CELL-DYN Sapphire™ – Reticulocyte/IRF Analysis

Reticulocyte analysis is now an accepted part of the repertoire of tests performed by automated hematology analyzers. A variety of methods are used for reticulocyte analysis, including light absorbance, scatter and fluorescence. The choice of detection technologies is determined by the stain used.

Despite this variation in methods, there are common needs among automated methods. Broadly, these are:

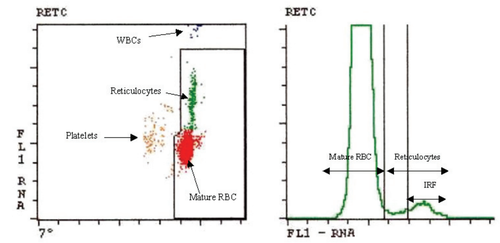
1. Rapid staining to sustain instrument throughput and reduce the impact of measurement of a kinetic process
2. High yield of fluorescence to give high sensitivity and precision even at low reticulocyte concentrations
3. Good differentiation of DNA and RNA staining to preclude WBC and NRBC interference
4. RNA concentration-dependent proportionality of fluorescence staining to ensure validity of the immature reticulocyte fraction (IRF)

For the validation of the CELL-DYN Sapphire™ method, we have characterized each of these needs in the method. Importantly, cross-method comparability between different manufacturers' IRF methods has yielded poor correlation between some methods. We therefore wanted to demonstrate the validity of our own method by showing comparability with a different analytical means of measuring reticulocyte age. For this we developed a co-staining method by which the RNA staining of cells by the CELL-DYN reticulocyte reagent and CD71 (transferrin receptor) expression on the developing cells could be compared.

The CELL-DYN Sapphire™ Reticulocyte Method

The CELL-DYN Sapphire™ has the capability to perform reticulocyte counts (expressed as percentage or absolute values) in addition to an immature reticulocyte fraction. The nucleic acid stain used is an asymmetrical cyanine dye. It is excited at 488 nm and emits fluorescence at 530 nm upon binding to DNA or RNA. The instrument uses two parameters for derivation of the reticulocyte data. These are IAS (intermediate angle scatter [7°]) and green fluorescence (FL1) **[Figure 1].**

Figure 1. Fluorescent reticulocyte analysis. WBCs and platelets are excluded from the analysis using 7° scatter and green fluorescence staining of nucleic acids. The IRF and reticulocyte populations are determined using FL1 histogram analysis of the RBCs.



Our studies have demonstrated that the Sapphire™ reticulocyte method is rapid and achieves steady state staining prior to analysis. The dye showed equivalent fluorescence in cells stained for only 20 seconds and for up to ten minutes prior to fluorescence flow cytometry.

Previous reports have demonstrated the potential for leukocyte interference in reticulocyte counts. Our experiments have shown that the CELL-DYN reticulocyte stain shows an almost four-fold fluorescence yield in DNA staining compared with RNA staining. Furthermore we have shown a markedly higher degree of fluorescence yield compared with Thiazole Orange (another widely used reticulocyte stain) **(Figure 2a).**

We have shown that the fluorescence yield of the CELL-DYN stain is progressive and proportionate to RNA concentration. **Figure 2b** shows the yield of fluorescence generated in response to staining of varying amounts of RNA with a constant amount of the CELL-DYN reticulocyte stain.

Additionally, we have demonstrated that the staining of the most immature reticulocytes defined by RNA staining is correlated with an alternative measure of reticulocyte maturity, namely CD71 expression **(Figures 2c and 2d)**. CD71 or the transferrin receptor is most highly expressed in developing reticulocytes due to their high demand for iron during Hb synthesis. As a consequence, the CD71 expression is a measure of cell maturity. Evidence of correlation (r=0.735) between the two parameters provides confirmation that the IRF is truly a measure of reticulocyte immaturity.

Finally, external scientific studies have demonstrated the superior precision of the CELL-DYN method over non-fluorescent methods which use light absorbance or scatter **(Figure 3).**

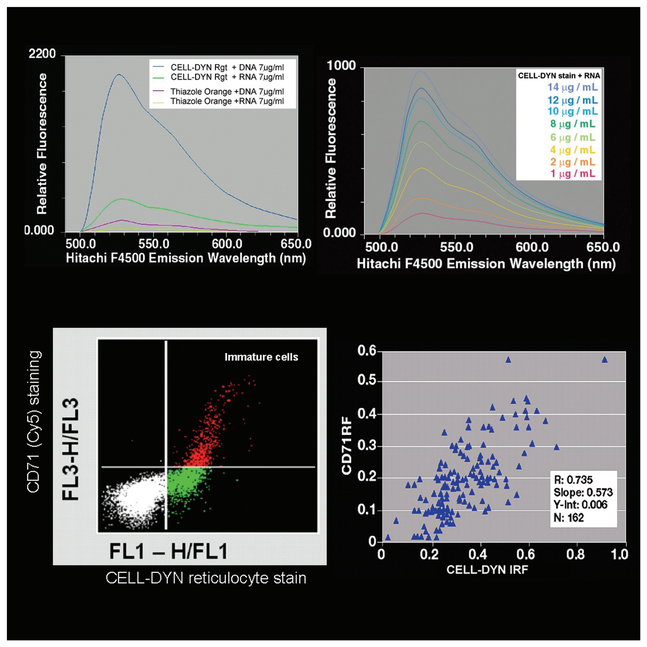


Figure 2. There is little agreement between the IRF values of different manufacturers' analyzers. The CELL-DYN Sapphire™ IRF method has been well-characterized to demonstrate **(a)** high differential fluorescence yield between DNA and RNA staining as well as **(b)** progressive and quantitative staining of RNA (upper plates). Additionally CD71 transferrin receptor staining has shown that the IRF derived from RNA staining correlates to an IRF derived from the number of CD71 receptors (an alternate measure of reticulocyte immaturity). This is seen in the flow cytometic analysis of the reticulocytes of a single study subject **(c)** and overall among 162 study subjects **(d)**.

Validation of a Fluorescent Nucleic Acid Stain Used for Reticulocyte Analysis

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International Society of Laboratory Hematology Scientific Meeting, Poster Abstract A89 2005

Figure 3. Paired precision data (replicate 1 vs. replicate 2) for IRF **(a)** and reticulocytes **(b)** in reticulocytopenic samples measured by CELL-DYN Sapphire™ and two alternative automated hematology analyzers. Note that for the reticulocytes and IRF, the closest agreement (expressed as a correlation coefficient) is seen in the CELL-DYN Sapphire™ data.

Conclusions

- Reticulocyte staining on the CELL-DYN Sapphire™ is rapid and achieves endpoint reaction prior to being read.
- The stain used is very bright giving high signal-to-noise ratios as well as good separation between DNA and RNA staining. This minimizes interferences and maximizes precision.
- Compared with two other automated reticulocyte counts, precision of the reticulocyte count and IRF were highest on the CELL-DYN Sapphire™.
- Fluorescence staining is progressive and proportionate to RNA concentration.
- The CELL-DYN IRF data correlates with a surrogate measure of reticulocyte maturity based on CD71 expression.
- The discordance which is seen between IRF values of different manufacturers is not caused by a failure of CELL-DYN Sapphire™ to measure reticulocyte maturity.