The LH Reticulocyte Count and Associated Parameters

The reticulocyte count has historically been used as a tool for providing information regarding red cell production, or erythropoiesis. Typically, the most common clinical indicator for performing a reticulocyte count is to diagnose or monitor the treatment of anemia. The emergence of flow cytometric methods of reticulocyte counting has resulted in improvements in accuracy and precision, as well as provide non-traditional parameters associated with reticulocyte maturation and size. Independent clinical studies have begun to establish clinical utility for these non-traditional parameters as potentially useful tools in the diagnosis and treatment of many anemic states.

The traditional method of manual reticulocyte counting can be limited in its usefulness as a monitoring test because of inherent imprecision, especially in the low and high ends of the counting range. Blood smear variability, staining differences, the limited number of cells evaluated, and inter-technologist bias have all been cited as contributors to coefficients of variation ranging from 25% to over 50%. In recent years, automated reticulocyte analysis has been incorporated into many of the medium and high volume routine hematology analyzers. What originally began as an outside the instrument pre-preparation method has now developed into second generation hematology instrumentation that aspirate, prepare, and analyze reticulocyte information automatically. This total automation of the reticulocyte analysis process has removed the variables associated with manual blood smears, staining, and inter-technologist bias, resulting in more standardization within the laboratory. In addition, the 30,000+ cells analyzed with the typical automated reticulocyte method, as opposed to the 1000 cells analyzed manually, lends the automated method to improved precision and reproducibility.

The Reagent System

The Beckman Coulter procedure of reticulocyte analysis uses new methylene blue stain, a non-fluorochrome dye, to precipitate the residual RNA within the reticulocytes. The function of the reticulocyte stain is to identify and delineate the reticulocytes from mature red cells.
Blood is mixed with the new methylene blue stain and allowed to incubate for a short period of time. An acidic, hypo-osmotic, ghosting solution is then introduced, clearing the hemoglobin while preserving the stained RNA within the reticulocytes. The function of the ghosting solution is two-fold – to permit hemoglobin to leak from the red cells and to swell the red cells to a spherical shape without bursting them.

The irregular shapes of mature red cells and reticulocytes produce unpredictable light scatter information when subjected to a laser light beam at angles from 0° to 90°. The sphering of the red cells provides reproducible light scatter information that forms the basis for determining the reticulocytes in the sample. Therefore, the ghosting solution also has a fixative property so that the cells maintain the resulting spherical shape caused by the swelling. The removal of the hemoglobin from the sphered red cell is essential as the decrease in the hemoglobin content enhances the definition of the reticulum and the consequent determination of the reticulocytes.

**VCS Technology**

Once the cells have been stained and cleared, the VCS Technology method of reticulocyte analysis is employed, utilizing a unique flow cytometric means of cell interrogation to count and classify reticulocytes. Cells are identified and classified by simultaneous three-dimensional analysis using Volume, Conductivity, and Light Scatter.

Volume, as measured by direct current, is used to identify the size of the cell. Reticulocyte analysis is highly dependent on size information, as immature red cells, by definition, are larger than mature red cells. Volume is displayed on the y-axis of the DataPlot.

Conductivity, or radio frequency measurements, provide information about the internal characteristics of the cell. Conductivity is displayed on the z-axis of the DataPlot, but is only seen in the rotating three-dimensional mode.

Light scatter measurements, obtained as cells pass through the helium-neon laser beam, provide information about cell surface characteristics and cell granularity. Reticulocyte analysis is also highly dependent on light scatter information as the red cells containing residual RNA scatter more light than mature red cells. Light scatter is displayed on the x-axis of the DataPlot.

**DataPlot Development and Contour Analysis**

The raw data events collected from volume, conductivity and light scatter are plotted three-dimensionally on a DataPlot.

Using the valleys that separate the populations, the platelets and white cells are segregated, leaving only mature red cells and reticulocytes for further analysis.
Contour analysis involves a series of steps designed to separate the reticulocyte from the mature red cells in a non-linear mode that follows the contour of the populations, as opposed to a straight line.

**Reticulocyte Positional Parameters**

The reticulocyte positional parameters are displayed at the Workstation as troubleshooting tools and to aid in DataPlot interpretation.

Based on 256 channels of histogram data for each measurement of volume, conductivity, and light scatter, the mean channel of each measurement for each population is established. The mean channels determine the actual position of the subpopulations on the DataPlot. Additionally, the standard deviation is calculated for each population to determine the degree of variability for each measurement. Both the mean channel and the standard deviation can be affected by instrument optimization, gain adjustments, reagents, or may be related to a specific patient sample.

**Additional Reticulocyte Information**

In addition to the positional parameters, the Workstation displays a series of temperature, count, and time parameters associated with the reticulocyte count.

The displayed temperature refers to the ambient temperature inside the instrument, at the flow cell. Extremes in ambient temperature due to fluctuations in the laboratory environment trigger changes in the incubation time of the reagents. The displayed setting indicates the temperature level currently set by the service representative. The settings can be low, normal, or high. The typical recommendation is normal.
The total counts refers to the total number of events that pass through the flow cell. The maximum total count is 32,767. The analyzed count refers to the number of mature red cell events, reticulocyte events, and white cell events. The displayed count refers to all mature red cell and reticulocyte events. The actual time is the length of time required by the instrument to count 32,767 events. Whereas the low and high time are calculated by the instrument as minimum and maximum limits.

Typical Reticulocyte DataPlot

The figure at the left illustrates a typical reticulocyte DataPlot. The positional, temperature, count, and time parameters for this DataPlot are displayed above.

Reticulocyte Flagging

Using complex algorithms, the positional parameters are used in combination with other population statistics to ensure integrity of results and to trigger reticulocyte flagging. Certain clinical conditions can generate DataPlots that are inconsistent with known mature red cell and reticulocyte patterns. These atypical patterns will then generate either a Verify Retic or Abnormal Retic Pattern message to alert the laboratory that further review is necessary. All reticulocyte flags, codes, and messages are listed and defined at the end of the monograph.

Examples of the Abnormal Retic Pattern Message

During the process of contour gating, a subpopulation of high light scatter events is observed with a volume mean more consistent with mature red cells than with reticulocytes. This pattern is consistent with the high light scatter properties of incompletely lysed (ghosted) red cells commonly seen with thalassemia. This pattern may be subtle or pronounced based on the heterogeneity of the disease and the condition of the patient.

Sickle cell samples containing multiple shapes and sizes of red cells will present with an increased volume mean and increased volume standard deviation for both reticulocytes and mature red cells. Again, the pattern may be subtle or pronounced based on the heterogeneity of the disease and the condition of the patient.

During the process of DataPlot development, a subpopulation of high volume events is observed with light scatter mean more consistent with white cells than
with reticulocytes. This population has been found to be a low volume continuum of the white cell population, perhaps due to fragile or deteriorating white cells.

**Reticulocyte Associated Parameters**

Automation of reticulocyte analysis and the use of flow cytometric light scatter measurements have enabled Beckman Coulter to provide more detail about the reticulocyte population. The reticulocyte population is further divided into ten regions of maturity. Those reticulocytes with the most RNA are found in the highest light scatter regions, three through ten.

In addition to the Reticulocyte Percent (RET%) and Reticulocyte Absolute Number (RET#), the Immature Reticulocyte Fraction (IRF), Mean Reticulocyte Volume (MRV), Mean Sphered Cell Volume (MSCV), and High Light Scatter Reticulocyte parameters (HLR% and #) are products of VCS Technology reticulocytes.

The following table defines the reticulocyte parameters available on the COULTER® LH 700 Series Hematology Systems. It should be noted that reference ranges may differ significantly between the various technologies used for reticulocyte analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Derivation</th>
<th>Unit</th>
<th>Reference Range Beckman Coulter</th>
<th>Reference Range Fourcade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retic %</td>
<td>All Retic Events</td>
<td>%</td>
<td>0.45 – 2.28</td>
<td>0.5 – 2.0</td>
</tr>
<tr>
<td>Retic %</td>
<td>All Red Cell Events</td>
<td>%</td>
<td>0.02 – 0.11</td>
<td>0.02 – 0.10</td>
</tr>
<tr>
<td>Retic #</td>
<td>Retic % x CBC Red Count</td>
<td>10^6 cells/µL</td>
<td>0.02 – 0.11</td>
<td>0.02 – 0.10</td>
</tr>
<tr>
<td>IRF</td>
<td>Retic Events (3-10)</td>
<td>Ratio</td>
<td>0.163 – 0.362</td>
<td>0.20 – 0.40</td>
</tr>
<tr>
<td>MRV</td>
<td>Average of All Retic Events</td>
<td>FL</td>
<td>102.73 – 124.89</td>
<td>100 – 125</td>
</tr>
<tr>
<td>@ MSCV</td>
<td>Average of all Red Cell Events</td>
<td>FL</td>
<td>84 – 104</td>
<td></td>
</tr>
<tr>
<td>@ HLR %</td>
<td>Retic Events (3-10)</td>
<td>%</td>
<td></td>
<td>0.07 – 0.71</td>
</tr>
<tr>
<td>@ HLR %</td>
<td>All Red Cell Events</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>@ HLR #</td>
<td>HLR % x CBC Red Count</td>
<td>10^6 cells/µL</td>
<td>0.003 – 0.050</td>
<td></td>
</tr>
<tr>
<td></td>
<td>All Retic Events</td>
<td>%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Research Use Only*
While all manufacturers have a common goal of defining reticulocytes and their sub-populations of immature reticulocytes, methodologies and algorithms vary. It is always advisable to establish individual laboratory reference ranges.

The Immature Reticulocyte Fraction (IRF) is defined as the ratio of young or immature reticulocytes to the total number of reticulocytes. Immature reticulocytes are larger and are classified by light scatter methods as those reticulocytes having the greatest light scatter properties, therefore, the highest level of RNA.

Clinical Utility of IRF

Immature reticulocytes, also referred to as shift or stress cells, normally constitute less than five percent (5%) of the total number of reticulocytes. Immature reticulocytes are released into the peripheral blood during periods of intense erythropoietic stimulation, such as hemorrhage, certain anemias, or in response to therapy designed to stimulate bone marrow production. The IRF may be called the red cell equivalent of the “left shift” typically associated with neutrophilic white cells, providing additional red cell information that may shorten the time from diagnosis to therapy or shorten therapy itself.

The literature suggests that the IRF, in combination with the reticulocyte count, might be useful in improving the classification of anemias, monitoring bone marrow recovery, and monitoring anemia therapies. Further and more specific clinical uses of the IRF from these references include:

- Monitoring stem cell regeneration after bone marrow transplant
- Monitoring bone marrow regeneration after intensive chemotherapy
- Monitoring iron, vitamin B₁₂, or folate therapy
- Monitoring affects of toxic drugs on the bone marrow
- Monitoring kidney transplant engraftment via erythropoiesis
- Classifying and evaluating anemias
- Monitoring neonatal transfusion requirements
- Detection of aplastic crisis

Improving the Classification, Diagnosis, and Treatment of Anemias

Davis, et al suggests that an improvement in the classification of anemias can be accomplished when the IRF is used in combination with the reticulocyte count. The following table provides a summary of the interaction between the IRF and the reticulocyte count in different clinical conditions.

<table>
<thead>
<tr>
<th>Clinical Condition</th>
<th>IRF</th>
<th>Reticulocyte Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone Marrow Engraftment</td>
<td>Elevated</td>
<td>Decreased</td>
</tr>
<tr>
<td>Hemolytic Anemia</td>
<td>Elevated</td>
<td>Elevated</td>
</tr>
<tr>
<td>Iron Deficiency Anemia</td>
<td>Elevated</td>
<td>Normal to Decreased</td>
</tr>
<tr>
<td>B₁₂ or Folate Deficiency</td>
<td>Elevated</td>
<td>Normal to Decreased</td>
</tr>
<tr>
<td>Recent Hemorrhage</td>
<td>Elevated</td>
<td>Normal to Elevated</td>
</tr>
<tr>
<td>Thalassemia</td>
<td>Normal to Elevated</td>
<td>Elevated</td>
</tr>
<tr>
<td>Myelodysplastic Syndromes</td>
<td>Normal to Elevated</td>
<td>Normal to Decreased</td>
</tr>
<tr>
<td>Hypoplastic Anemia</td>
<td>Normal to Elevated</td>
<td>Decreased</td>
</tr>
<tr>
<td>Aplastic Crisis</td>
<td>Normal to Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Myeloid-Lymphoid Malignancies</td>
<td>Decreased</td>
<td>Normal</td>
</tr>
<tr>
<td>Aplastic Anemia</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
</tbody>
</table>
Monitoring Bone Marrow Recovery Following Bone Marrow Transplant

Bone marrow transplants are widely performed and generally accepted for some hematologic disorders and solid tumor therapy. Prior to the marrow transplant, the patient receives high doses of chemotherapy and radiation in an effort to irradiate the bone marrow. This process leaves the patient with little or no red cell, white cell, or platelet production, requiring extensive monitoring to determine the amount and duration of administration of prophylactic antibiotics and blood products.\textsuperscript{15}

After the transplant, the CBC, differential and reticulocyte count are used to monitor the patient for early signs of bone marrow recovery. The absolute neutrophil count is generally accepted as a primary indicator of returning bone marrow production of myeloid cells and a successful bone marrow engraftment. A post transplantation increase in neutrophils and immature myeloid cells to 0.5\times10^9/L or greater typically defines successful myeloid engraftment.\textsuperscript{5,15,16}

Platelet counts, although useful indicators of megakaryocyte production, are less frequently monitored. Since patients require frequent platelet transfusions, an increase in the platelet count may be due to the transfusions and may not reflect actual engraftment.\textsuperscript{3,5}

An increase in the reticulocyte percent of greater than 1\% or an increase in the absolute reticulocyte count of 50\times10^9/L or greater is used as an indicator of erythroid engraftment.\textsuperscript{3,15} Although increased reticulocyte counts represent marrow regeneration, they are less sensitive and later indicators than the absolute neutrophil count. Davis performed a study of twelve bone marrow transplant patients that showed a one to seven week lag in reticulocyte count response when compared to the response of absolute neutrophil counts.\textsuperscript{15}

Davis and others have also evaluated the IRF in cases of bone marrow transplants.\textsuperscript{3,17} In contrast to the reticulocyte count, they found that the IRF response closely tracks, and often precedes the absolute neutrophil count response to returning bone marrow activity. A substantial benefit of the IRF is that it is less likely to be affected by events that add variability to the absolute neutrophil counts, such as clinical or subclinical infections. An increase of the IRF of more than 20\% from the post marrow transplant suggests successful erythroid engraftment.

The IRF has been shown by Davis and others to indicate successful engraftment in half the time from transplantation when compared to the reticulocyte count. Davis has concluded that the IRF is the most sensitive indicator of successful bone marrow engraftment since a rise in the IRF is the earliest response detectable by currently available laboratory methods.\textsuperscript{14}

Monitoring Erythropoiesis Following Kidney Transplant

Erythropoietin (EPO) is a hormone that regulates red cell production in the bone marrow. During erythropoietic stress generated by an anemic state, erythropoietin levels rise, red cell production increases, and reticulocytes are released from the bone marrow into circulation.\textsuperscript{23}

Since the kidneys are the primary source of EPO, chronic renal failure results in a chronic anemia associated with the lack of erythropoietin production and release. While traditional therapy may involve blood transfusions to maintain hemoglobin levels, kidney transplant is the most effective physiological treatment of anemia caused by chronic renal failure.
Moulin et al.\textsuperscript{27} followed 74 renal transplant patients using serial measurements of serum erythropoietin, hematocrit, reticulocyte values, and serum creatinine. After transplant, the IRF increased by 25% over the pre-transplant values and at an average of seven days before an increase in the absolute reticulocyte count. Moulin concluded that the IRF was a more sensitive, early indicator of erythropoiesis after renal transplant than the absolute reticulocyte count.

**Monitoring EPO Therapy in Patients with Chronic Renal Failure**

As all patients with chronic renal failure are not candidates for kidney transplant, recombinant human erythropoietin (r-HuEPO), an alternative to blood transfusions, has become available that reduces the risks associated with continuous transfusions.\textsuperscript{24}

r-HuEPO is an effective treatment in patients with anemia associated with chronic renal failure, compensating for the kidney’s lack of erythropoietin production and maintaining hemoglobin levels. r-HuEPO has also proved to be effective in treating anemia caused by an insufficient response to EPO that may be associated with acquired immune deficiency syndrome, cancer, prematurity, or in patients that deny blood transfusions.\textsuperscript{24}

As the costs related to treating chronic renal failure are rising, there is a significant potential for an increase in cost savings by monitoring the effectiveness of r-HuEPO.\textsuperscript{25,26} The IRF is likely to be an important and sensitive parameter for evaluating a patient’s response to EPO therapy.\textsuperscript{12,19}

After staining with new methylene blue, the red cells are treated with an acidic, hypo-osmotic, ghosting solution that spheres the cells and clears the hemoglobin. The resulting sphered cells are then classified as either mature red cells or reticulocytes using VCS Technology. The average volume of all events classified as reticulocytes is referred to as the Mean Reticulocyte Volume (MRV). The average volume of all red cell events - both mature red cells and reticulocytes, is referred to as the Mean Sphered Cell Volume (MSCV).

**Clinical Utility of MSCV**

As part of an instrument evaluation, Chiron, et al.\textsuperscript{28} assessed the correlation between the Mean Corpuscular Volume (MCV) from the CBC and the MSCV of 286 samples. As expected with the biconcave structure of the red cell, the overall correlation indicated a positive bias of the MSCV. There was, however, an unexpected subpopulation of 48 samples with the MSCV less than the MCV. This subpopulation contained the entire group of 31 samples from patients with hereditary spherocytosis (HS).

HS is a relatively common disorder related to red cell structural protein defects. The actual red cells of HS exhibit a decreased surface to volume ratio in the absence of the biconcave structure that leads to an increased osmotic fragility. Based on testing, Chiron discusses the possibility that the red cells of HS reach a critical level of osmotic volume expansion and then fragment, resulting in a lower than expected MSCV. When using a simple cutoff of MSCV < MCV, Chiron obtained a sensitivity of 100% and specificity of 93.3% for HS. The conclusion to the evaluation suggests that the relationship of the reticulocyte MSCV to the CBC-MCV may be a highly indicative test of hereditary spherocytosis.
Recent advances in technology have provided clinicians with more precise reticulocyte counts and new reticulocyte parameters that may prove to be of great value. One of the most promising new parameters is the immature reticulocyte fraction which has practical implications in the practice of medicine.\textsuperscript{25} Additionally, the relationship between the MSCV and the MCV has generated much interest as an indicator of hereditary spherocytosis.

Possible clinical relevance makes it reasonable to assume that research involving reticulocyte associated parameters will increase. Beckman Coulter currently provides a number of reticulocyte associated parameters and will continue to support research using these parameters for the future.

**Reticulocyte Flags, Codes, and Messages**

<table>
<thead>
<tr>
<th>Definitive Messages</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Reticulocytosis %</td>
<td>Laboratory-defined as RET % &gt; high action limit</td>
</tr>
<tr>
<td>Reticulocyctosis #</td>
<td>Laboratory-defined as RET # &gt; high action limit</td>
</tr>
</tbody>
</table>

**Suspect Messages**

<table>
<thead>
<tr>
<th>Verify Retic</th>
<th>Triggered by a number of internal algorithm checks.</th>
</tr>
</thead>
</table>

**Abnormal Retic Pattern**

The Abnormal Retic Pattern message is triggered by any one of the following Research Suspect Messages:

- RET Interference
- Sickle
- Thalassemia
- Low Volume WBC

**Research Suspect Messages**

- RET Interference: Both the mature red cell population and the reticulocyte population exhibit high light scatter and high volume means.
- Sickle: Both the mature red cell population and the reticulocyte population exhibit high volume means and standard deviations.
- Thalassemia: A population of events with high light scatter and low volume characteristics.
- DDD: Data Discontinuity Detector: Indicator that flow rate abnormalities due to flow cell blockage were detected during analysis.
- Low Volume WBC: A population of high volume events with light scatter consistent with white cells.

**Instrument Flags and Codes**

- The Incomplete Computation (.....) code is triggered by any one of the following conditions:
  - If RBC incomplete (.....), then RET# and HLR# will display (.....)
  - If RBC voteout (-----), then RET# and HLR# will display (.....)
  - If RET# >999.9 x 10\textsuperscript{6} cells/L, then all reticulocyte parameters except RET# will display (.....)
  - If RET% >100% then all reticulocyte parameters except RET% will display (.....)
  - If RET% =0.00, then IRF will display (.....)

- The Exceeds Reportable Range code (+) is triggered by one of the following conditions:
  - If with RET%, then the RET% > 100%
  - If with RET#, then the RET# > 0.7500 x 10\textsuperscript{6} cells/L
Reticulocyte Flags, Codes, and Messages (continued)

<table>
<thead>
<tr>
<th>Instrument Flags and Codes (continued)</th>
</tr>
</thead>
<tbody>
<tr>
<td>triggered by (exceeds printable range)</td>
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<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>R (review results)</td>
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</tbody>
</table>

References

29. Fourcade CH, Jary L, Belaouni H. Reticulocyte Analysis Provided by the Coulter GEN·S: Significance and Interpretation in Regenerative and Nonregenerative Hematologic Conditions. Laboratory Hematology. 1999