# Automated counting of nucleated red blood cells (NRBC): evaluation of the Sysmex XE-2100 system

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## Introduction and background

The identification of circulating NRBCs provides important clinical clues in: 1) para-physiologic states (premature babies); 2) conditions of intense erythropoietic stimulation (immune hemolytic anemia, hemolytic crisis in congenital anemias, microangiopathic anemia, severe thalassemia or sickle cell anemia, recovery from aplasia or bone marrow transplantation); 3) severe disorders of hematopoiesis (infiltration by tumor cells, lymphoma or leukemia; fibrosis and extramedullary hematopoiesis); 4) hypoxic states (severe hemorrhages, cardiac and pulmonary disorders, fetal asphyxia) (1-4). Automated blood cell counters cannot easily distinguish NRBCs from white blood cells (WBC). NRBCs are variably and unpredictably included within lymphocytes or the noise component, according to their heterogeneous size and sensitivity to lysis. NRBCs are a cause of pseudolymphocytosis and can mimic lymphoproliferative disorders in instrument reports. Most blood cell counters flag circulating NRBCs through increased noise or disappearance of the separation between lymphocytes and noise. The specificity of NRBC flag was reported above 90%; sensitivity, on the other hand, has shown a much wider range of variability (20-90%) (5). Cytometric NRBC count can be obtained semi-automatically using monoclonal antibodies and multidimensional flow-cytometry (6). Fully automated methods based on nuclear fluorescence are available in the Abbott Cell-Dyn 4000 (4,6,7) and in the new Sysmex XE-2100 blood cell analyzer. We report here the first results of the evaluation of NRBC counting with the Sysmex XE-2100 in our ReCAMH.

## Methods

***The Sysmex XE-2100 NRBC counting method***. The Sysmex XE-2100 hematology analyzer performs white cell differential count measuring: 1) radiofrequency and direct current resistance (indicators of cell content density and cell size); 2) forward and lateral scatter of light emitted by a semiconductor laser beam (indicators of cell size and internal components); 3) lateral fluorescent light. NRBC identification and counting is carried out after red cell lysis. Intact WBCs and NRBCs are stained with a fluorescent dye (Stromatolyser-NR). In the two-dimensional cytogram the intensity of lateral fluorescent light is represented on the x-axis, while the intensity of the forward-scattered light is represented on the y-axis. NRBCs are identified as a well-separated cell cluster on the left side of the WBC population (FIG 1). NRBC count is expressed as a proportion per 100 WBC and as absolute number per unit volume of blood. A flag NRBC present is printed in samples with a measured NRBC proportion of 2.0% or more.

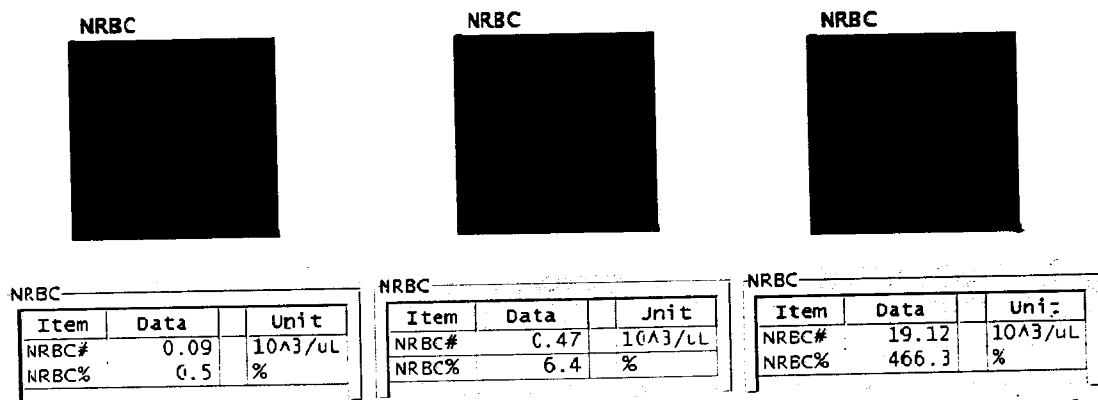
***Experimental design of the evaluation.*** We have assessed:

1) imprecision of the Sysmex XE-2100 NRBC counting using linear regression and the NCCLS-H20A method of differences between duplicates (8);

2) inaccuracy, throughout comparison with NRBC count obtained with the reference NCCLS-H20A microscope method; comparability was assessed by linear regression and analysis of differences between reference and the XE-2100 method;

3) clinical sensitivity and specificity of the Sysmex XE-2100 NRBC method according to the NCCLS-H20A, NCCLS GP-10P (9) and Galen and Gambino (10) methods: the capability to identify samples containing NRBCs was assessed using the microscope as the reference method on a selected sample population with high prevalence of abnormalities.

*Study samples.* All samples were venous peripheral blood collected in K2-EDTA, analyzed within 6 hours from venipuncture using the XE-2100 under routine operation and current quality control procedures. Microscope analysis was carried out by two hematologists (GZ, GMi) on May-Grünwald-Giemsa stained peripheral blood smear, assessing the proportion of NRBCs out of 100 leukocytes. A total of 400 leukocytes per film was counted.




NRBC

| Item | Data | Unit |
|---|---|---|
| NRBC# | 0.09 | 10^3/uL |
| NRBC% | 0.5 | % |

NRBC

| Item | Data | Unit |
|---|---|---|
| NRBC# | C.47 | 10^3/uL |
| NRBC% | 6.4 | % |

NRBC

| Item | Data | Unit |
|---|---|---|
| NRBC# | 19.12 | 10^3/uL |
| NRBC% | 466.3 | % |

FIG 1



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## Results

### Imprecision

Reproducibility of the XE-2100 NRBC measurement was assessed through duplicate analysis of 666 samples in which microscope routine examination had found at least one NRBC. Instrument results ranged from 0.0 to 6.26 $x10^9/l$ (mean 0.42) for the absolute count and from 0.0 to 81.9% (mean 5.9) for the proportion. Out of the total of 666 samples, 357 samples had less than 2% NRBC and 309 samples had 2% or more NRBC (2% NRBC is the actual instrument threshold for reporting a NRBC PRESENT flag). Results are reported in Figure 2 (A,B) for NRBC absolute count and in Figure 3 (A,B) for NRBC proportion. ***Mean differences between duplicate runs were 0.01 x $10^9/l$ NRBC and 0.11% NRBC, respectively. Coefficients of variation were 12.6% for the absolute count and 12.8% for the proportion.*** Imprecision of NRBC measurement in the 309 samples flagged for NRBC (with 2% or more NRBC) was significantly improved, with CVs of 8.8% both for the absolute and the proportional count.

**Figure 2A.** **Imprecision of NRBC absolute count: correlation of 666 duplicates ($x10^9/l$ - log scale)**

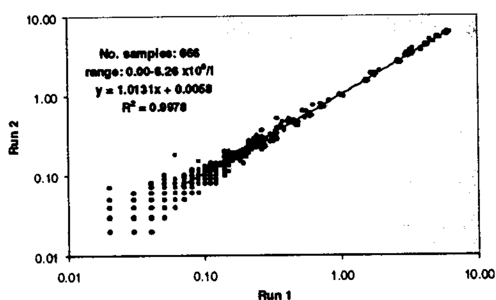



**Figure 2B.** **Imprecision of NRBC absolute count : analysis of differences of 666 duplicates ($x10^9/l$ - semilog scale)**

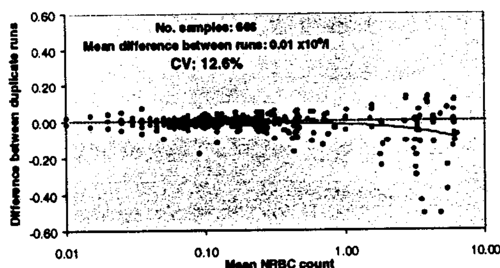



**Figure 3A.** **Imprecision of NRBC proportion: correlation of 666 duplicates (log scale)**

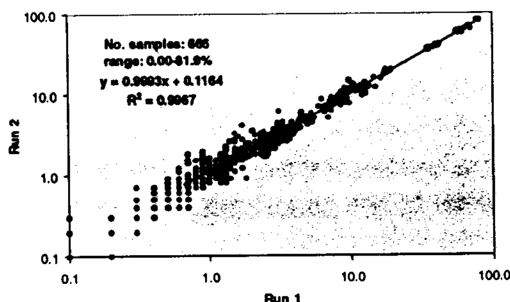
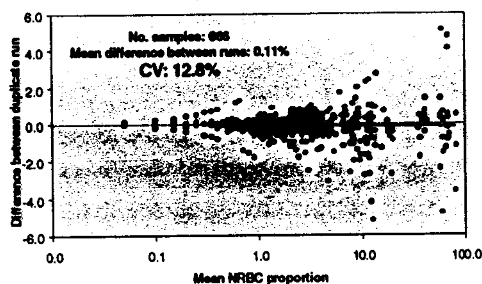



**Figure 3B.** **Imprecision of NRBC proportion: analysis of differences of 666 duplicates (semilog scale)**



**Inaccuracy (comparison with microscope)**
We compared the proportion on NRBC counted by the XE-2100 with the microscope count using linear regression and analysis of differences on 140 samples containing from 0.1 to 466.3 NRBCs per 100 leukocytes. Results are displayed in Figure 4 (A,B). ***Correlation between XE-2100 and the reference method is excellent ($R^2$ = 0.985)*** (Figure 4A). Analysis of differences indicates almost perfect agreement in the great majority of samples, with **a *mean difference (XE-2100 versus microscope) of –1.55.*** There is a tendency of the XE-2100 to slightly underestimate samples with very high number of NRBC (above 50-100 out of 100 leukocytes) (Figure 4B).

**Figure 4A.** Inaccuracy of NRBC proportion: comparison with microscope, linear regr. (log scale)

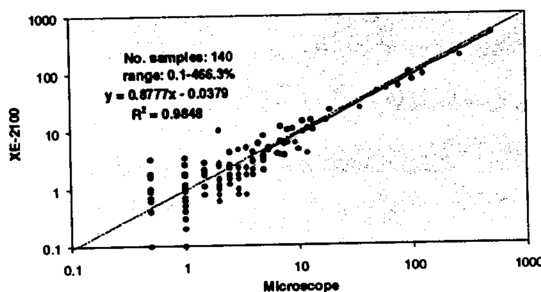
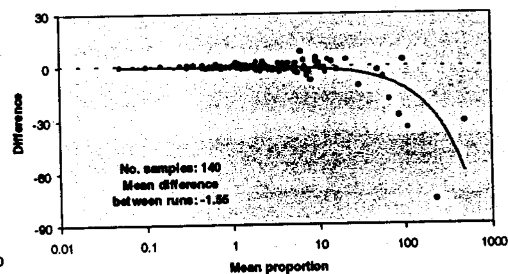



**Figure 4B.** Inaccuracy of NRBC proportion: comparison with microscope, analysis of differences (semilog scale)




**Clinical sensitivity and** specificity
The study of clinical sensitivity and specificity was carried out on 300 samples, selected as to include a wide range of abnormal cell types (Table 1) and a very high prevalence of positive samples containing NRBC of the stained film. Samples with NRBCs at the microscope were 134 (44.7%). The summary of this study is shown in Table 2. In 93.3% of the samples there is agreement between methods. False positives are 13 (4.3%), all with very low automated NRBC count (Table 3). False negatives are only 7 (2.3%): they were obtained in samples with 0,5-1,5% NRBC discovered on the peripheral blood film. *Sensitivity* of the automated NRBC count (any result above 0) is ***94.8%*, and *specificity*** is ***92.2%. The predictive value of a NRBC count > 0 is 90.7% for the presence of NRBC on the film, while predictive value of a zero NRBC report is 95.6% for the absence of NRBC***. Since XE-2100 NRBC PRESENT flag is reported only for samples having more than 2% NRBC, we also assessed the performance of this flag on our sample population. If samples with less than 2% NRBC (not flagge) are considered as negative, false positives drop to 0, with 100% specificity, but fale negatives rise to 62 (20.7%), with an unacceptably loss of sensitivity. Therefore, in our experience the diagnostic value of the reported NRBC count and proportion are clearly superior to the simple interpretive flag.



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| Table 1. Specimen types for the sensitivity/specificity study. | |
|---|---|
| **Healty, CBC normal, subjects** | 80 |
| **Newborn and premature babies** | 61 |
| **Thalassemia** | 9 |
| **Acute leukemia with blasts** | 21 |
| **Chronic myelod leukemia and SMP** | 14 |
| **CLL and lymphoproliferative disorders** | 33 |
| **Acute immunohemolytic anemia** | 5 |
| **Malaria** | 6 |
| **Leukopenia after CHT and/or BMT** | 35 |
| **Miscellaneous (post-surgery, ICU, ER...)** | 36 |

| Table 2. XE-2100 NRBC count: summary of the clinical sensitivity/specificity study (300 samples, any NRBC count, including <2%) | |
|---|---|
| **True negatives (TN)** | **153** |
| **True positives (TP)** | **127** |
| **Agreements (TP+TN):** | **280/300 = 93.3%** |
| **False negatives (FN)** | **7 (2.3%)** |
| **False positives (FP)** | **13 (4.3%)** |
| **False negative ratio [(FN/TP+FN) * 100]** | **5.2%** |
| **False positive ratio [(FP/TN+FP) * 100]** | **7.8%** |
| **Sensitivity [(TP/TP+FN) * 100]** | **94.8%** |
| **Specificity [(TN/TN+FP) * 100]** | **92.2%** |
| **Predictive value of a negative result [(TN/TN+FN) * 100]** | **95.6%** |
| **Predictive value of a positive result [(TP/TP+FP) * 100]:** | **90.7%** |

| Table 3. Details of false negative and false positive results (see Figure 5) | |
|---|---|
| False negatives (n = 7) | |
| Diagnosis | Microscope (/100 WBC) |
| AML, M5 with 50 x $10^9$/l blasts | 1.5 |
| AML, M1, leukopenic with 20% blasts | 0.5 |
| CML, 57 x $10^9$/l WBC | 0.5 |
| CLL, 22 x $10^9$/l WBC | 0.5 |
| CLL, 57 x $10^9$/l WBC | 1.5 |
| Premature | 1.5 |
| post-surgery | 1.0 |
| False positives (n=13) | |
| Mean XE-2100 NRBC count | 0.6/100 |
| Range (only 1 sample out of 13 had more than 0.9% NRBC) | 0.1-1.3% |

## Conclusions

Our study demonstrates excellent performance of the SysmexXE-2100 method for NRBC counting. Level of imprecision is very low, due to the very high reproducibility (CV 12.8%) even in samples with very low NRBC count. Results of XE-2100 are also fully comparable with the microscope reference method (R2 = 0.985; mean difference –1.55), indicating a high level of accuracy. There is only a slight underestimation of NRBC proportion in specimens with very high NRBC count, which is however devoid of clinical significance. The solid capability of the XE-2100 to identify specimens with circulating NRBC is indicated by the sensitivity of 94.8% and predictive value of a negative result of 95.6%, due to the very low frequence of false negatives, and by the very small proportion of NRBC in these specimen: no case with more than 1.5% NRBC escaped recognition. Specificity is also excellent (92.2%), with a predictive value of a positive results of 90.7%. On the other hand, specificity of the NRBC PRESENT flag, triggered by a NRBC proportion of 2% or more, is 100%, but its sensitivity is decreased to unacceptable levels: so the chosen flagging threshold of 2% should probably be lowered. In conclusion, oury results do indicate that the Sysmex XE-2100 method for NRBCs on peripheral blood is precise and reproducible, shows an excellent correlation with the other methods and is both sensitive and specific for clinical use.



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