usually disappear within one week after birth[9, 10]. NRBCs have been described as rare events in the peripheral blood of pregnant women, and although in most cases they likely arise by transplacental hemorrhage of fetal blood, they may also be of maternal origin[11].

It is of interest that although humans, like all mammals, have non-nucleated RBCs in the peripheral blood, fish reptiles and birds have nucleated RBCs in the peripheral blood. The evolutionary advantage of non-nucleated RBCs in the peripheral blood is unclear. It may related to increased flexibility of the red cell enabling it to traverse the narrow sinusoids of the spleen, or the availability of more space in the RBC for hemoglobin, thus maximizing the oxygen transport function of the cell.

The exact mechanism that prevents NRBC from being released into the peripheral blood in the bone marrow in mammals has not been completely elucidated. It is unlikely that the nucleus is a major impediment for release of cells from the marrow, since nucleated granulocytes and lymphocytes are routinely released from the marrow. It is more likely that the assembly of an advanced cytoskeleton[12] and decreased adhesion of reticulocytes to the marrow stroma[13] permit their release into the peripheral blood. The actual movement of cells from the bone marrow compartment into the peripheral blood may be due to a pressure gradient between the two compartments[14].

The presence of NRBC in peripheral blood in association with certain disease states has been known for over a century[15]. Many of the early studies reported the presence of NRBC in both hematological and non-hematological diseases, and emphasized the poor prognosis associated with the presence of NRBC in the peripheral blood[8, 16, 17]. One study of 1496 cases demonstrated a 50% mortality among the patients with NRBCs in the peripheral blood[8]. A large number of different disease states have been associated with peripheral blood NRBCs[8, 16, 17]. These include: solid tumors, with or with metastases to the bone, hematological malignancies, both acute and chronic, benign hematological conditions such as hemolysis, nutritional anemia, hemorrhage, infectious mononucleosis, myelodysplasia; a range of non-hematological diseases such as septicemia, inflammatory bowel disease, chronic lung disease, fractures, myocardial infarction, liver disease and a host of other conditions.

In the perinatal period, when NRBC can routinely be found in peripheral blood, increases in these cells have been associated with chronic hypoxia and anemia from various causes, viral infections, congenital heart disease, pulmonary failure and many other conditions[9]. Even in children, NRBCs in peripheral blood are associated with many different disease states, including both neoplastic and non-neoplastic conditions[18].

Traditional evaluation of NRBC in peripheral blood

The traditional method for identification and enumeration of NRBC is by the morphological eye count[19]. This entails placing a drop of peripheral blood on a glass slide, spreading it into a thin layer, staining it with a Romanowski type stain and examining it under the microscope. This process is time consuming and requires a highly trained observer. Even with trained observers, it has been shown that 15% of cases of NRBCs in the peripheral blood may be missed which detracts from its use as a screening tool. Enumeration of NRBCs by this method entails counting 100-200 white blood cells (WBC) and noting the percentage of NRBCs to WBC. Because of the small number of cells counted, this enumeration is associated with a high degree of imprecision.

Instrumental identification and enumeration of NRBC

Automated hematology analyzers are now used in most laboratories for performing a complete blood count and differential count. It is now accepted that for enumeration of normal WBC in peripheral blood, automated hematology analyzers have better accuracy and precision than the morphological eye counts[20]. Many of the previous generations of instruments also had the ability to signal the possible presence of NRBCs by sensing an abnormal population of cells and generating a "suspect flag"[21]. Further advancements in technology have resulted in the current generation of cells analyzers, such as the Sysmex XE-2100 that can identify and enumerate NRBCs in the peripheral blood in an automated fashion.

MATERIALS AND METHODS

Sysmex XE-2100

The Sysmex XE-2100, an automated hematology analyzer is a modern, sophisticated cell analysis system that combines features of both CBC analyzers and flow cytometers[10, 22, 23]. It employs multiple technologies to produce a 30 parameter analysis of peripheral blood at throughputs of up to 150 specimens per hour. The technologies utilized include electronic aperture impedance using both direct current and radiofrequency current, differential cell membrane lysis, laser light induced forward and orthogonal light scatter and fluorescence emission. For identification and enumeration of NRBCs a combination of differential cell lysis, forward light scatter and fluorescence emission are utilized. A lysing reagent lyses all RBCs (including NRBCs) and permeabilizes and stabilizes (but does not lyse) WBC membranes. A polymethine fluorescence RNA/DNA binding dye binds to the NRBC nuclei and also to the WBC nuclei and cytoplasmic RNA. The sample is exposed to a semi-conductor laser in an optical block and analyzed for forward angle light scatter and fluorescence intensity. NRBCs are distinguished from WBC by their lower scatter and fluorescence intensity and from RBC ghosts and platelets by their higher fluorescence intensity and forward light scatter. Results are

presented as a percentage of the WBC and also as an absolute count. Simultaneously the total WBC and lymphocyte counts are corrected to exclude the NRBC.
The analyzer can also be run in a discreet CBC/ differential mode. In this mode a complete CBC and differential WBC is produced but a NRBC count is not performed, thus conserving on expensive reagents needed for performing a NRBC count. However, in this mode, if NRBCs are present, the instrument can sense an abnormal population of cells and produce a "NRBC present" flag. The sample can then be reanalyzed to include an NRBC count.

Performance characteristics of XE-2100 NRBC count

We have evaluated the performance characteristics of the XE-2100 NRBC count in a busy tertiary care hospital. Blood used for analysis were residual material from patient samples sent to the clinical laboratory for routine testing. All blood samples were collected in K_2EDTA anticoagulant, kept at room temperature and analyzed within 4 hours of collection. Blood samples were analyzed in closed mode, except when only small quantities were available, in which case they were analyzed in capillary mode. The XE-2100 instrument was calibrated by the manufacturer and 3 levels of quality control were run throughout the evaluation process.
Imprecision was determined using commercial quality control material (*e*-CHECK, Sysmex Corporation, Japan). Three different levels of analyte were run in duplicate, three times a day over a period of 21 days. The within run and total precision were calculated according to the National Committee for Clinical Laboratory Standards (NCCLS) document EP5-A[24].
Linearity was evaluated using six serial dilutions of fresh whole blood using phosphate buffered saline (PBS) as the diluent.
A comparison between the XE-2100 NRBC count and manual morphology count was performed according to the NCCLS H20-A document. A total of 262 samples were evaluated. The patients had a wide variety of diagnoses including cancer, leukemia, prematurity, sepsis, hemorrhage, arthritis, inflammatory bowel disease, bone marrow fibrosis, hemolysis, metastatic cancer, etc. All samples were run on the XE-2100 to obtain a complete CBC and differential which included a NRBC percentage and absolute count. A peripheral blood film was made and stained in all patients. A 200 cells differential count was performed on each blood film by 2 different observers and an average percent NRBC count obtained. The percentage count was multiplied by the corrected WBC count from the XE-2100 to derive an absolute NRBC count. The XE-2100 NRBC count and the manual morphology counts were compared by linear regression for both percentage and absolute counts. The sensitivity of the XE-2100 NRBC count was analyzed by constructing a matrix table.
The sensitivity of the "NRBC?" suspect flag obtained when the analyzer was run in CBC/differential mode was evaluated in 69 specimens. The sensitivity of the XE-2100 "NRBC?" suspect flag was analyzed at a threshold of 2% (since the "NRBC present" message appears in the CBC/differential mode at a threshold of 2%) by constructing a matrix table.

Comparison to flow cytometry (FCM)

The manual morphology count is associated with a high degree of imprecision. A CV of up to 45% for manual NRBC precision has been reported[25]. The main reason for this is the small number of cells counted (usually 100 in a routine clinical lab) and the small numbers of NRBCs encountered in abnormal specimens. In such a situation it can be predicted that the imprecision will be high. Therefore a reference flow cytometric method has been recommended for the enumeration of NRBCs[25]. In this method PI is used as a nuclear stain and CD45 used to separate NRBCs from leucocytes (***Fig. 1***). We have evaluated the XE-2100 in a comparison to a reference FCM NRBC counting method utilizing 121 neonatal blood specimens.

Normal range

Normal blood was obtained from 23 male and 23 female volunteers. These specimens were run on the XE-2100 in both CBC/differential and discreet mode.

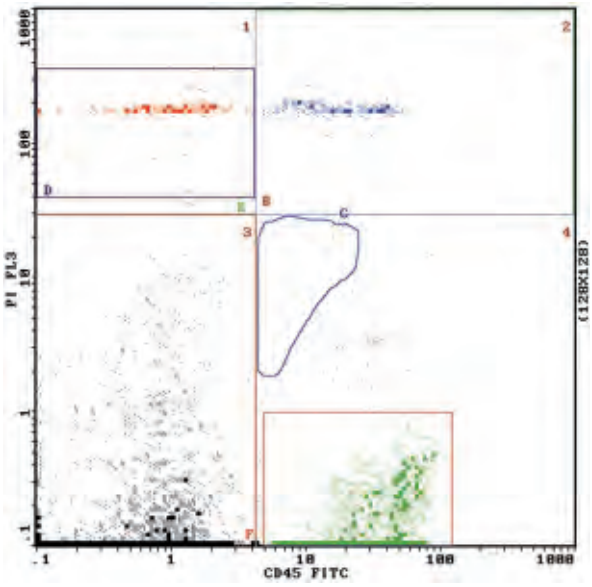

Fig. 1 *Enumeration of NRBC by flow cytometry*
Quadrant 1 are NRBC, 2 and 4 are WBC and 3 is RBC and platelet debris. Platelet aggregates may accumulate in amorphous gate C and are excluded from the WBC count.

RESULTS

Table 1 summarizes the results for imprecision of the XE-2100 NRBC count.
The results for linearity of NRBC are shown in ***Fig. 2***.
The results of linear regression comparing the XE-2100 percentage and absolute counts with the morphology counts are shown in ***Figs. 3 and 4***.
The matrix table analysis of the XE-2100 NRBC count sensitivity is shown in ***Table 2***.

Table 1 *Imprecision of XE-2100 NRBC count*

	Low	Middle	High
Mean NRBC ($\times 10^{12}$/L)	2.26	7.20	15.97
Within Run Imprecision (CV)	2.9	1.84	1.21
Within Run Imprecision (SD)	0.0657	0.1330	0.1928
Total Imprecision (CV)	2.81	1.74	1.25
Total Imprecision (SD)	0.0635	0.1254	0.1996

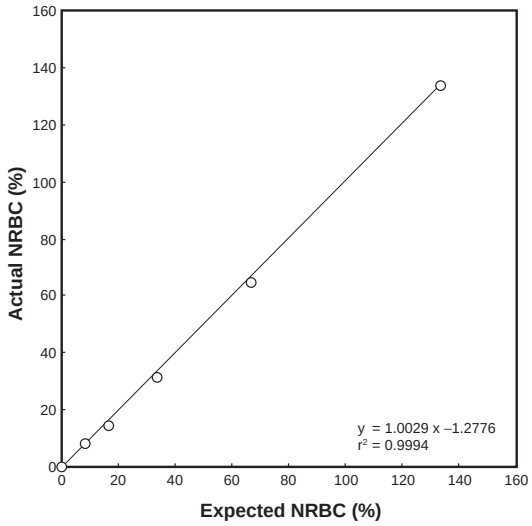

Fig. 2 *Linearity of XE-2100 NRBC count*

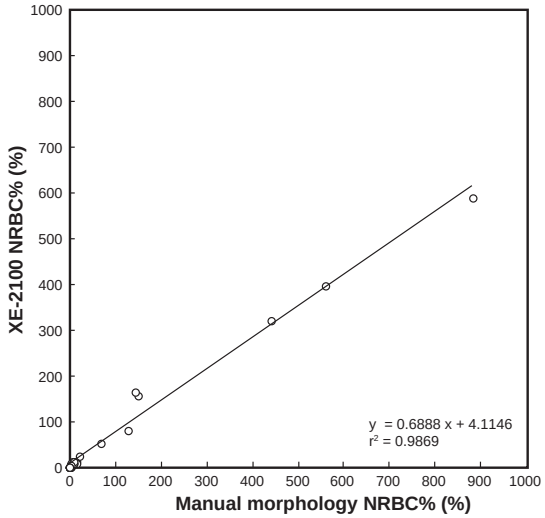

Fig. 3 *Comparison of XE-2100 and manual morphology percentage counts*

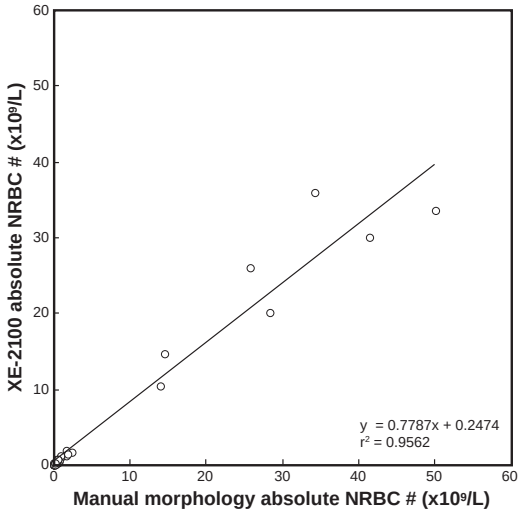

Fig. 4 *Comparison of XE-2100 and manual morphology absolute counts*

Table 2 *Sensitivity of the XE-2100 NRBC count*

NRBC Count		Manual Morphology	
		Positive	Negative
XE-2100	+	53	13
	–	8	188

Efficiency: $\frac{TP + TN}{TN+TP+FN+FP} \times 100 = 91.98\%$

Sensitivity: $\frac{TP}{TP + FN} \times 100 = 86.89\%$

Specificity: $\frac{TN}{TN + FP} \times 100 = 93.53\%$

False Negative Rate: $\frac{FN}{FN + TP} \times 100 = 13.1\%$

False Positive Rate: $\frac{FP}{FP + TN} \times 100 = 6.47\%$

Table 3 *Sensitivity of "NRBC?" flag*

NRBC Flag		Manual Morphology	
		Positive	Negative
XE-2100	+	15	2
	–	0	52

Efficiency: $\frac{TP + TN}{TN+TP+FN+FP} \times 100 = 97.1\%$

Sensitivity: $\frac{TP}{TP + FN} \times 100 = 100\%$

Specificity: $\frac{TN}{TN + FP} \times 100 = 96.3\%$

False Negative Rate: $\frac{FN}{FN + TP} \times 100 = 0\%$

False Positive Rate: $\frac{FP}{FP + TN} \times 100 = 3.7\%$

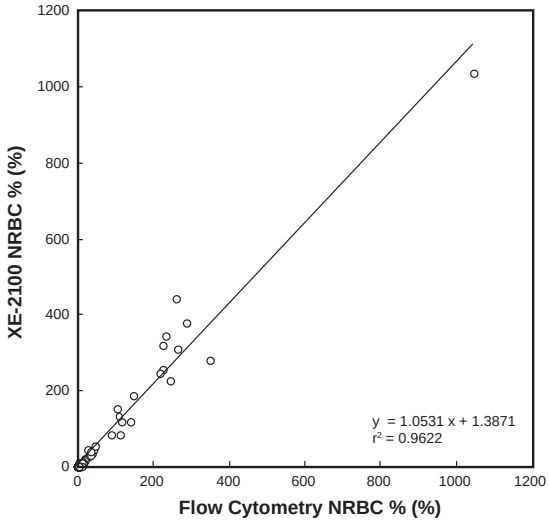

Fig. 5 *Comparison of proposed reference flow cytometric and XE-2100 NRBC percentage counts*

The matrix table of the "NRBC?" suspect flag sensitivity is shown in ***Table 3***.
The results comparing the XE-2100 NRBC count with a proposed flow cytometric reference NRBC count method are shown in ***Fig. 5*** for 121 neonatal specimens.

DISCUSSION

The traditional manual morphology method for identifying and counting NRBC requires a skilled observer and is time consuming. However the greatest limitation is the lack of precision and accuracy[26] because of the low proportion of NRBCs usually encountered in the peripheral blood even in abnormal states, and the small number of cells counted to generate a differential count in the typical clinical laboratory (usually 100-200 cells). It is now clearly recognized that automated cell analysis has a much higher degree of precision than manual methods because of the large number of cells counted (usually in excess of 10,000). Previous models of hematology cell analyzers could only identify and enumerate cells normally found in the peripheral blood. The availability of a robust and reliable automated analysis of cells not normally present in the peripheral blood, such as NRBCs would offer significant benefits to the clinical laboratory.
The lack of precision of manual morphology NRBC counts is widely recognized and CV's as high as 40% (range 20-110%) have been reported[25]. The XE-2100 produces a count with a high degree of precision: Within run imprecision CV = 2.9% (low range), 1.84% (mid range), 1.21% (high range), and total imprecision CV= 2.81% (low range), 1.74% (mid range) and 1.25% (high range). This level of precision exceeds recommendations

for precision goals for most CBC parameters[27].

The linearity of the NRBC count was evaluated using doubling dilutions of a very high count. The Sysmex XE-2100 NRBC count demonstrated linearity (R^2=0.99) over a range of 0-140 nRBC/100 WBC. The slope was 1.0 and the intercept –1.28. The extended dynamic range of linearity obviates the need to perform manual counts at low count levels or dilutions of specimens at very high NRBC counts.

We have previously demonstrated that the XE-2100 NRBC count is stable up to 24 hours in specimens stored at both room temperature and in the refrigerator[10]. This is a valuable feature in laboratories where specimens may be transported from great distances.

The XE-2100 NRBC count was compared with a manual morphology count performed by two experienced technologists, each performing a 200 WBC differential count according to the NCCLS protocol. There was good correlation between the XE-2100 and manual morphology for both percentage (R^2=0.98) and absolute (R^2=0.95) counts. At very high counts, a positive bias was noted for the manual morphology NRBC counts. This bias was not observed when the XE-2100 NRBC count was compared to the proposed reference flow cytometric method (***Fig. 5***). The positive bias of the manual morphology counts at high levels appears to be due to the frequent presence of damaged or smudged white blood cells on glass slides in patients with very high NRBC counts resulting in an overestimation of the NRBCs. It should be noted that the absolute NRBC count produced by the XE-2100 is a single platform count which is inherently more accurate than the dual platform absolute count derived from the manual morphology count.

The analysis of the sensitivity of the XE-2100 NRBC count against manual morphology showed a false negative ratio of 13.1% and a false positive ratio of 6.47%. Two of the false negative cases had a manual morphology count of 2%. The rest were less than 1% and would likely not be picked up in a 100 cell differential count. It would be preferable to decrease the false negative ratio by decreasing the threshold settings, but this would likely increase the false positive ratio.

It has been known for many years that the precision of manual morphology counts for cells that are present in small numbers (<5%) is extremely poor[20, 26]. This is largely because in routine practice, only 100-200 cells are counted to establish differential count. In contrast, automated instruments count more than 10,000 cells when performing a differential count, resulting in much improved precision. Thus it may be inappropriate to compare 2 different methodologies which such widely varying imprecision. An alternative to manual morphology counts is conventional flow cytometric counting, since the large number of events counted results in a high degree of precision. A proposed reference method for NRBC counting based on flow cytometric methods has been published and found useful for evaluating automated CBC instrumentation NRBC counts[25]. In this method, Propidium Iodide is used to separate NRBCs from RBC debris and platelets, and CD45 to separate NRBC from white cells (***Fig. 1***). We have compared the XE-2100 NRBC count to the proposed reference flow cytometric count in 121 neonatal specimens (***Fig. 5***). It demonstrates excellent correlation (R^2=0.96) with a slope of 1.05 and intercept of 1.4. This validates the use of a flow cytometric based reference method for comparison of automated CBC NRBC counts, and we would advocate such methodology for evaluation of all automated CBC analysis of low frequency events.

In automated CBC and differential analyzers, the presence of NRBC interferes with the lymphocyte count resulting in erroneous counts for total WBC and lymphocytes. When the XE-2100 performs a NRBC count it also generates an automated corrected WBC count and corrected lymphocyte count. We have previously demonstrated that the automated XE-2100 corrected WBC and lymphocyte counts correlates well with manually derived corrected WBC and lymphocyte counts[10]. The availability of automated corrected counts obviates the need for any secondary manual manipulation of data from the analyzer.

The XE-2100 enables discreet test selection, of a CBC/differential test so that a CBC and differential count can be performed without a NRBC count. In this mode, NRBC counting reagents are not utilized resulting in reduced operational costs. However, a “NRBC?” suspect flag is generated whenever NRBCs are present, giving the operator the opportunity to re-run the specimen selecting a CBC, differential and NRBC count test. We tested the sensitivity of this flag in 69 specimens run in the discreet CBC and differential mode. The results (***Table 3***) showed a sensitivity of 100% and a false negative rate of 0%. However, in routine operation of the instrument, we have encountered occasional specimens with a false negative “NRBC?” suspect flag, indicating that false negatives do occur with this flag, although at low frequency. It would also be desirable to reduce the threshold for this flag, although this would likely reduce the specificity of the flag.

In order to generate a reference range, we performed NRBC counts on 46 normal adult volunteers (23 males and 23 females). In none of the specimens was a “NRBC?” flag or NRBC count generated by the instrument. The normal range for NRBC number in our adult population was 0×10^9/L for both males and females.

In conclusion, the automated hematology analyzer XE-2100 NRBC count has a high degree of precision, demonstrates excellent linearity over a wide range of counts and shows good correlation with both manual morphology and a proposed flow cytometric based reference NRBC count. The “NRBC?” suspect flag works well as a screening tool when the instrument is run in a discrete testing mode. The availability of automated NRBC identification and enumeration represents a significant advance in analytical capabilities in the clinical hematology laboratory and should be translated into improved patient care and outcomes and greater efficiencies in the workflow within the clinical laboratory.

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