Application Note

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Osmotic Fragility of Red Blood Cells Quantified on the Agilent NovoCyte Flow Cytometer

Authors

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Abstract

In this application note, we analyzed cellular osmotic fragility on the Agilent NovoCyte flow cytometer, investigating the resistance of red blood cells (RBCs) to changes in salinity and incubation temperature. The results demonstrate the utility of this research method for accurate quantitative assessment of membrane integrity in RBC samples.

Introduction

Among the red blood 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 one in every 1,000 to 2,000 individuals, resulting from genetic mutations in RBC membrane proteins.

The typical hallmark of HS, although not specific, is the presence of spherocytes in a blood smear; however, spherocytes can be rare in certain people, and require microscopic detection by a skilled operator. Therefore, assays were developed that exploit the surface area-to-volume ratio, which is 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 the investigation of HS, which includes a 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 is therefore rapidly becoming the HS research test of choice.

Red blood cell hemolysis occurs from saline concentration alteration

An RBC in an isotonic saline solution will undergo hemolysis when placed in a hypotonic environment, by exposing it to de-ionized 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 RBCs to obtain a final concentration of anywhere from 30 to 100% PBS (Figure 1). After incubating for three minutes, hemolysis was analyzed on the NovoCyte flow cytometer 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 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 to allow for the automatic determination of absolute cell counts. Determination of the cell concentration reveals that dramatic hemolysis occurs at 50% PBS with a ~3.5 fold decrease in cell concentration relative to the 60% PBS solution (Figure 1C). These data confirm the hemolytic effect of DI water on RBC membrane integrity and determine the conditions for the FCM OF on the NovoCyte flow cytometer.

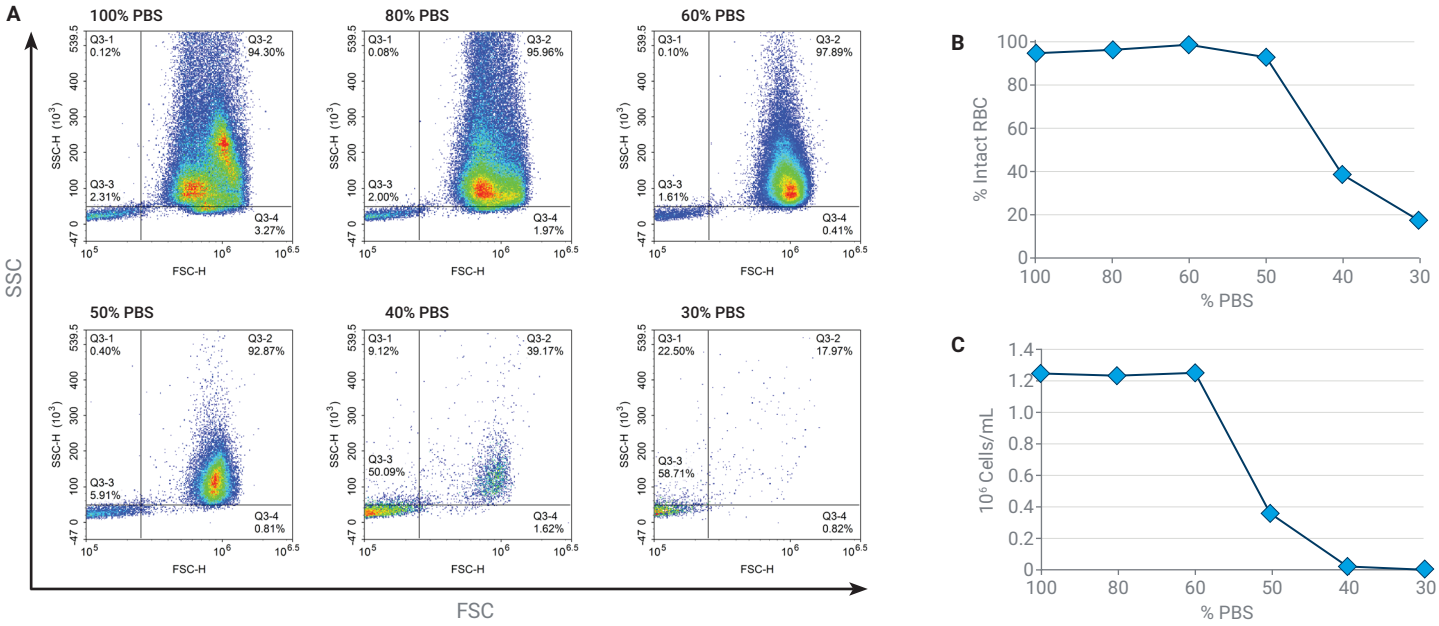
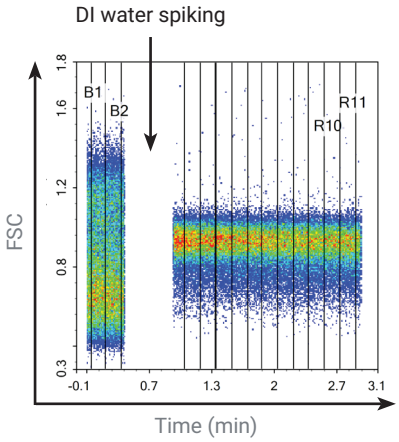


Figure 1. Hemolysis-induced changes in forward and side scatter. Whole blood was diluted in 30 to 100% PBS and incubated for three minutes before analysis. Intact RBCs are plotted on the right upper quadrant in the scatterplot of SSC versus FSC (A). The frequency of intact RBC for each sample is graphed in (B). Cell concentration (millions of cells/mL) for each sample is graphed in (C).

Flow cytometry osmotic fragility test is affected by incubation time and temperature

Next, RBC osmotic fragility measurements were performed on normal donor blood using the NovoCyte flow cytometer. 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). Next, DI water is added to the sample to induce hemolysis (final concentration 55%) (Figure 2, table). The degree of osmotic hemolysis is calculated as %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 people with HS. As previously reported, RBCs analyzed from normal donor 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, 25, 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. There was 38% RRC at 4 °C incubation, 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.



	Count	Y Mean
B1	18,689	802,550
B2	18,237	796,784
R10	7,093	886,906
R11	7,225	887,681

Figure 2. FCM OF test from a healthy donor. The FCM OF test was performed on healthy human blood with a modified protocol first described by (Won and Suh, 2009).[1] Blood samples were collected in EDTA tubes, and analyzed within two hours of blood collection. The red cell suspension is prepared by a two-step dilution with normal saline. In the first step, 20 µL of blood was diluted in 1 mL of PBS. Second, 10 µL of the diluted blood was diluted again in 1.1 mL PBS, resulting in a final 55% PBS solution. A baseline of 20 µL of the final RBC suspension was analyzed at a flow rate of 50 µL/minute. Next, 0.9 mL of DI water was spiked into the sample, and an additional 100 µL was acquired. Each region acquired is a 10 second segment of time. The degree of osmotic hemolysis was expressed as %Residual red cells (%RCC), calculated by the formula: %Residual red cells (%RRC)= mean event count of last two regions/mean event count of first two regions × 1.1/2.0 × 100 (%). The % residual red cells was, on average, 65% for samples acquired immediately after blood collection.

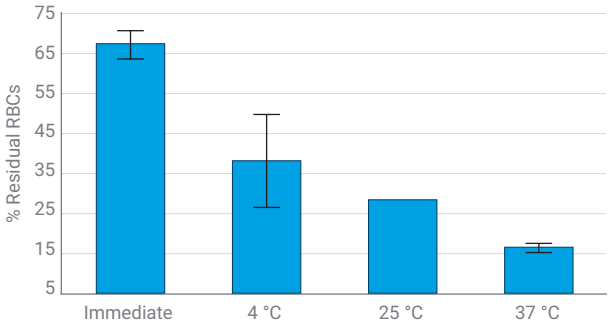


Figure 3. Effect of time and incubation temperature on osmotic fragility. The FCM OF test was performed on normal human blood immediately after collection, or after overnight incubation at 4, 25, or 37 ˚C. %RRC was calculated as described in Figure 2.

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 cellular dysfunction. This application note demonstrates the ease with which labs can obtain FCM OF test results on the NovoCyte flow cytometer.

References

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