

**Whole Blood – Immunolabeling Protocol for Orflo
Cytometers****Scope:**

This procedure applies to the antibody (e.g. anti-CD marker) labeling of white blood cells (WBC's) isolated from a peripheral whole blood sample by 1.) Performing a buffy coat isolation (*optional*), 2.) Applying an antibody label, and 3.) Lysing the residual red blood cells (RBC's).

Overview:

As RBC's outnumber the WBC's in a whole blood sample by ~1000x, it is necessary to eliminate the RBC interference prior to running on the Orflo Flow Cytometers. In our experience, RBC lysis buffers vary considerably in their efficacy at removing RBC 's. While the BioLegend RBC Lyse Buffer (used in this protocol) is better than most, we have also found that starting with a buffy coat isolation dramatically reduces the starting RBC concentration, improving the overall results.

Reagents:

- Purified Water (Milli Q or Distilled)
- 10x RBC Lyse Buffer ([Biolegend cat#420301](#))
- Cell Staining Buffer ([BioLegend cat # 420201](#) or PBS with 0.5% BSA, 0.1% Azide)
- Labeled Antibodies:
 - **Moxi Flow:** Phycoerythrin (PE) labeled Antibody (e.g. PE anti-human CD4 - BioLegend cat # [300507](#))
 - **Moxi GO 488nm or 532nm systems with 561nm/LP Filter installed:** Phycoerythrin (PE) labeled Antibody(e.g. PE anti-human CD4 - BioLegend cat # [300507](#)) or other excitation/emission compatible fluorophore-conjugated Antibody
 - **Moxi GO 488nm system with 525/45nm filter** – FITC or Alex Fluor 488nm – conjugated Ab (e.g. FITC anti-human CD3, [BioLegend cat #300306](#))
- (*Optional*) Moxi Cyte Flow Reagent ([Orflo MXA079/MXA080](#))

Pre-Prep:

- Warm water and RBC Lyse Buffer to room temperature
- Dilute RBC lyse buffer to 1x, E.g.
 - Add 9ml of purified water to a 15mL centrifuge tube
 - Add 1ml of 10x RBC Lyse Buffer to the above tube

1. Buffy Coat Isolation (optional):

- 1.1. Obtain a fresh Whole Blood sample into an anti-coagulant (e.g. Sodium Citrate)-treated tube.
- 1.2. Spin the whole blood at 1000 x g for 10 minutes with the BRAKE OFF
- 1.3. Remove and discard the majority (all but 2-3mm) of the top (amber/translucent) plasma layer, taking care not to disturb the buffy coat layer (at the interface of the red, RBC layer).
- 1.4. Use a large pipette (cut tip to widen if necessary) to extract the leukocyte band (the buffy coat). Notes:
 - The buffy coat is the (cloudy/white) interface layer between the RBC's (red) and the plasma (amber/translucent).

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- To ensure minimal loss of WBC's, it is necessary to include the residual plasma as well as a small portion of the RBC layer in the extracted buffy coat sample.

1.5. Aliquot buffy coat into a 15ml centrifuge tube

1.6. Dilute cells to a concentration of $\sim 5 \times 10^6$ cells/ml. (e.g. for 3ml starting volume of whole blood, add ~ 2 ml of staining buffer to extracted buffy coat sample)

2. Antibody Label:

2.1. Quick spin (e.g. 500 x g, 5 seconds) antibody vials for maximum volume.

2.2. Prepare 15ml centrifuge tubes with the antibody labels with the manufacturer-recommended test volume for 100 μ L of sample/blood. Note: Prepare Isotype and compensation controls as necessary (CellSimple Only)

2.3. Aliquot 100 μ l of the cell sample ($\sim 5 \times 10^5$ total cells) to each prepared centrifuge tube. Pipette up and down to mix sample and antibody well.

2.4. Incubate for 15-20 min at room temperature, protected from light.

3. RBC Lyse:

3.1. Add 2ml of the room temperature, 1x RBC lysis buffer to each tube. Note: Immediately after adding it to the sample, vortex the sample (e.g. level 3-4) for 1-2 seconds

3.2. Incubate at 37°C in the dark for 15 minutes.

3.3. Add 3ml Staining Buffer and invert 2x.

3.4. Spin the sample at 350xg for 5 minutes with the BRAKE OFF.

3.5. Carefully remove the supernatant.

3.6. Add 5ml staining buffer and pipette to re-suspend cell pellet (Note: If using a 1mL Pipette, add just 1ml staining buffer, pipette the sample, then add 4ml additional staining buffer with a fresh tip)

3.7. Spin the sample at 350xg for 5 minutes with the BRAKE OFF.

3.8. Carefully remove the supernatant.

3.9. Re-suspend sample to an expected $\sim 1 \times 10^5 - 5 \times 10^5$ cells/ml by adding $\sim 1-2$ ml of Staining Buffer or PBS.

3.10. (Optional) Add 20 μ l of Moxi Cyte Flow Reagent per/ml of sample and invert 10x to mix.

3.11. Analyze with the Moxi Flow or Moxi GO Flow Cytometers using the "Open Flow Cytometry" Assay.