Osmotic fragility of red blood cells quantified on the NovoCyte® flow Cytometer

Among the red cell membrane disorders, hereditary spherocytosis (HS) is one of the most common causes of inherited hemolytic anemia. It is more frequent in Caucasians, affecting approximately 1 in every 1000–2000 individuals resulting from genetic mutations in red blood cell (RBC) membrane proteins. The typical hallmark of hereditary spherocytosis, although not specific, is the presence of spherocytes in a blood smear, however, spherocytes can be rare in certain patients and requires microscopic detection by a skilled operator. Therefore, new assays were developed that exploit the surface area-to-volume ratio which are typically reduced in spherical erythrocytes (NaCl osmotic fragility, glycerol lysis test, and others). Unfortunately, these assays proved insufficiently sensitive, commonly missing mild cases of HS. Current guidelines recommend the use of flow cytometry assays for diagnosis of HS, that include flow cytometry osmotic fragility test (FCM OF). There are certain advantages of the FCM OF test: it is quantitative and objective, easy and inexpensive to perform, does not require pre-incubation of blood samples, and has high test efficiency. The FCM OF test has been demonstrated to accurately determine the clinical severity of HS patients therefore, the FCM OF test is rapidly becoming the HS diagnostic test of choice. In this application note, we performed the FCM OF test on the NovoCyte flow cytometer, investigating the resistance of RBC to changes in saline concentration and incubation temperature.

Red blood cells hemolysis occurs from saline concentration alteration

A RBC in an isotonic saline solution will undergo hemolysis when placed in a hypotonic environment, by exposure it to deionized water (DI Water). The FCM OF test is based on the susceptibility or resistance of RBCs to lysis when exposed to DI water. First, the effect of hypotonic solution was investigated, where DI water was added to red blood cells to obtain a final concentration of anywhere from 30-100% PBS (Figure 1). After 3 minute incubation, hemolysis was analyzed on the NovoCyte by changes in forward scatter (FSC), side scatter (SSC), and cell concentration (Figure 1A). Intact RBCs are larger with a higher FSC and SSC; however, during hemolysis RBCs shrink causing a decrease in FSC and SSC. As has been previously reported, a dramatic decrease in the frequency of intact RBCs occurs between 40% and 50% PBS solution (Figure 1B). The NovoCyte flow cytometer uses a high precision syringe pump allowing for the automatic determination of absolute cell counts. Determination of the cell concentration reveals that dramatic hemolysis actually occurs at 50% PBS with a ~3.5 fold decrease in cell concentration relative to the 60% PBS solution (Figure 1C). This data confirms the hemolytic effect of DI water on RBC membrane integrity and determines the conditions for the FCM OF on the NovoCyte flow cytometer.

Figure 1: Hemolysis-induced changes in forward and side scatter
Whole blood was diluted in 30%-100% PBS and incubated for 3 minutes prior to analysis. Intact red blood cells (RBCs) are plotted on the right upper quadrant in the scatter plot of side scatter (SSC) y-axis versus forward scatter (FSC) x-axis (1A). Frequency of intact RBC for each sample is graphed in (1B). Cell concentration (millions of cells/mL) for each samples is graphed in (1C).
Flow cytometry osmotic fragility test is affected by incubation time and temperature

Next we performed RBC osmotic fragility measurements on the NovoCyte with normal patient blood. FCM OF measurements take place in two parts: first, diluted blood is analyzed on the flow cytometer to generate a baseline count (Figure 2,left side). Next, DI water is added to the sample to induce hemolysis (Final concentration 55%) (Figure 2,right side). The degree of osmotic hemolysis is calculated as % residual red cells (%RRC) which is the percentage of remaining RBCs after DI water addition compared to the baseline count. Increased osmotic fragility is indicated by a low %RRC and is significantly decreased in patients with HS. As previously reported, RBCs analyzed from normal patient blood have ~65% RRC.

Both temperature and incubation time affect cell membrane integrity. Therefore, To examine the effect of time and temperature on osmotic fragility, RBCs were incubated at 4˚C, 25˚C, and 37˚C overnight followed by the FCM OF test (Figure 3). A dramatic decrease in %RRC is observed with increased temperature. The %RRC decreased as the incubation temperature increased. At 4˚C incubation there were 38% RRC, 28% RRC at 25˚C, and only 16% RRC at 37˚C. This indicates that incubated RBCs are not stable at any temperature overnight and when possible, the FCM OF test should be performed immediately after blood collection.

Conclusion

Increased osmotic fragility is found in hereditary spherocytosis, other RBC membrane disorders and in idiopathic acquired hemolytic anemias. Therefore, it is essential to have the capacity to determine osmotic fragility. RBC analysis by flow cytometry is increasingly becoming the test of choice for osmotic fragility as well as other characteristics of clinical cellular dysfunction. In this application note, our data demonstrates how easily the FCM OF test can be run on the NovoCyte flow cytometer and obtain accurate results.

References


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