After EasySep™ Separation

Performance Analysis

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Learning Objectives

In this session, you will learn:

How to assess the separation performance after EasySep™

- Purity analysis based on flow cytometry
  - General considerations for fluorochrome choices
  - General considerations for staining controls
  - General considerations for gating
  - Specific considerations for flow cytometry analysis of EasySep™ separated cells
- Recovery analysis

Purity Analysis Based on Flow Cytometry

Purity Analysis Based on Flow Cytometry

EasySep™ Human Memory B Cell Isolation Kit

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Notes and Tips

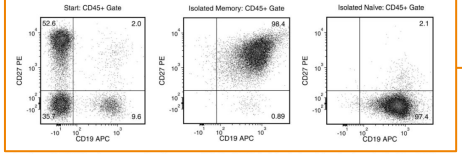
ASSESSING PURITY

For purity assessment of CD19+CD27+ cells by flow cytometry, use the following fluorochrome-conjugated antibody clones:

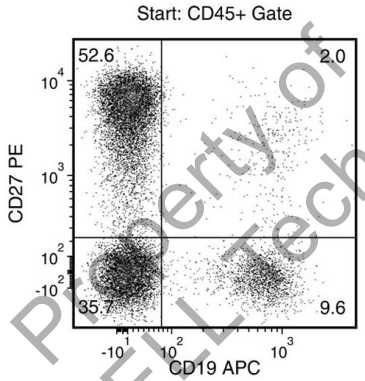
- Anti-Human CD19 Antibody, Clone HIB19 (Catalog #60005), and
- Anti-Human CD45 Antibody, Clone HI30 (Catalog #60018) or Clone 2D1 (Catalog #60123), and
- Anti-Mouse CD27 Antibody, Clone LG.3A10 (Catalog #60160), which cross-reacts with human CD27

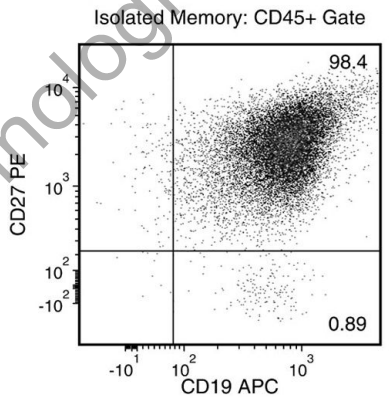
NOTE: Brilliant Violet™ antibody conjugates should be carefully titrated on EasySep™ Release-isolated cells prior to analysis by flow cytometry or fluorescence microscopy. For purity assessment with Brilliant Violet™ antibody conjugates, use of BD Horizon Brilliant™ Stain Buffer is recommended to reduce non-specific interactions. For more information, refer to the manufacturer's instructions or contact us at techsupport@stemcell.com.

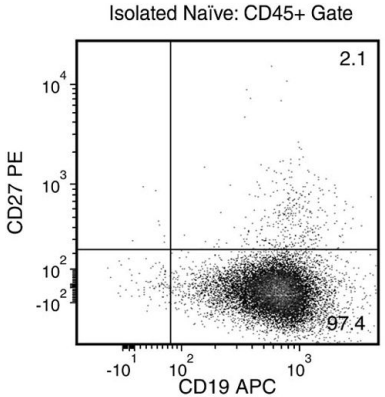
Data



Starting with PBMCs, the memory B cell content (CD19+CD27+) of the isolated fraction is typically 97 ± 2% (mean ± SD using the purple EasySep™ Magnet). Using the optional protocol, the naïve B cell content (CD19+CD27-) of the isolated fraction is typically 93 ± 5% (mean ± SD).







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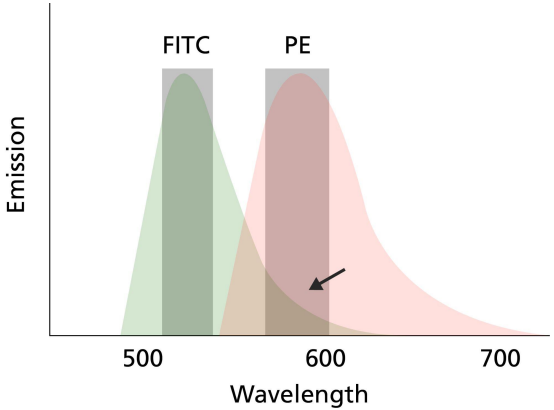
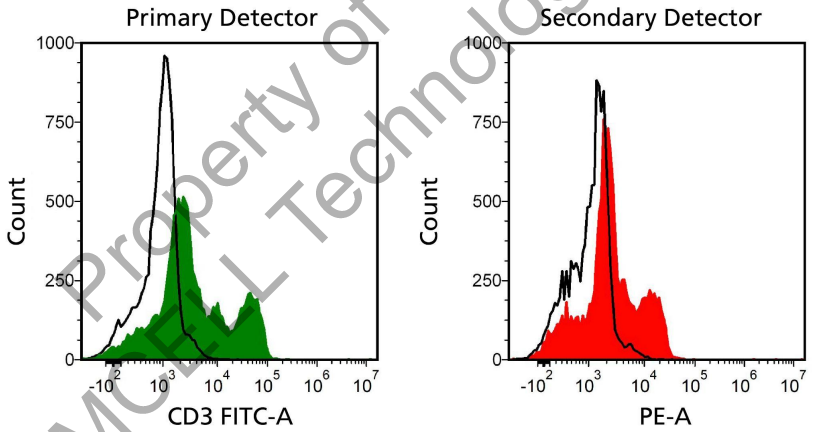
Flow Cytometry - Considerations for Fluorochromes

- **Spectrum**
  - Compatibility with your flow cytometer
  - Overlap between the fluorochromes





Solid peaks: Cells are stained with mouse anti-human CD3 FITC, clone UCHT1.
Left: Signal in FITC channel
Right: Signal in PE channel
Black outlined peaks: Unstained cells

- **Brightness**
  - Bright fluorochrome for a target marker with a low expression level or target cells with a low frequency

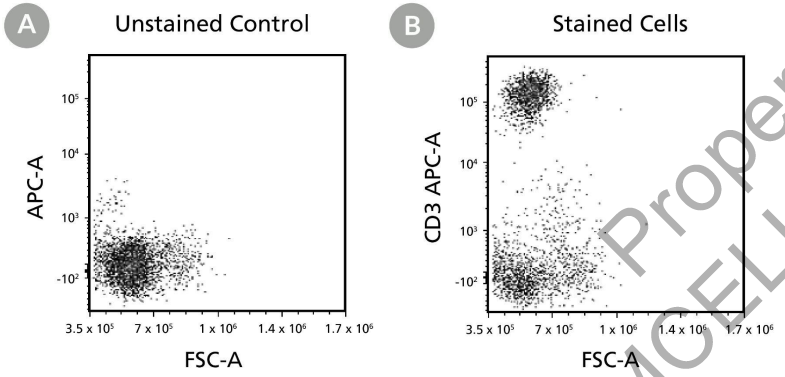
Flow Cytometry - Staining Controls

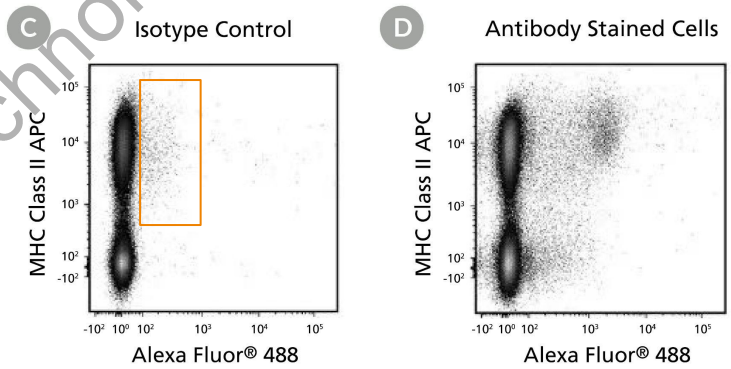
- **Unstained control**

  Unstained control shows the background autofluorescence.

- **Isotype control**

  Isotype controls show non-specific binding and allow optimization of staining.





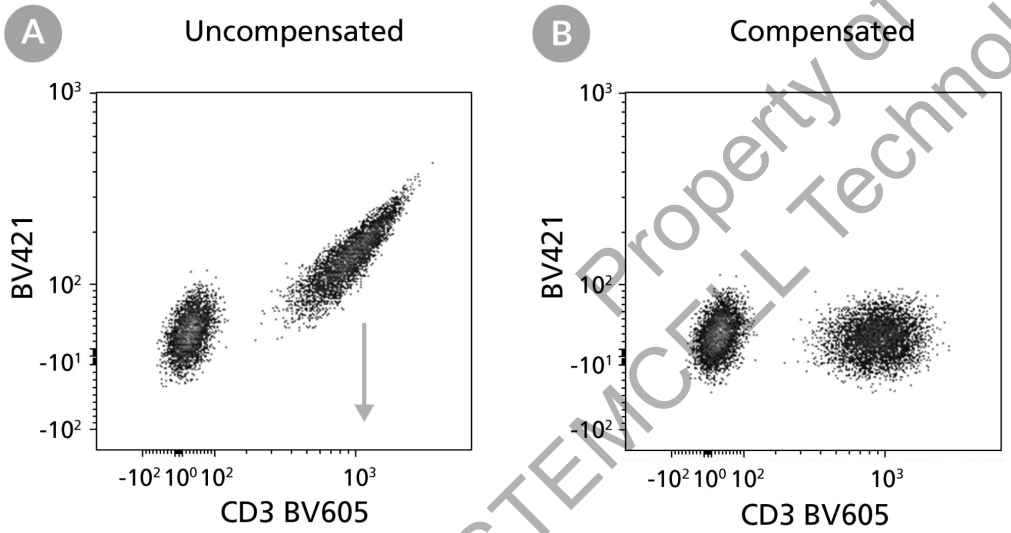
(A) Unstained PBMCs;
(B) PBMCs stained with mouse anti-human CD3 (clone UCHT1) APC

(C) Mouse splenocytes labeled with an Armenian hamster IgG Alexa Fluor® 488 isotype control antibody and an anti-mouse MHC class II antibody, APC.
(D) Mouse splenocytes labeled with Anti-Mouse CD11c Antibody, Clone N418, Alexa Fluor® 488 and an anti-mouse MHC class II antibody, APC.

Flow Cytometry - Considerations for staining controls

- **Single stained control**

  Single stained controls show spectral overlap and allow fluorescence spillover to be corrected.



PBMCs stained with mouse anti-human CD3 (clone UCHT1) BV605. (A) Uncompensated; (B) Compensation applied.

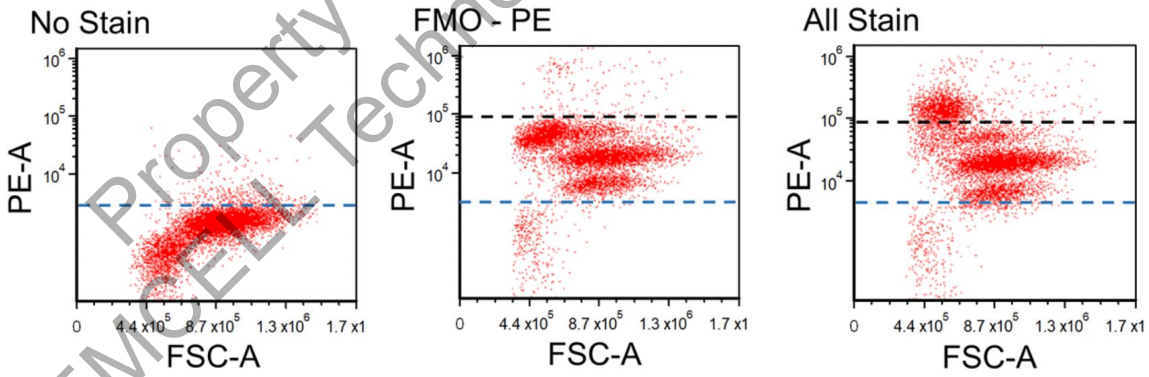
Flow Cytometry - Considerations for Staining Controls

- **Fluorescence Minus One (FMO) Control**

  FMO controls show the fluorescence spillover from all other fluorophores to the one being analyzed and help you determine where to set your gates.

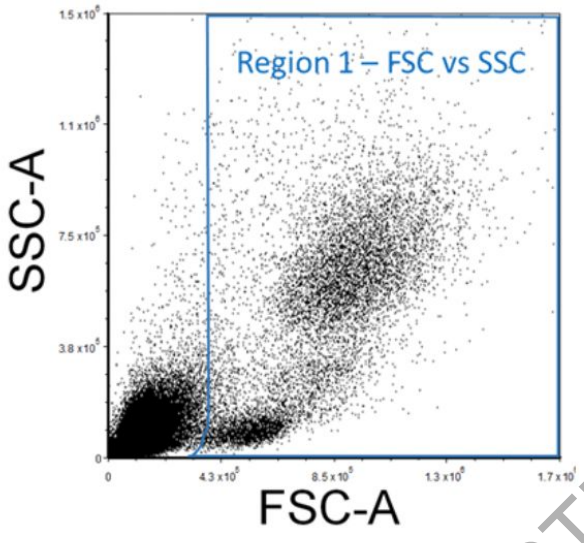
Dot plots of multicolor flow cytometry showing the fluorescence spread into the PE channel (FMO control compared to a no stain control). Black dotted line represents the FMO gating boundary compared to the no stain boundary in blue.

The sample for FMO-PE control is stained with anti-CD45-FITC, anti-CD56-APC antibodies, and PI; the sample for the All Stain group is stained with anti-CD3-PE, anti-CD45-FITC, anti-CD56-APC antibodies, and PI.

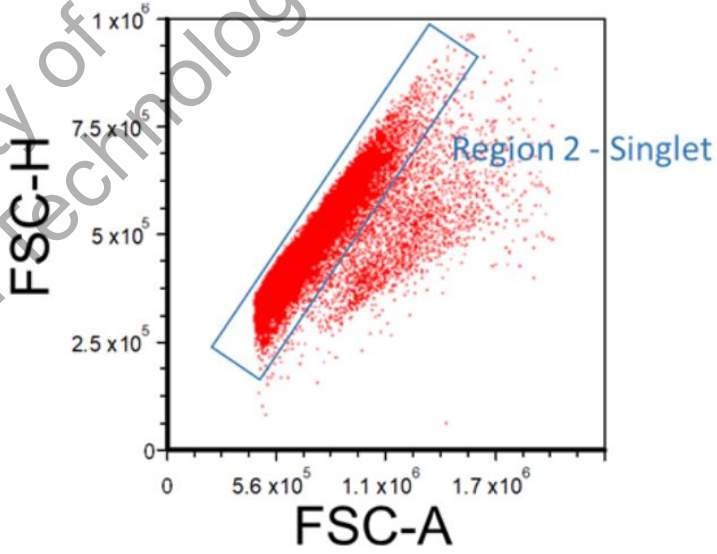


Flow Cytometry Analysis - Considerations for Gating

1. Check FSC-SSC plot and exclude debris



2. Exclude doublets or multiplets



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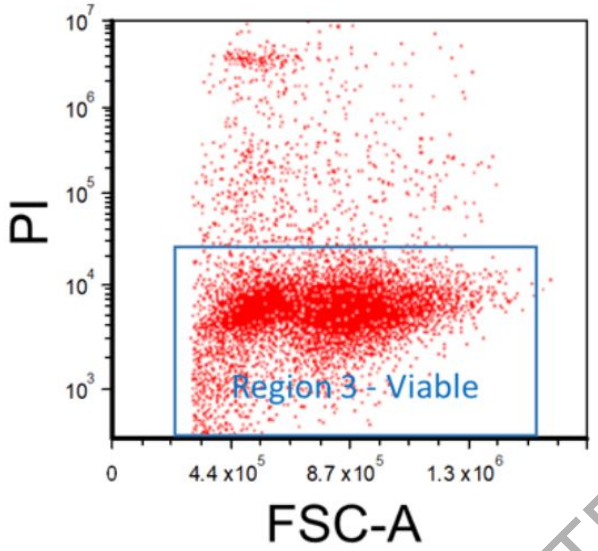
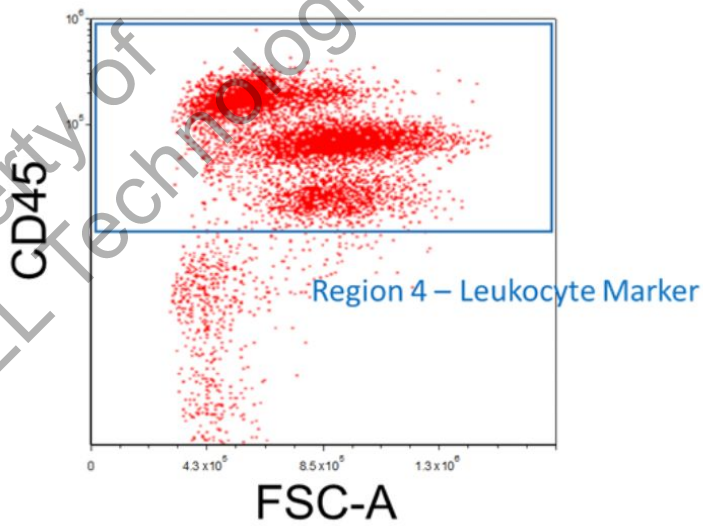
Flow Cytometry Analysis - Considerations for Gating

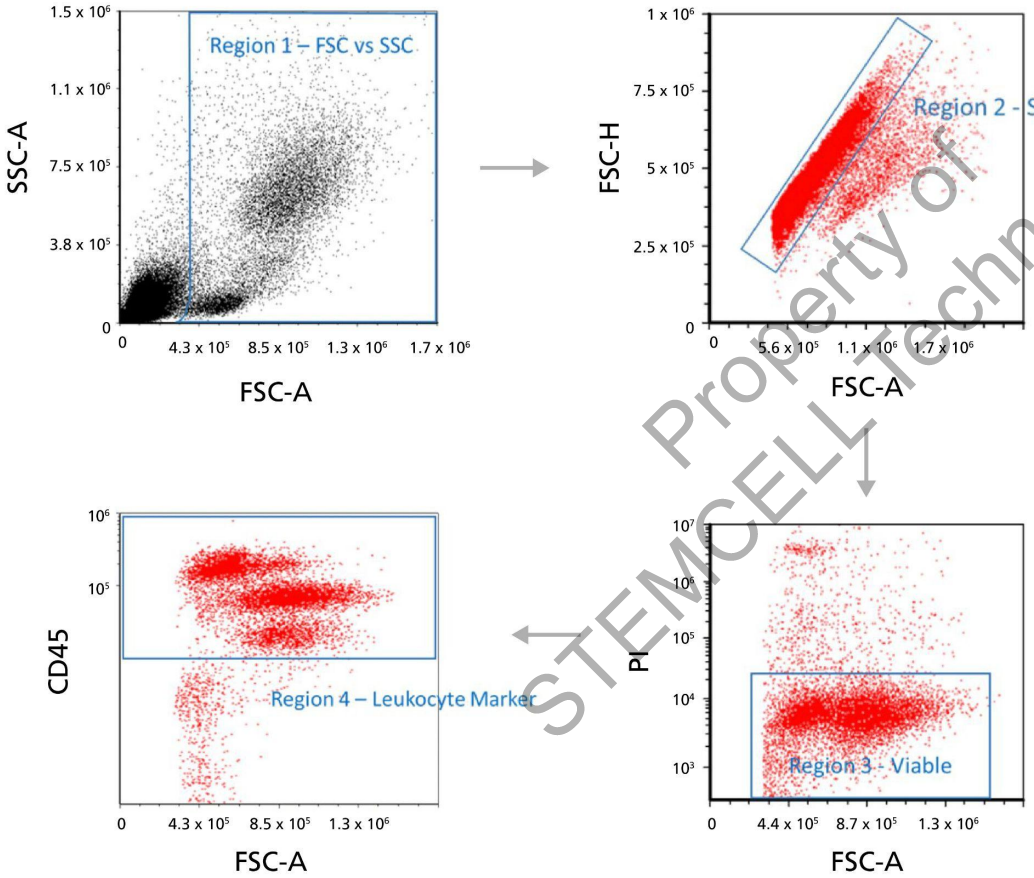
3. Exclude non-viable cells



4. Include a leukocyte marker



Flow Cytometry Analysis - Gating strategy



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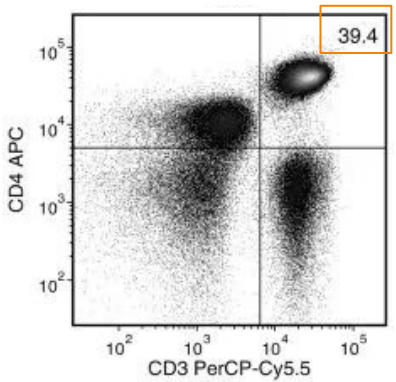
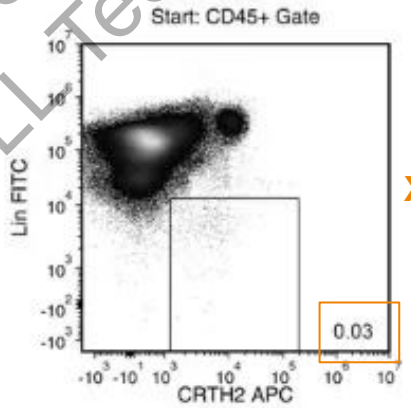
Flow Cytometry Analysis - Percentage of the Target Cells

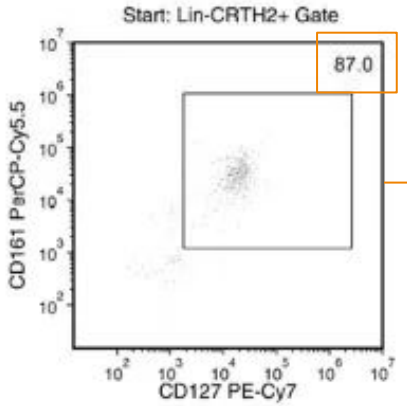
The percentage of target cells among live single cells is used to measure the cell percentage. Measuring the percentage of target cells among live single leukocytes provides more accurate data when working with immune cells.

- For target cell types that are defined by one or two markers, you can use the percentage of cells gated based on the markers among live and single leukocytes.
- For target cell types that are defined by more than two markers, the purity is the product of multiplying all the percentages of each marker.





X



→ 0.026%

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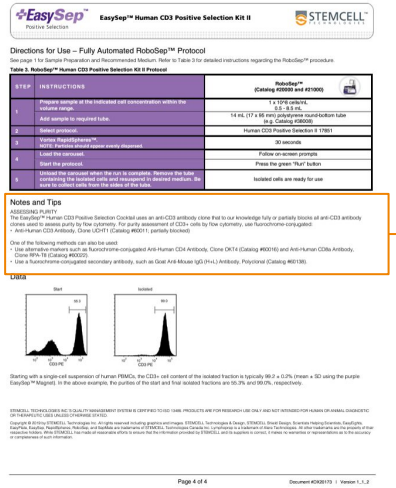
Flow Cytometry Analysis for EasySep™ Separated Cells

- Check the Notes and Tips in the Product Information Sheets and use recommended antibody clones



EasySep™ Positive Selection — EasySep™ Human CD3 Positive Selection Kit II — STEMCELL™ Technologies

Directions for Use – Fully Automated RoboSep™ Protocol

See page 1 for Sample Preparation and Recommended Medium. Refer to Table 3 for detailed instructions regarding the RoboSep™ procedure.

Table 3. RoboSep™ Human CD3 Positive Selection Kit II Protocol

STEP	INSTRUCTIONS	RoboSep™ (Catalog #20000 and #21000)
1	Prepare sample at the indicated cell concentration within the volume range.	1 x 10^8 cells/mL, 0.5 - 8.5 mL
	Add sample to required tube.	14 mL (17 x 95 mm) polystyrene round-bottom tube (e.g. Catalog #38008)
2	Select protocol.	Human CD3 Positive Selection II 17851
3	Vortex RapidSpheres™. NOTE: Particles should appear evenly dispersed.	30 seconds
4	Load the carousel.	Follow on-screen prompts
	Start the protocol.	Press the green "Run" button
5	Unload the carousel when the run is complete. Remove the tube containing the isolated cells and resuspend in desired medium. Be sure to collect cells from the sides of the tube.	Isolated cells are ready for use

Notes and Tips

ASSESSING PURITY

The EasySep™ Human CD3 Positive Selection Cocktail uses an anti-CD3 antibody clone that to our knowledge fully or partially blocks all anti-CD3 antibody clones used to assess purity by flow cytometry. For purity assessment of CD3+ cells by flow cytometry, use fluorochrome-conjugated:

- Anti-Human CD3 Antibody, Clone UCHT1 (Catalog #60011; partially blocked)

One of the following methods can also be used:

- Use alternative markers such as fluorochrome-conjugated Anti-Human CD4 Antibody, Clone OKT4 (Catalog #60016) and Anti-Human CD8a Antibody, Clone RPA-T8 (Catalog #60022).
- Use a fluorochrome-conjugated secondary antibody, such as Goat Anti-Mouse IgG (H+L) Antibody, Polyclonal (Catalog #60138).

Data

Starting with a single-cell suspension of human PBMCs, the CD3+ cell content of the isolated fraction is typically 99.2 ± 0.3% (mean ± SD) using the purple EasySep™ Magnet. In the above example, the purities of the start and final isolated fractions are 55.3% and 99.0%, respectively.

Notes and Tips

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- Use a fluorochrome-conjugated secondary antibody, such as Goat Anti-Mouse IgG (H+L) Antibody, Polyclonal (Catalog #60138).

- If cells are isolated with **EasySep™ Release** products: Brilliant Violet™-conjugated antibodies should be carefully titrated on the cells before analysis. The BD Horizon Brilliant™ Stain Buffer is recommended to reduce non-specific interactions.

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Recovery Analysis

Recovery Calculation

- To accurately calculate the recovery of a cell isolation procedure, you will need the following four pieces of data. Accurate determination of each of these components is critical:

#1 Total # of cells that will be separated	**Started with 5 x 10^7 cells**
#2 Starting % of desired cells	**The frequency of desired cells in the starting sample is 30%**
#3 Total # of cells in enriched fraction	**Enriched 7.5 x 10^6 cells**
#4 % Purity of enriched fraction	**The purity of desired cells in the enriched fraction is 95%**

- Cell recovery is calculated using the following formula:

% Recovery = [(**#3**)x(**#4**)] / [(**#1**)x(**#2**)] X 100%

% Recovery = [(7.5 x 10^6) x 95%] / [(5 x 10^7) x 30%] = 47.5%

Summary

- There are a number of factors to consider and incorporate into your flow cytometry staining and gating strategy, including:
  - Using appropriate antibodies and dyes for staining
  - Setting necessary controls
  - Using an optimal staining protocol and flow cytometer
  - Applying appropriate gating strategy
- For cells isolated by EasySep™ positive selection products, it is important to analyze them with the antibody clone recommended in the Product Information Sheet.
- For cells isolated with EasySep™ Release products: Brilliant Violet™ conjugated antibodies should be carefully titrated on the cells before analysis. The BD Horizon Brilliant™ Stain Buffer is recommended to reduce non-specific interactions.
- You can determine the cell recovery of the isolation procedure by assessing the cell counts and frequencies of desired cells in the sample before and after isolation.

Questions?

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Thank you!

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Please complete the quiz and the final assessment.