

Immunophenotyping peripheral blood cells

A no-lyse, no-wash, no cell loss method for immunophenotyping nucleated peripheral blood cells using the Attune® Acoustic Focusing Cytometer

This application note describes a no-lyse, no-wash method for immunophenotyping whole blood that takes advantage of acoustic focusing technology. Previously described methods that use a no-lyse, no-wash staining protocol do so with a loss of light scatter resolution [1]. Loss of cell population identification using light scatter in these applications is a limitation of traditional flow cytometry, in which hydrodynamic focusing is used to orient cells in the flow stream. In contrast, the Attune® Acoustic Focusing Cytometer aligns cells in the core stream using acoustic forces independent of the fluid stream. Cells are precisely aligned in the core, resulting in much higher throughput than is possible with traditional flow cytometry.

With the Attune® cytometer, it is now practical to process stained whole-blood without the need to either lyse red blood cells (RBCs) or incorporate wash steps for removal of RBC fragments and platelets. Whole blood is simply stained with a premixed cocktail of directly conjugated antibodies, diluted 1:400 in phosphate buffered saline (PBS), and analyzed on the Attune® cytometer without sacrificing light scatter resolution. Vybrant® DyeCycle™ Ruby Stain is used to fluorescently select nucleated cells from the core stream, avoiding the need to collect large data sets.

Human blood is used here for demonstration purposes, but this technique may be used with any mammalian blood. In this application, 10 µL whole blood is stained in a 50 µL total volume and then diluted 400-fold to 4 mL final volume. A fluorescence threshold is used to select the nucleated cell population from the much more abundant non-nucleated red blood cells.

This method delivers additional time savings by reducing the number of sample preparation steps and by eliminating lysis and wash steps, thereby avoiding cell loss that can affect cell subpopulations of interest.

Basic protocol

Materials

- Vybrant® DyeCycle™ Ruby Stain (Invitrogen Cat. No. V-10273)
- Directly labeled anti-human antibodies
- Normal Mouse IgG (Invitrogen Cat. No. 10400C)
- Attune® Performance Tracking Beads (Applied Biosystems Cat. No. 4449754)
- Phosphate Buffered Saline (PBS)
- PBS-BSA (PBS + 1%BSA + 2 mM NaN₃, pH 7.2)
- 12 x 75 mm tubes or other flow cytometry tubes
- Attune® Acoustic Focusing Cytometer
- AbC™ anti-Mouse Bead Kit (Invitrogen Cat. No. A-10344)
- Human peripheral blood collected in anticoagulant

Titration antibodies

1. Titrate all antibody conjugates following this staining protocol to determine the optimal staining concentration.
2. Antibody conjugates may be used at the manufacturer's recommended staining concentration with the AbC™ anti-Mouse Bead Kit (see protocol notes on preparing antibodies, step 2).
3. For multicolor testing, pre-mix antibody conjugates in 1X PBS-BSA with Vybrant® DyeCycle™ Ruby Stain to provide titer in a 40 μ L total volume. Adjust the Vybrant® DyeCycle™ Ruby Stain concentration in the pre-mix to provide a 2.5 μ M concentration in the 50 μ L staining volume.

Preparing blood

1. Collect peripheral venous blood in blood collection tubes containing anticoagulant. Mix well.
2. [Optional] Block Fc binding receptors by pretreating with 67 μ L of 3 mg/mL normal mouse Ig per 1 mL whole blood and incubating for a minimum of 10 min prior to antibody labeling.

Staining protocol

1. Pipet all antibody conjugates into labeled sample tubes; final total volume should be 40 μ L.
2. Add 10 μ L of anticoagulated whole blood to the antibody mixture and mix well.
3. Incubate protected from light for 30 min at room temperature (or reagent manufacturer's recommendation).
4. Prepare PBS with 2.5 μ M Vybrant® DyeCycle™ Ruby Stain in sufficient quantity for 4 mL per tube.
5. Add 4 mL 2.5 μ M DyeCycle™ Ruby stain in PBS to the tubes immediately prior to loading on the Attune® cytometer.

Compensation controls

1. Prepare single-color compensation samples using the AbC™ anti-Mouse Kit.
2. Add one drop of AbC™ anti-Mouse capture bead (Component A) to a labeled sample tube for each conjugate antibody included in the panel.
3. Add the recommended amount of each mouse antibody conjugate to the AbC™ anti-Mouse capture bead.
4. Incubate for 15 min at room temperature, protected from light.
5. Add 3 mL PBS and centrifuge for 5 min at 200 x g.
6. Carefully remove the supernatant and resuspend the bead pellet by adding 1 mL PBS.
7. Prepare one AbC™ anti-Mouse negative control bead (Component B) sample by adding 1 drop to a labeled sample tube containing 1 mL PBS.
8. Prepare one tube of 20 μ L whole blood in 4 mL PBS with 2.5 μ M Vybrant® DyeCycle™ Ruby Stain for use as the BL3 compensation control. Incubate for a minimum of 15 min at room temperature.

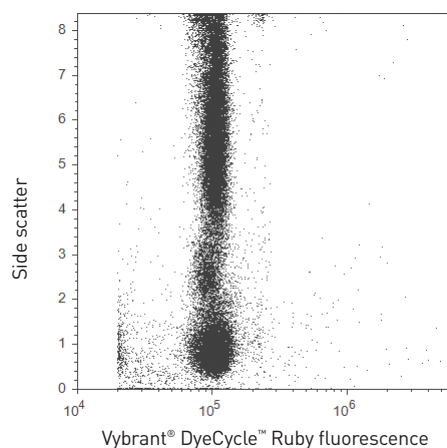


Figure 1. Dot plot of Vybrant® DyeCycle™ Ruby Stain vs. side scatter showing the fluorescence threshold level (20,000) on human peripheral whole blood.

Data collection

1. Verify instrument performance with the Attune® Performance Tracking Beads.
2. Create a new experiment using instrument settings optimized for no-lyse, no-wash staining.
3. In the experiment browser, right-click "Compensation" and select "Compensation setup."
4. In the compensation setup dialog box, select to compensate on height and select the parameters required for your panel.
5. Pulse height is used for both scatter and fluorescence signals, as it provides lower measurement standard deviations than pulse area (see Protocol notes on optimizing instrument settings, step 3).
6. There should be one single-color compensation sample matched to each parameter selected.
7. Click OK to create the compensation control samples.
8. Select the workspace for your specimen sample by double-clicking on the first tube for the specimen in the experiment browser.
9. Create the following plots:
 - Bivariate plot of the Vybrant® DyeCycle™ Ruby Stain threshold channel height vs. side scatter (for threshold adjustment)
 - Vybrant® DyeCycle™ Ruby Stain height vs. area (for cell singlet selection)
 - Bivariate plot of forward scatter vs. side scatter
 - Additional plots as needed for data analysis.
10. Place a polygon gate around the population of interest (Figure 2B).
11. Optimize BL3 fluorescence threshold to separate nucleated cells from non-nucleated events (e.g., RBCs and platelets).
12. Optimize remaining fluorescence PMT voltage levels.
13. Once instrument optimizations are complete, select the first

compensation control tube.

14. Enable forward scatter threshold for the compensation controls by changing the FSC threshold logic to "or".
15. Beads require a forward scatter threshold. You may also need to adjust the forward and side scatter voltages to place the bead singlets on scale. Do not, however, change fluorescence PMT voltages from those optimized for your cells. Compensation controls and the stained cell panel must be run at the same fluorescence voltages to obtain a valid compensation matrix.
16. Set the recording conditions to collect 5,000 R1 (bead singlet) events, acquisition volume to 200 μL , and flow rate to 200 $\mu\text{L}/\text{min}$.
17. Run the Component B compensation control, adjust the R1 gate to include only bead singlets and record the data.
18. Copy the R1 gate to the remaining compensation controls and run each tube in order.
19. For the BL3 Vybrant[®] DyeCycle[™] Ruby Stain compensation control, return the FSC threshold setting to "Ignore", and return the scatter voltages to those optimized for cells.
20. Select your first sample tube of stained cells.
21. Set the collection rate to 500 $\mu\text{L}/\text{min}$, acquisition volume and recording criteria to obtain the desired events (we recommend starting with 2,500 μL volume for 2,500 lymphocyte events).
22. Proceed with collecting data for samples.

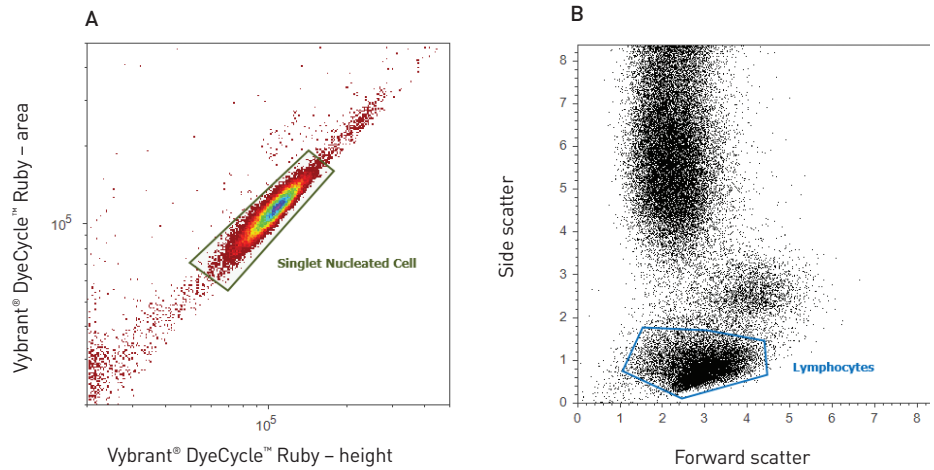


Figure 2. Density plot of Vybrant[®] DyeCycle[™] Ruby stained human peripheral blood. (A) Fluorescence height versus area with a polygon gate selecting single nucleated cells. **(B)** A daughter dot plot of the singlet nucleated cell gate is used to gate on the subpopulation of interest. In this case, a polygon gate is set around the lymphocyte population.

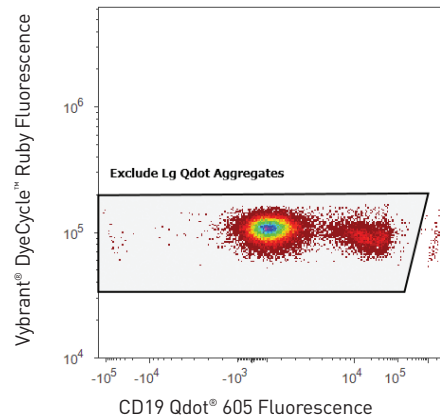


Figure 3. Density plot of human peripheral blood showing Vybrant[®] DyeCycle[™] Ruby Stain fluorescence versus mouse anti-human CD19 Qdot[®] 605 fluorescence. The polygon gate is drawn to exclude the brightly fluorescent Qdot[®] aggregates. These aggregates will appear about ten times brighter (1 log) than the normal targeted (CD19) stained cells. The Qdot[®] aggregate population will also orient at a diagonal to the cell staining axis of Vybrant[®] DyeCycle[™] Ruby.

Data analysis

The recommended gating strategy is to start with fluorescence height versus area in a bivariate plot to gate on singlets (Figure 2A) to select the population of interest in a forward scatter versus side scatter daughter plot.

When using Qdot® nanocrystals in the staining panel, it may be necessary to gate out nanocrystal aggregates using Qdot® fluorescence versus Vybrant® DyeCycle™ Ruby Stain. Qdot® aggregates will appear as events ten times brighter than the targeted population, with the population at a diagonal from that of the Vybrant® DyeCycle™ Ruby Stain (Figure 3).

The use of these gates to select only single cell population and exclude Qdot® aggregates provides a cleansing strategy to remove undesired events from the data set for analysis. These gates should be established in a hierarchical fashion: singlet nucleated cells → exclusion gate → lymphocyte scatter gate.

5-color example

In the following analysis example, whole human peripheral blood was stained with a 6-color panel composed of mouse anti-human CD3 FITC, mouse anti-human CD56 PE, mouse anti-human CD8 Pacific Blue® dye, mouse anti-human CD4 V500, and mouse anti-human CD19 Qdot® 605 dye. Titer for these five direct conjugates ranged from 0.031 µg to 0.25 µg (5 nM for CD19 Qdot® 605 dye). This example 5-color staining required five fluorescence-minus-one (FMO) control samples, and one 5-color panel sample tube. The FMO controls are used to determine appropriate marker placement.

The initial gating strategy shown in Figures 2 and 3 was used to remove undesired events and select the lymphocyte scatter population for immunophenotyping analysis. A series of density bivariate plots is generated to show the various immunophenotype populations (see Figure 4).

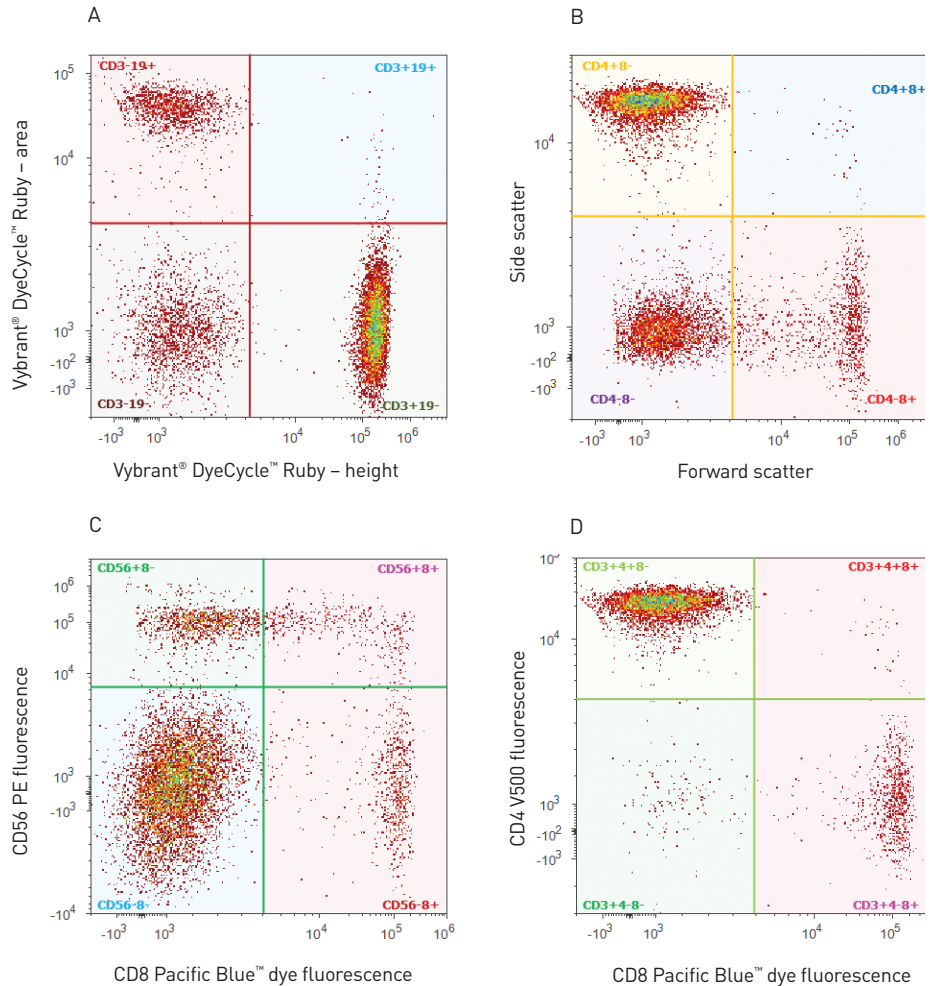


Figure 4. Five-color immunophenotyping analysis example. (A) T and B cell populations with mouse-human CD3 FITC vs. mouse anti-human CD19 Qdot® 605 dye density plot. (B) CD4 and CD8 cell subpopulations in a bivariate density plot with mouse anti-human Pacific Blue® dye vs. mouse anti-human CD4 V500. (C) Natural killer (NK) cells and CD8 population subsets in a bivariate density plot with mouse anti-human CD56 PE and mouse anti-human CD8 Pacific Blue® dye. (D) T helper and cytotoxic cells in a CD3 gated daughter bivariate density plot with mouse anti-human CD8 Pacific Blue® and mouse anti-human CD4 V500.

Protocol notes

Preparing antibodies

1. Successful results with this method depend on testing and titrating directly conjugated antibodies with this staining method prior to preparing mixed antibody cocktails. Indirect staining is not compatible with no-wash staining, and only the use of directly labeled or Zenon[®] dye-labeled antibodies is recommended. Antibody conjugates must be titered in single color following this method, and optimal titer selected where there is maximal separation of positive cells with minimal background of the negative population. We suggest using the staining index to determine titer. The staining index is calculated as the difference in median between the labeled and unlabeled cell populations divided by twice the standard deviation of the negative population.
2. The use of antibody capture beads as a compensation control is highly recommended. This not only saves valuable sample, but is also critical when the targeted population is rare or dim for the marker requiring compensation. It is not necessary to titrate the conjugate antibodies on the AbC[™] anti-mouse beads separately from the whole blood titration. You may use conjugate antibodies at the manufacturer's recommended concentration for cell staining. However, you can save on reagent consumption by titrating conjugate antibodies on AbC[™] anti-mouse beads. The correct titer will provide fluorescence median intensity of the AbC[™] anti-mouse bead within one log of the brightest stained whole blood [2]. Do not expect the titer determined for staining mouse blood to be appropriate for staining AbC[™] anti-mouse beads.

Optimizing instrument settings

1. Instrument PMT voltages and threshold values should be optimized during antibody titration and panel development. As these optimal instrument settings are determined, save the settings in a "gis" instrument settings file named for your panel. This settings file can then be loaded as your default instrument settings when creating new experiments. Loading predefined instrument settings at the start of your experiment saves you valuable time and sample when running your panel.
2. The recommended collection rate during data recording is a standard collection rate of 500 $\mu\text{L}/\text{min}$. A collection rate of 200 $\mu\text{L}/\text{min}$ will provide more time to observe changes while adjusting thresholds and PMT voltages.
3. Select pulse height in your parameter configuration. In this application, pulse height will provide better scatter and fluorescence precision than either pulse area or width. The use of fluorescence threshold along with collecting pulse height data minimizes the effect of coincidence on the cells of interest. Note that compensation must be configured with the pulse height selected to apply to pulse height fluorescence parameters.
4. Optimize the fluorescence threshold to separate nucleated events from cellular debris. Adjust fluorescence PMT voltages so that cell autofluorescence is well above system noise by placing the autofluorescence around a median fluorescence of 10^3 . Use a sample labeled with only Vybrant[®] DyeCycle[™] Ruby Stain so you can evaluate cellular autofluorescence for the other fluorescence parameters. Next, verify that all stained cells for each of the conjugate antibodies remain on scale at titer. Where events become off scale, you should lower PMT voltages

or reconsider what dyes are being used for your targets. In multicolor staining, use the brightest dyes (e.g., PE) for the lowest expressing targets. Save the optimized settings and use these in creating an experimental template to simplify instrument setup when running your study samples.

Acquisition volumes

You will need to determine the exact acquisition volume based on desired sample conditions and event count. Typically, 10 μL of whole blood will provide at a minimum 2 acquisitions for 15,000 nucleated events with acquisition times of approximately 1 min. A good starting point is to use a 2,000 μL acquisition volume and 500 $\mu\text{L}/\text{min}$ collection rate. You may choose to increase the total number of acquisition events in two ways. One is to acquire additional stained cells and append data to the sample file. Another is to decrease the effective blood dilution from 1:400 to 1:200 by using 20 μL of whole blood and preparing the premixed antibody conjugates in 30 μL volume to maintain the 50 μL staining volume. Decreasing the blood dilution will degrade the light scatter resolution of the cell populations.

Optimizing compensation with Vybrant® DyeCycle™ Ruby Stain

Vybrant® DyeCycle™ Ruby Stain has negligible spectral crossover when used in the standard filter configuration of the Attune® cytometer. The compensation matrix generated for Vybrant® DyeCycle™ Ruby Stain crossover will contain zero or near-zero values. Depending on the conjugate dye selection, there can be substantial spectral crossover into the Vybrant® DyeCycle™ Ruby Stain channel. Compensation correction can result in events spread out from the Vybrant® DyeCycle™ Ruby stained populations (see Figure 5A). Care must be taken when Vybrant® DyeCycle™ Ruby stained populations are used in the gating strategy to avoid excluding desired events from your gate. Spectral bleed over into BL3 can be minimized by substituting the standard 640LP filter with a 695/40 bandpass to provide a more optimal filter configuration when using Vybrant® DyeCycle™ Ruby Stain.

References

1. Weaver JL, McKinnon K, Germolec DR (2010) Phenotypic Analysis Using Very Small Volumes of Blood. *Curr Protoc Cytom* 6.30.1–6.30.8.
2. Roederer M (2002) Compensation in flow cytometry. *Curr Protoc Cytom* 22:1.14.1–1.14.20.