Comparison of a semi-automated new Coulter methylene blue method with fluorescence flow cytometry in reticulocyte counting

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A semi-automated new methylene blue method for reticulocyte counting was compared to the manual method and fluorescence flow cytometry. Over a wide range of reticulocyte counts (0.3–12.7%) the new methylene blue method was found to be comparable to that of fluorescence flow cytometry, with high precision. Between-run precision studies showed coefficients of variation (CVs) of 4.8, 6.9 and 14.5% for reticulocyte counts of 9.5, 2.4 and 0.7%, respectively. Within-run precision studies showed CVs of 8.6, 8.1 and 6.1% for reticulocyte counts of 0.9, 5.3, and 18.4%, respectively. The correlation coefficient between the Coulter method and the manual method was 0.83, and 0.85 between the Coulter method and flow cytometry. The possibility of extending the incubation time to at least 4 h and the insignificant changes observed with samples stored for up to at least 72 h at 4–8°C make this new method most convenient and allow any laboratory having Coulter STKS® or MAXM® cell counters to perform reticulocyte counts in an efficient, reliable manner.

Key words: automated counting; flow cytometry; methylene blue; reticulocytes

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A reliable reticulocyte count can offer valuable clinical information with regard to the haemopoietic status of the patient. Unfortunately, the manual method using supravital stains, which is tedious, lacking in precision, and having great intra-observer variability, is not reliable enough [1–4]. During the past few years, automated methods using flow cytometry have tremendously increased the accuracy and precision of reticulocyte counting [5–9]. These methods have allowed reticulocyte counting to be reliably performed in the routine clinical laboratory.

Recently, the Coulter Corporation (Miami, Florida, USA) has developed a semi-automated method, utilizing the new methylene blue procedure, for use with their STKS and MAXM haematology analysers. We compared this method to the standardized manual method and to fluorescence flow cytometry, and studied the effects of incubation and storage times on this new technique.
MATERIALS AND METHODS

Blood sampling

Blood samples were collected into tripotassium EDTA anticoagulant from unselected patients for whom reticulocyte counts had been requested, and were analysed within 4 h of being drawn.

Manual method for reticulocyte counting

Equal amounts of blood and new methylene blue were mixed and incubated at room temperature for 10 min. Wedge smears were prepared from the remixed dilutions. Experienced technologists, unaware of the automated reticulocyte count results, examined 1000 red blood cells and reported reticulocyte count as a percentage of total red blood cells. The criterion for definition of a reticulocyte was taken from the National Committee for Clinical Laboratory Standards H16-P and H44-P protocols [10, 11].

Fluorescence flow cytometry for reticulocyte counting

Samples of 5 μl of whole blood were added to 1 ml of thiazole orange reagent (Retic-COUNT, Becton Dickinson, Rutherford, New Jersey, USA), incubated at room temperature in the dark for 60 min, and analysed.

Analysis was performed on a flow cytometer (FACScan, Becton Dickinson) and data acquisition and analysis performed automatically (Retic-Count Enumeration Software, Becton Dickinson). With this method, 10000 red cells were analysed in the instrument, which was equipped with an argon-ion laser, with 15-mW excitation at 488 nm. Green fluorescence was measured using a 530-nm bandpass filter with a bandwidth of 30 nm in front of a photomultiplier tube.

Coulter method for reticulocyte counting

The method is an extension of the VCS technology (Coulter). Briefly, 50 μl of well mixed whole blood are incubated in a test tube with 4 drops (about 130 μl) of a proprietary formulation of new methylene blue reagent. After 5–60 min of incubation at room temperature a 2-μl aliquot of the first dilution is transferred into another test tube and further diluted with 2000 μl of reagent B (0.08% sulphuric acid).

The diluted sample is aspirated and cells measured for volume, conductivity and laser light scatter. A maximum of 32000 red cells are analysed and the instrument (STKS, Coulter), using customized gating for each sample, separates the mature red cells, reticulocytes, white blood cells, and, on the lower threshold, the platelets. Results can be reported as absolute reticulocyte number and/or a percentage.

Evaluations

The between-run precision of the Coulter method was measured by analysing the results from control material which was run daily for a month. The control material is commercially prepared stabilised human erythrocytes, with an added reticulocyte-like component at three concentration levels, 0.7, 2.4 and 9.5%. The within-run precision of the Coulter method was evaluated by 20 consecutive analyses of each of three samples which had normal, intermediately raised and very raised reticulocyte numbers, as determined by the manual method. Each of the replicate samples was prepared completely as a separate sample with separate staining and dilution steps.

The manufacturer specifies a maximum of 60 min for sample incubation with methylene blue reagent. We tested the possibility of extending the incubation time for up to 4 h, which would make batch testing even more convenient. Thus, 21 samples were analysed after 1 h of incubation and re-analysed after 4 h of incubation.

A random group of 32 samples was evaluated for the effect of storage. These samples were analysed upon receipt in the laboratory and at 24, 48 and 72 h of storage in a refrigerator at 2–8°C.

A random group of samples was analyzed by the Coulter method as compared to the manual method and flow cytometry. A total of 66 were compared to both manual and flow cytometry methods, and an additional 34 to flow cytometry only.

Statistical methods

All statistical calculations were performed using commercially available software run on an
IBM personal computer. Tests for significance between data sets were calculated by performing a paired t test using RS/1 software (BBN Software Products, Cambridge, Massachusetts, USA). All other calculations, and the display of graphical data, were performed using Lotus 1-2-3 software (Lotus Development, Georgia, USA).

To establish the agreement between different methods of reticulocyte enumeration, the standard error of the binomial distribution (SE$_p$) was calculated using the following formula:

$$\text{SE}_p = \sqrt{\frac{p \times q}{n_1} + \frac{p \times q}{n_2}}$$

where $p$ = probability of identifying a reticulocyte; $q$ = 1 - $p$; $n_1$ = percentage of reticulocytes counted (method 1); $n_2$ = percentage of reticulocytes counted (method 2).

Reticulocyte data from samples analysed by alternative techniques was shown graphically, and compared by the method of least-squares regression, and the correlation coefficient, $r$, was calculated.

RESULTS

Table I shows the results of between-run precision studies of control samples analysed once daily for 30 days. Mean reticulocyte counts of 0.8, 2.4 and 9.3% were obtained with coefficients of variation (CVs) of 14.5, 6.9 and 4.8%, respectively. Table II shows the results from the reticulocyte within-run precision analysis. At all reticulocyte levels studied, the coefficient of variation, did not exceed 14%, which is well within the performance specifications quoted by the manufacturer [12].

**Table I. Between run precision analysis of Coulter method at three concentration levels.**

<table>
<thead>
<tr>
<th>Level I</th>
<th>Level II</th>
<th>Level III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assigned values, %</td>
<td>0.7</td>
<td>2.4</td>
</tr>
<tr>
<td>Number of analyses</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Mean value, %</td>
<td>0.8</td>
<td>2.4</td>
</tr>
<tr>
<td>2 SD</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>CF, %</td>
<td>14.5</td>
<td>6.9</td>
</tr>
</tbody>
</table>

**Table II. Within-run precision analysis of the Coulter method.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean, %</th>
<th>Range, %</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.90</td>
<td>0.78-0.99</td>
<td>13.6</td>
</tr>
<tr>
<td>2</td>
<td>5.30</td>
<td>4.89-5.69</td>
<td>8.1</td>
</tr>
<tr>
<td>3</td>
<td>18.40</td>
<td>16.78-19.89</td>
<td>6.1</td>
</tr>
</tbody>
</table>

**Table III. Effect of storage time at 4–8°C on reticulocyte count (n=32).**

<table>
<thead>
<tr>
<th>Storage time, h</th>
<th>Mean difference*</th>
<th>SD</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>0.00</td>
<td>0.52</td>
<td>NS</td>
</tr>
<tr>
<td>48</td>
<td>-0.01</td>
<td>0.63</td>
<td>NS</td>
</tr>
<tr>
<td>72</td>
<td>0.21</td>
<td>0.59</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Mean difference = reticulocyte % at 0 h-reticulocyte % at 24, 48 and 72 h respectively NS, not significant at a significance level of 0.05.

Increase of incubation time with methylene blue from 1 h to 4 h did not seem to affect the results employing samples with reticulocyte values of 0.8 to 10.2%. The overall mean difference between an incubation period of 1 h and 4 h was $-0.24\%$, with a standard deviation of differences of 0.62. A paired t test confirmed that the two data sets were similar, there being no significant differences at a significance level of 0.05.

The effect of storage on 32 random samples at 4–8°C for up to 72 h is seen in Table III. The mean differences between data sets indicated that very little change in the reticulocyte percentage occurred over the 72 h, the maximum difference being only 0.21%. The standard deviation of the differences did not exceed 0.65 and the paired t test did not show any significant difference between all data sets at a significance level of 0.05. These data confirm those found in a previous study [13].

Figures 1, 2 and 3 show the reticulocyte values recovered from the same sample when employing different techniques. Figure 1 shows that there was good agreement between manual and Coulter STKS methods ($r=0.829$). Figure 2 shows the good correlation between the Coulter method and the fluorescence flow cytometry method ($r=0.85$), although higher values were observed in the fluorescence flow cytometric method, especially at reticulocyte values of less than 4%. This phenomenon can also be seen in Figure 3.
where a large proportion of the samples show higher values when analysed by fluorescence flow cytometry compared with the manual method.

**DISCUSSION**

Reticulocyte counts, an important technique in the investigation of erythropoietic activity, have suffered in the past from the imprecision and inaccuracy of the manual method. In addition, the labour intensiveness of the manual method made it very difficult for laboratories to absorb the workload associated with performance of reticulocyte counts. This study has shown that the new methylene blue method for counting reticulocytes on the Coulter STKS provides an opportunity to improve the precision of reticulocyte enumeration and has demonstrated that
a within-run precision CV of 6–14% over a range of reticulocyte values of 0.9 to 18.4% may be achieved. Similar results have been found in a recently published study [13]. This is superior to the manual technique which has previously been reported to have a within-run precision CV of 30%, compared to a CV of 8.4% with the semi-automated Coulter method, with reticulocyte values between 0.5 and 19.3% [14].

A previous study, using a FACScan and RETIC-count software to enumerate reticulocytes, has shown this technique to produce good agreement with the manual method, producing a correlation coefficient, r, of 0.86 [15], though data from the present study achieved an r value of only 0.76 (Fig. 3). In the present study, correlation analysis of flow cytometry and the Coulter STKS produced an r value of 0.85 (Fig. 2), indicating that a similar degree of accuracy may be achieved from both fluorescence flow cytometric and Coulter STKS methodologies.

The observation that flow cytometry gives higher reticulocyte numbers than methylene blue is a subject which should certainly be investigated in future studies. Davis et al. [9] observed the same phenomenon in a multi-institutional study and attributed it to the extremely high fluorescence increase in the presence of RNA shown by thiazole orange. Thus flow cytometry, utilizing thiazole orange, might be recognizing mature, weakly fluorescing reticulocytes. Whether this difference is clinically significant or not is not known; however, it is important to realize that normal laboratory values for reticulocytes will depend on methodology.

Davis et al. [9] compared results of reticulocyte analyses from eight different laboratories employing many different automated or semi-automated flow cytometric methods. The sites included some using thiazole orange or ethidium bromide with multipurpose flow cytometers from different manufacturers, and sites using auramine O with the dedicated Sysmex R-1000 reticulocyte counter. All flow cytometric methods showed a precision superior to that of the classical manual methods, with the dedicated instrument producing CVs of less than 10% with samples containing greater than 0.5% reticulocytes. In addition, the inter-site precision of the dedicated instrument was extremely high, with correlation coefficients of greater than 0.98.

Although our study did not specifically address the problem of interference by leukocytes, nucleated red blood cells or platelet clumps, this should be greatly diminished by the Coulter technology based upon volume, conductivity and light-scatter measurements [13].

The effect of small red cell inclusions, such as Howell–Jolly bodies, although usually present
in small numbers, was not investigated. Also, the diagnostic usefulness of a reticulocyte maturity index (RMI), which is available with the newer Coulter reticulocyte algorithms, should be investigated in the future.

The Coulter method offers certain advantages compared with fluorescence flow cytometry. First, it uses the supravital stain, new methylene blue, as does the classical manual method, which might make results closer to the reference reticulocyte definition. In addition, the ability to perform the reticulocyte counts on the same instrument with which the patients' cell counts were performed allows for a simple integration of the results producing a single patient test report. Finally, the use of methylene blue method, unlike flow cytometry, allows slides to be prepared for review directly from the incubation mixture.

Our study demonstrated that the 1-h incubation, as prescribed by the manufacturer, could be extended to at least 4 h. This would allow batching of samples which come into the laboratory at irregular intervals and permit analyses to be performed much more conveniently. The results showing no significant changes in samples stored at 4–8°C for up to at least 72 h mean that laboratories could delay analysis of samples, if necessary. This would ease the pressure on haematology laboratories, especially those with many requests for reticulocyte counts.

In conclusion, we have found the long-awaited new Coulter method for reticulocyte counting to be precise and accurate, allowing this most important test to be conveniently performed in the clinical haematology laboratory.

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REFERENCE


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