Preparing Cells for EasySep™

Frequently Used Processing Methods for Human Blood Samples

Presenter
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Learning Objectives

In this session, you will learn:

- Which common anticoagulants are used for blood sample collection
- How to isolate PBMCs, cryopreserve them, and thaw them for isolation
- How to prepare a buffy coat
- How to remove red blood cells (RBCs)
- How to remove platelets

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Common Anticoagulants for Blood Sample Collection

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Common Anticoagulants for Blood Sample Collection

1. **Irreversible**
   - Heparin: Activates antithrombin III, but can interfere with the polymerase chain reaction

2. **Reversible**
   - Chelates cations, need additional anticoagulant in subsequent washing or dilution media to prevent clotting
     - Acid Citrate Dextrose (ACD)
     - Ethylenediaminetetraacetic acid (EDTA)
     - Sodium citrate, Citrate Phosphate Dextrose (CPD), Citrate Phosphate Dextrose Adenine (CPDA), Oxalate, etc.

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PBMC Isolation

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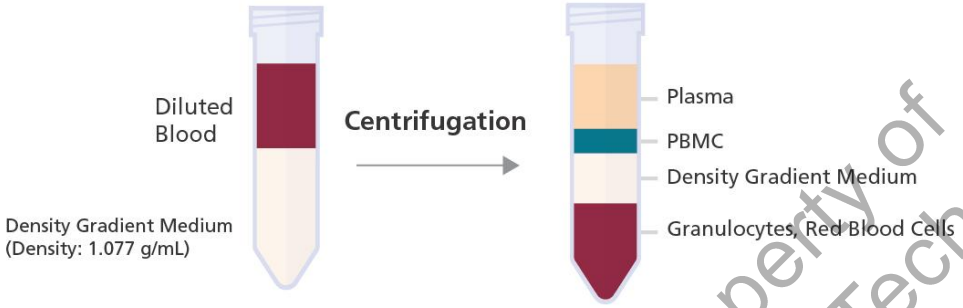
PBMC Isolation

Basic Density Gradient Centrifugation

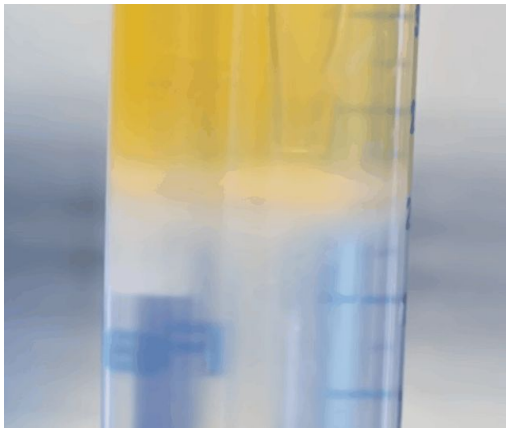


1. Dilute blood samples with PBS + 2% FBS or other suitable medium.
2. Add density medium to the tube, tilt the tube, and slowly add the diluted blood from about 2 cm above density media.
3. Centrifuge at 800 x *g* for 20 - 30 minutes at room temperature with the brake OFF.
4. Harvest the cells by inserting the pipette through the upper plasma layer to the mononuclear cells at the interface.
   Optional: remove the plasma layer to reduce platelet contamination.
5. Wash the cells with the appropriate buffer.
   Optional: Add a wash step with reduced centrifuge speed to remove platelets.

- RBCs and granulocytes may be present in the isolated cells and the contamination can be more significant when the blood is old (>48 hours).
- When working with patient samples, immature and low-density granulocytes may also be present in the isolated PBMC fraction.

Step 2. Add diluted blood above density media.

Step 4. Harvest the cells.



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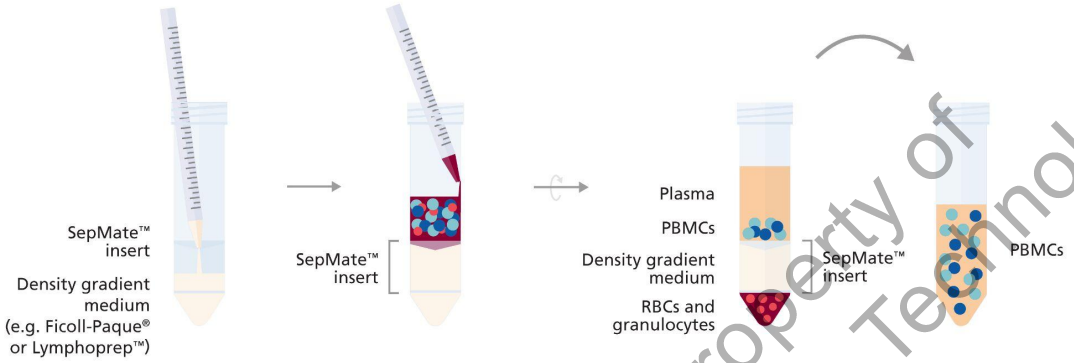
PBMC Isolation

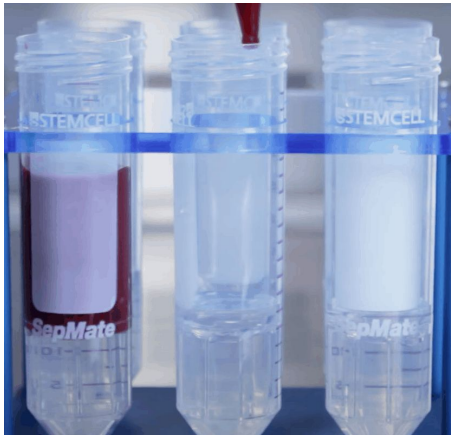
Density Gradient Centrifugation with SepMate™



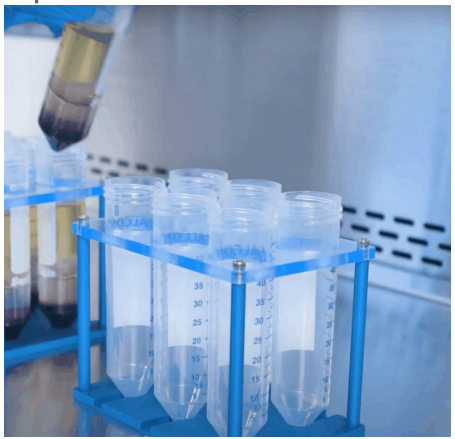
1. Add density gradient medium under the insert through the central hole.
   - Measure the volume by serological pipette
   - Lift pipette tip to dispense the last 0.5-1 mL and avoid injecting air below the insert.
2. Keep the tube vertical and add the diluted blood.
3. Centrifuge at 1200 x *g* for 10 - 20 minutes at room temperature with the brake ON.
4. Harvest the cells by pouring or pipetting.
   - When a “cloudy” or diffuse suspension of cells are observed above the insert, pour off the entire supernatant to collect the cells.
   - Optional: when a clear PBMC layer can be seen, remove the plasma layer first to reduce platelet contamination.
5. Wash the cells with the appropriate buffer.

RBCs may be present in the isolated PBMC fraction and can be more significant when the blood is old. Increasing the centrifuge time by 10 minutes may help reduce the RBC contamination.

Step 2. Add diluted blood.



Step 4. Harvest the cells.



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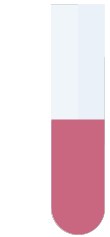
PBMC Isolation

Immunomagnetic Separation with EasySep™

Blood



Buffy Coat

Apheresis Blood Samples

EasySep™

PBMCs

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PBMC Cryopreservation and Thawing

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PBMC Cryopreservation

Isolated PBMCs can be stored for future cell isolations.

1. PBMCs can be pelleted and resuspended in cryopreservation medium.
   - It is recommended to freeze isolated PBMCs at a concentration of 0.5 - 10 x 10^6 cells/mL. However, you should try freezing the cells at multiple concentrations to determine which concentration gives the desired viability, recovery, and functionality after thawing.

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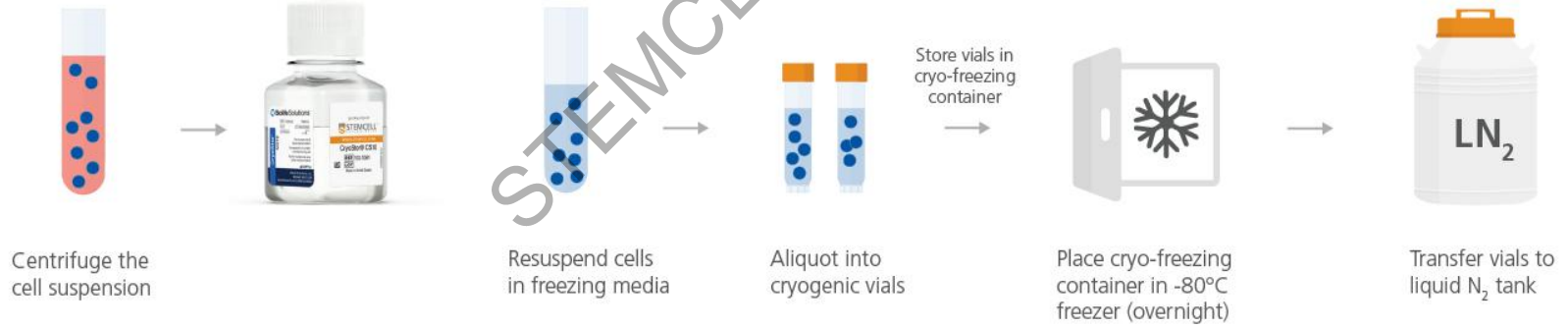

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   - It is recommended to freeze isolated PBMCs at a concentration of 0.5 - 10 x 10^6 cells/mL. However, you should try freezing the cells at multiple concentrations to determine which concentration gives the desired viability, recovery, and functionality after thawing.
2. Slow rate-controlled cooling to -80°C or using an isopropanol freezing container.
   - Do not let cells sit in cryopreservation medium at room temperature. Keep on ice and transfer rapidly.
3. Transfer the vials to liquid nitrogen (below -135°C) and store until needed.
   - Long-term storage at -80°C is not recommended.

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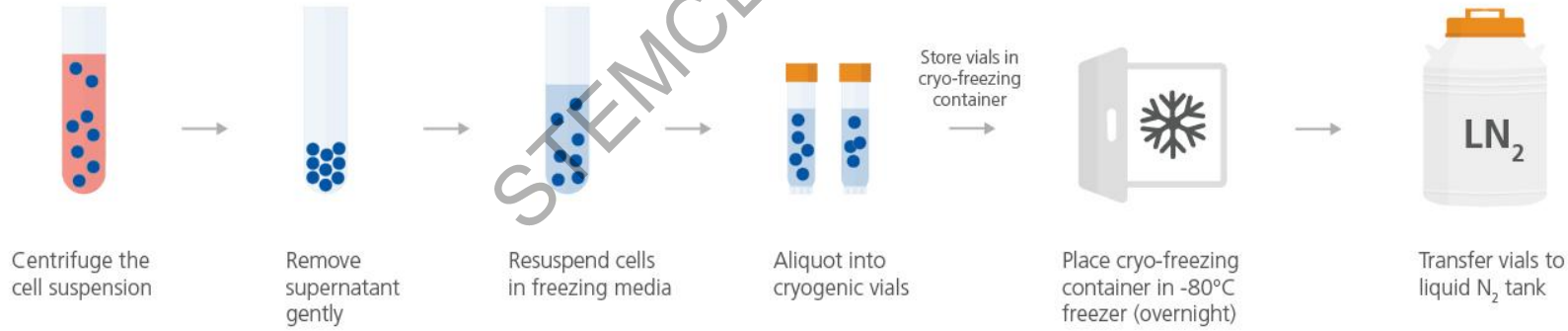
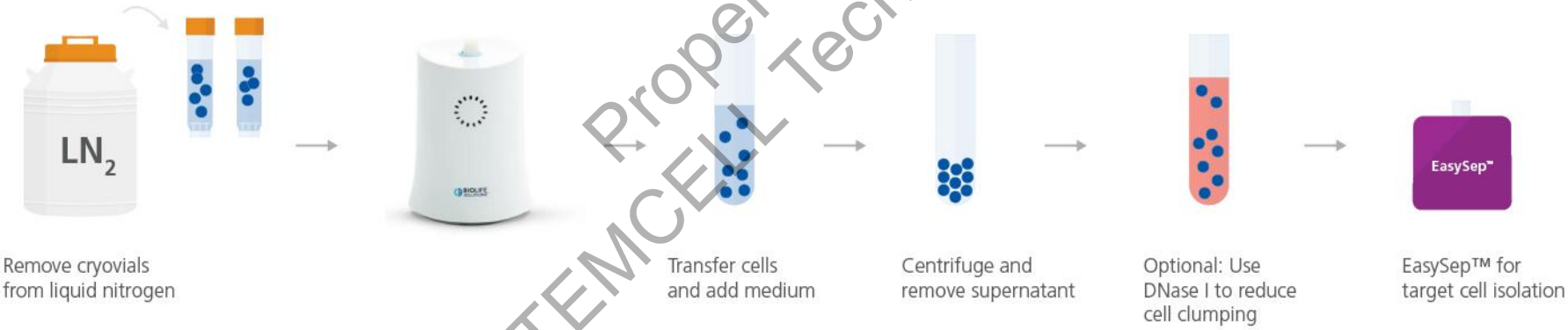


PBMC Thawing

Thawed PBMCs can be used with EasySep™

1. Warm the medium in a 37°C water bath and thaw cryogenic vials at 37°C or with the ThawSTAR® CFT2.
    - Get all the reagents and consumables ready before thawing a vial to minimize its exposure to room temperature. Place the cells on dry ice or in a liquid nitrogen container until thawing.



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PBMC Thawing

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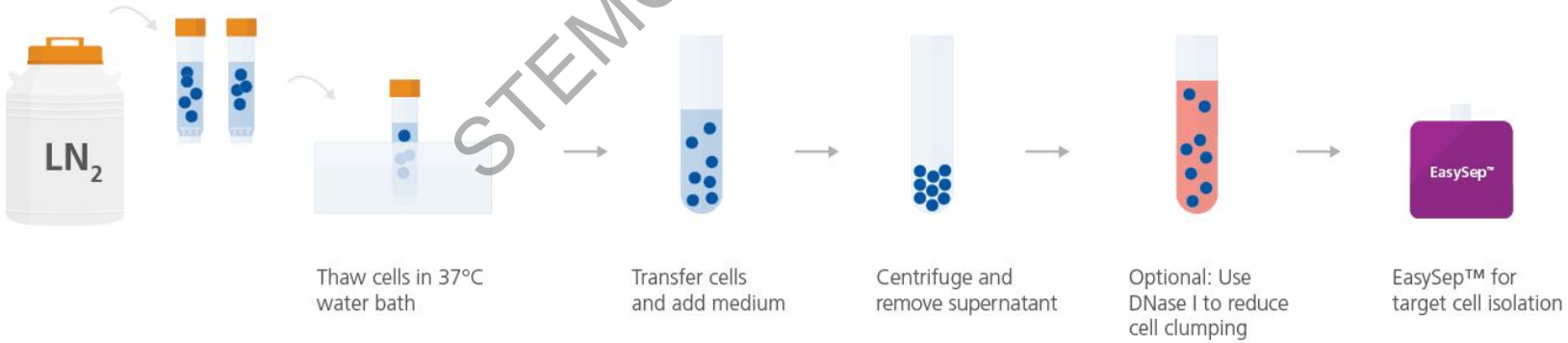
1. Warm medium in a 37°C water bath and thaw cryogenic vials at 37°C or with ThawSTAR® CFT2.
   - Get all the reagents and consumables ready before thawing a vial to minimize its exposure to room temperature. Place the cells on dry ice or in a liquid nitrogen container until ready to proceed with thawing.
   - Remove the vial from the water bath when a small amount of ice remains.
2. Transfer the cells to a 50 mL conical tube. Use the warm medium to rinse the vial and add it dropwise to the cells while gently swirling the tube. Add 15 - 20 mL more medium dropwise to the tube for washing.
   - Measure the volume and save an aliquot of cells for counting and viability assessment.
3. Centrifuge the tube to pellet the cells. The cell pellet can be reconstitute at desired concentrations for isolation.
   - To prevent cell aggregation, add 100 µg DNase I Solution per mL of cell suspension and incubate at room temperature for 15 minutes, and/or filter through a 37 - 70 µm cell strainer.



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Buffy Coat Preparation

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Buffy Coat Preparation

Concentrate whole blood by making a buffy coat

1. Dilute whole blood at 1:1 ratio with PBS + 2% FBS or other suitable medium.
2. Centrifuge at 800 x *g* for 10 minutes at room temperature with the brake off.
3. Harvest the concentrated leukocyte band (the buffy layer), plus a small portion of the plasma and concentrated RBCs.
   - The target is to concentrate the leukocytes approximately 5-fold.

The cellular composition of buffy coat is similar to the whole blood.
RBC content levels: Whole Blood > Buffy Coat > PBMCs.
Check the **Product Information Sheet (PIS)** before using buffy coat with an EasySep™ product.

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Step 3. Harvest the leukocyte band.

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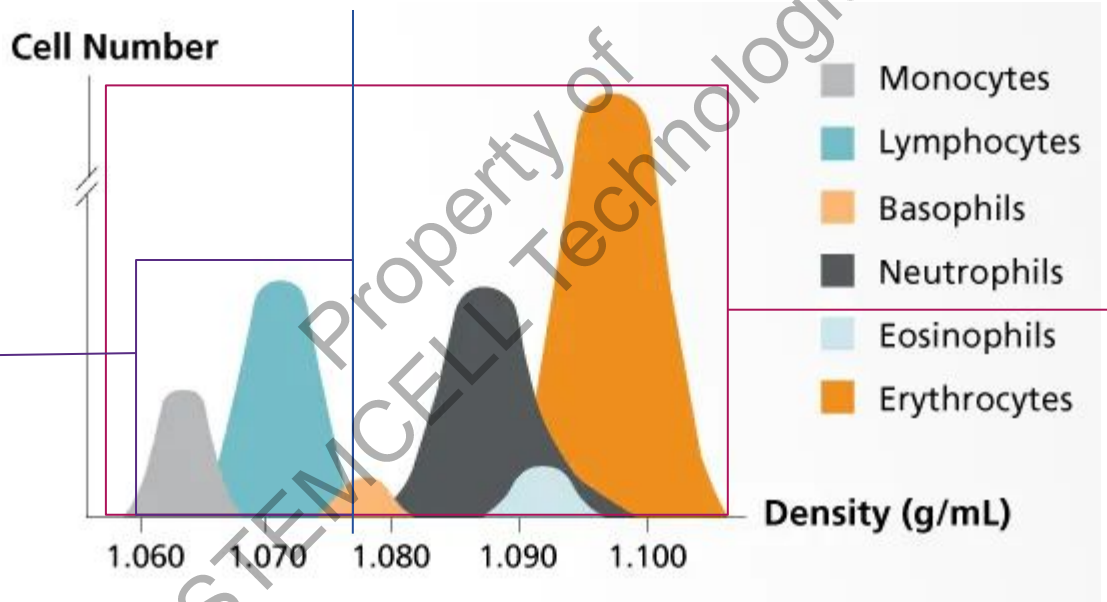
PBMCs and Buffy Coats Are Different

Density gradient media: 1.077 g/mL



PBMCs

- Lymphocytes (T cells, B cells, and NK cells)
- Monocytes, Dendritic cells

Buffy Coat

- PBMCs
- Granulocytes
- RBCs
- Platelets

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Red Blood Cell (RBC) Removal

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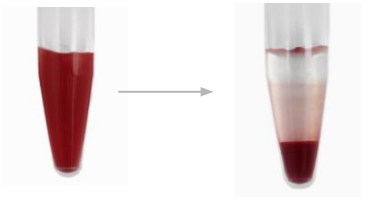
Red Blood Cell (RBC) Removal

Lysis

- Ammonium Chloride lysis
  - Easy to use and can be repeated to remove RBCs. Use **cold and fresh** lysis buffer and incubate **on ice**.
- Hypotonic lysis
  - Lysis with water. Concentrated media should be added **immediately** after incubation to stop the lysis.

Aggregation Sedimentation

- HetaSep™
  - Sediment RBCs, often used for RBC debulking.
  - Performs optimally with fresh blood samples. The interface between the RBC pellet and plasma may appear hazy when HetaSep™ is used with older blood.



Immunomagnetic Separation

- EasySep™ RBC Depletion Reagent
  - Target RBC surface antigen Glycophorin A for removal; high depletion efficiency and easy to use.
  - EDTA must be added when monocytes are desired.

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Red Blood Cell (RBC) Removal

	Ammonium Chloride Lysis	Hypotonic Lysis	HetaSep™	EasySep™ RBC Depletion Reagent
Mechanism	Lysis	Lysis	Sedimentation	Separation
Residual RBCs	5 - 50%	Variable	1 - 5%	<1%
Advantage	Minimal effect on leukocytes and easy to use	No chemical introduced	Removes large amounts of RBCs	High depletion efficiency and easy to use
Disadvantage	Harsh on sensitive cells (e.g. HPCs), can cause cell activation (e.g. Granulocytes)	Can burst nucleated cells if the incubation period is not timed accurately	Performance relies on subjective determination and is reduced with old samples and nucleated RBCs	More expensive and not recommended with Positive selection kits
Applicable Samples	Whole blood, LP, LRSC, buffy coat, PBMCs, etc	Whole blood, LP, LRSC, buffy coat, PBMCs, etc	Whole blood, buffy coat, etc	Whole blood, LP, LRSC, buffy coat, PBMCs, etc

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Platelet Removal

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Platelet Removal

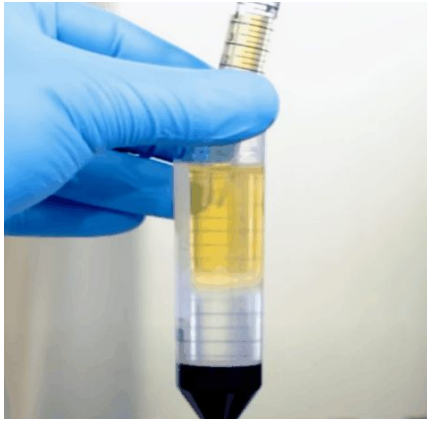
Remove Plasma Layer

After density gradient centrifugation, remove and discard the plasma layer.

Wash Cells

1. Wash the cells with PBS + 2% FBS.
2. Centrifuge at 120 - 150 x *g* for 10 minutes at room temperature with the brake OFF.
3. Carefully remove supernatant by aspiration.
    - The washing step can be repeated if desired, but cell loss is expected during the wash steps.

Remove plasma layer



Remove supernatant by aspiration

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Summary

- Anticoagulants should be used when collecting blood samples for cell isolation.
- PBMCs can be isolated by density gradient centrifugation with a medium that has a density of 1.077 g/mL (e.g., Lymphoprep™).This separation can be facilitated by the use of SepMate™ tubes.
- PBMCs can be cryopreserved and thawed for cell isolation with EasySep™.
- Buffy coats can be prepared by centrifuging diluted whole blood and contain granulocytes and RBCs in addition to PBMCs.
- RBCs can be removed from samples using various methods and protocols.
- Platelets can be removed from samples by discarding the plasma and slow spin wash.

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Questions?

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Thank you!

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Please complete the quiz before moving onto Module 3.