truculture®

APPLICATION NOTES

Flow Cytometry

Protocols for Processing TruCulture Samples at Clinical Sites

myriad® RBM

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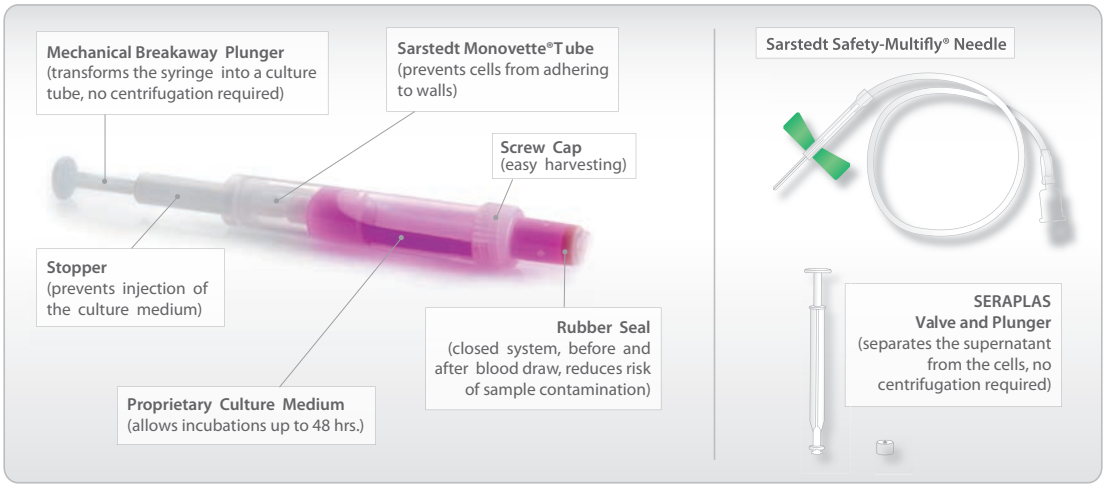
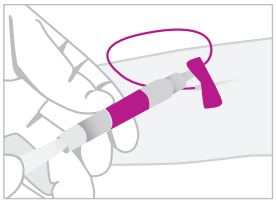
Introduction

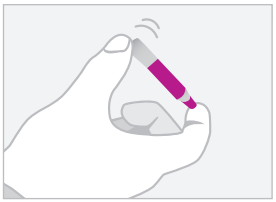
Pharmacodynamic analysis encompasses several techniques, including protein measurements and flow cytometry analysis. TruCulture® is a whole blood collection and culturing system that is easily implemented at clinical trial sites and provides access to samples for both secreted protein and cell population analysis. After a 37°C stationary incubation with or without targeted stimulants, supernatants and cells can be separated and individually analyzed. This application note describes various protocols for multi-color flow cytometry analysis of TruCulture cell samples that can be adopted at any collection site.





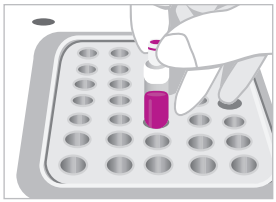
01. COLLECT

Draw 1 mL of blood directly into the TruCulture Tube and break off the plunger.



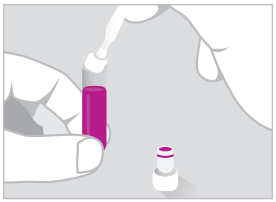
02. MIX

Gently invert tube to mix 3 to 5 times.



03. INCUBATE

Place tube in 37°C heat block for up to 24 or 48 hours.



04. SEPARATE

Manually insert valve to separate supernatant from the cells. Collect supernatant and cell layer for downstream analysis.

Protocol Overview

The flow cytometry protocols in this application note are intended for the analysis of TruCulture cell samples. A step-by-step instructions for use (IFU) for the TruCulture system can be found online at **https://myriadrbm.com/products-services/truculture/**. After incubation of whole blood in the TruCulture tubes, the supernatant and cell layer are separated using the seraplas valve. The supernatant is removed. Next, the seraplas valve is removed to access the cell sample.

The antibody staining panel used for all protocols is listed in Table 1. All antibodies were purchased from BioLegend and used at 2.5µL per sample in staining buffer of 1% bovine serum albumin (BSA) in 1x Dulbecco's phosphate buffered saline (PBS). Analysis of stained cell samples was performed using the Beckman Coulter CytoFLEX LX. Note that modifications may be necessary depending on deviations in the marker panel.

Table 1

Antibody (anti-human)	Catalog Number
CD45-FITC	368508
CD3-Pacific Blue	300431
CD4-APC/FIRETM750	300560
CD8-PerCP/Cy5.5	344710
CD19-Brilliant Violet 785TM	302240
CD11b-Brilliant Violet 650TM	301336
CD66b-APC	305118
CD161-PE	339904
HLA-DR-PE/Cy7	361612

1 *No Lyse/No Wash Protocol*

This protocol is for staining fresh cell samples, without the need for a centrifuge or additional processing, at the collection site. After staining, the samples can be fixed and stored and/or shipped at 4°C to a central lab for analysis.

a. *Prepare flow staining tubes:*

   i. *Prepare antibody staining panel (2.5µL/antibody/sample) – total volume with staining buffer should be 50µL/sample/tube.*

   ii. *Aliquot 50µL of antibody panel into staining tubes.*

b. *Take 100µL of the cell sample from the TruCulture tube and put into a staining tube*

c. *Vortex for 1 second to mix*

d. *Incubate at room temperature for 15 to 20 minutes*

e. *Add 300µL/tube of 4% paraformaldehyde (PF) in 1xPBS.*

f. *Store at 4°C until analysis (<1 week)*

g. *Dilute samples 1/3 in 1xPBS prior to analysis.*

Results

Analysis of the above stained samples were gated using CD45 and SSC. Violet side scatter can be used if desired, but it is not necessary if CD45, which is expressed on all leukocyte populations, is used. **Figure 1** demonstrates the gating strategy used to delineate the major lymphocyte populations, and **Figure 2** demonstrates the gating strategy for the neutrophil and monocyte populations.

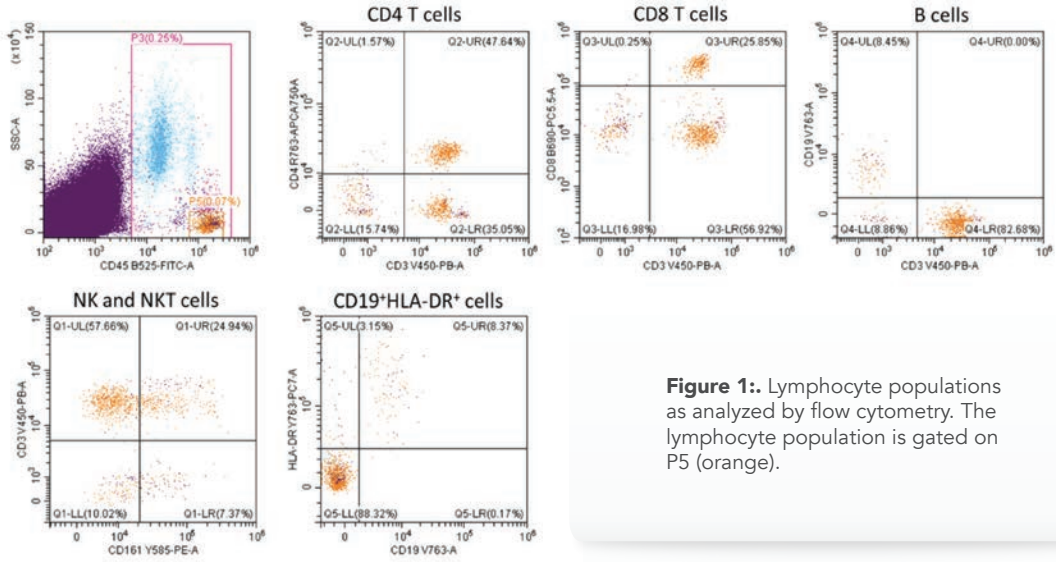


Figure 1:. Lymphocyte populations as analyzed by flow cytometry. The lymphocyte population is gated on P5 (orange).

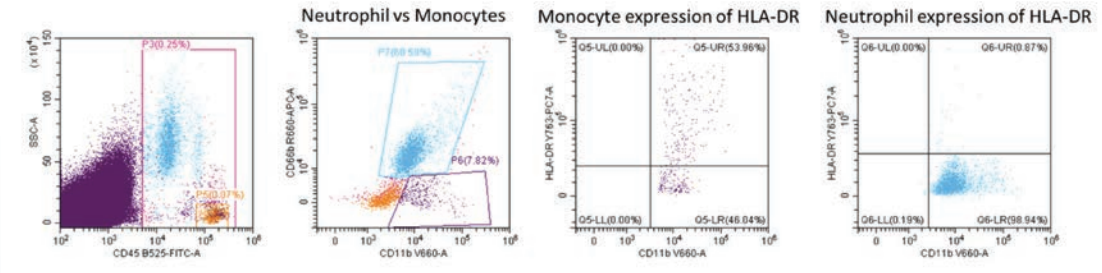


Figure 2: Granulocyte and Monocyte populations as analyzed by flow cytometry.

2 *Stain/Lyse RBC Protocol*

This protocol is intended for the staining of fresh cell samples, followed by RBC lysis and fixation. After staining, the samples can be stored and/or shipped at 4°C to a central lab for further processing and analysis.

a. *Prepare flow staining tubes:*

   i. *Prepare antibody staining panel (2.5µL/antibody/sample) – total volume with staining buffer should be 50µL/sample/tube.*

   ii. *Aliquot 50µL of antibody panel into staining tubes.*

b. *Take 100µL of the cell sample from the TruCulture tube and put into a staining tube*

c. *Vortex 1 second to mix*

d. *Incubate at room temperature for 15 to 20 minutes*

e. *Add 500µL of OptiLyse C Lysing solution (Beckman Coulter Cat# A11895)*

f. *Vortex for 1 second*

g. *Incubate at room temperature for 10 minutes*

h. *Add 500µL of 2% PF with 2% BSA in 1xPBS*

i. *Vortex for 1 second*

j. *Incubate at room temperature for at least 5 minutes*

k. *Store samples at 4°C until analysis (< 1 week)*

l. *Before analysis, centrifuge samples at 400 RCF for 5 minutes*

m. *Resuspend samples in 500µL of 1%BSA in 1xPBS*

Results

Analysis of the above stained samples were gated using CD45 and SSC. **Figure 3** demonstrates the gating strategy used to delineate the major lymphocyte populations, and **Figure 4** demonstrates the gating strategy for the neutrophil and monocyte populations.

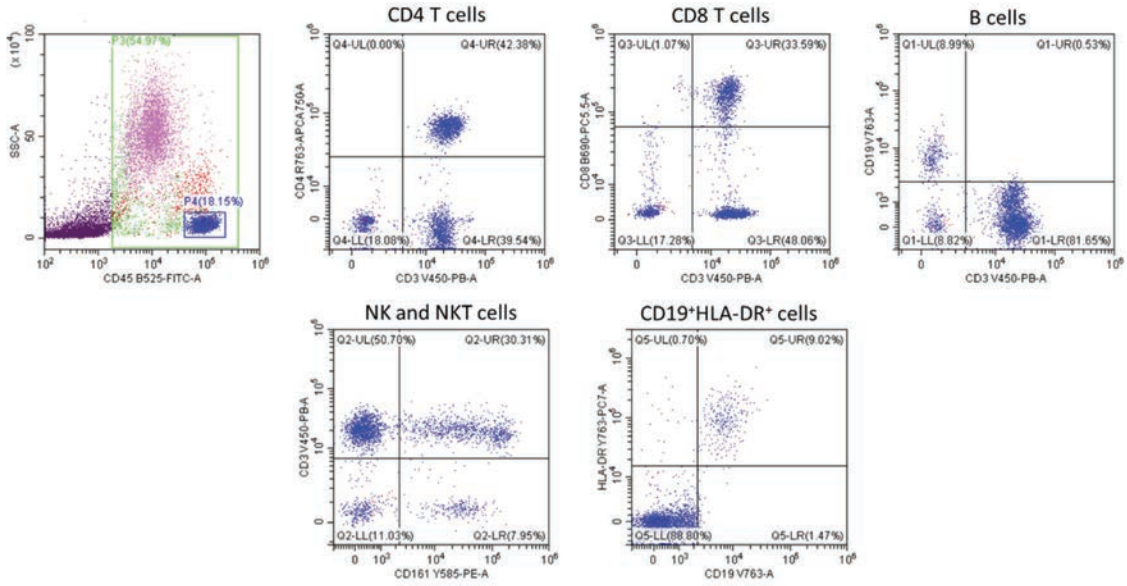


Figure 3: Lymphocyte populations as analyzed by flow cytometry. The lymphocyte population is gated on P4 (blue).

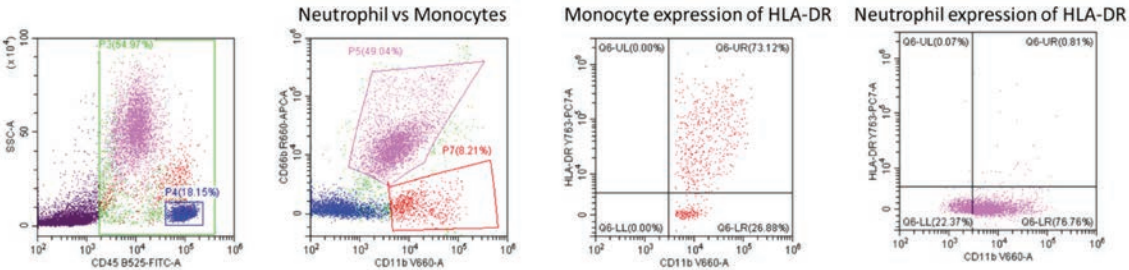


Figure 4: Granulocyte and Monocyte populations as analyzed by flow cytometry.

3 *Fix/Stain/No Lyse/No Wash Protocol*

This protocol is intended for fixing of cell samples at the collection site and shipping to a central lab for staining and analysis.

a. *Take 100µL of the cell sample from the TruCulture tube and put into a staining tube*

b. *Add 500µL/sample of CytoLast buffer (BioLegend Cat# 422501)*

c. *(Alternatively: Other fixative solutions suitable for flow cytometry can be used.)*

d. *Store and ship at 4°C (<72 hours)*

e. *Centrifuge fixed samples at 400 RCF for 5 minutes*

f. *Remove supernatant.*

g. *Stain samples for 30 minutes at room temperature:*

   i. *Prepare antibody staining panel (2.5µL/antibody/sample) – total volume with staining buffer should be 50µL/sample/tube*

   ii. *Aliquot 50µL of antibody panel into each sample*

h. *Add 400µL of 1% PF 1% BSA in 1xPBS*

i. *Dilute samples1/3 in 1xPBS prior to analysis.n 1xPBS*

Results

Analysis of the above stained samples were compared to similar samples stained prior to fixation and were gated using CD45 and SSC. **Figure 5** demonstrates the gating strategy used to delineate the major lymphocyte populations. As clearly seen, there is an obvious decrease in the expression intensity of CD8 in the staining of the post-fixed samples (as evident with the differences in the quadrant gating). Additionally, there was also an increase in staining intensity of CD11b of the granulocyte population (data not shown) in the post-fixed samples. In order to stain post-fixed cell samples for flow cytometry, the antibody clones need to be carefully selected to minimize differences that can occur between fixed and non-fixed samples.

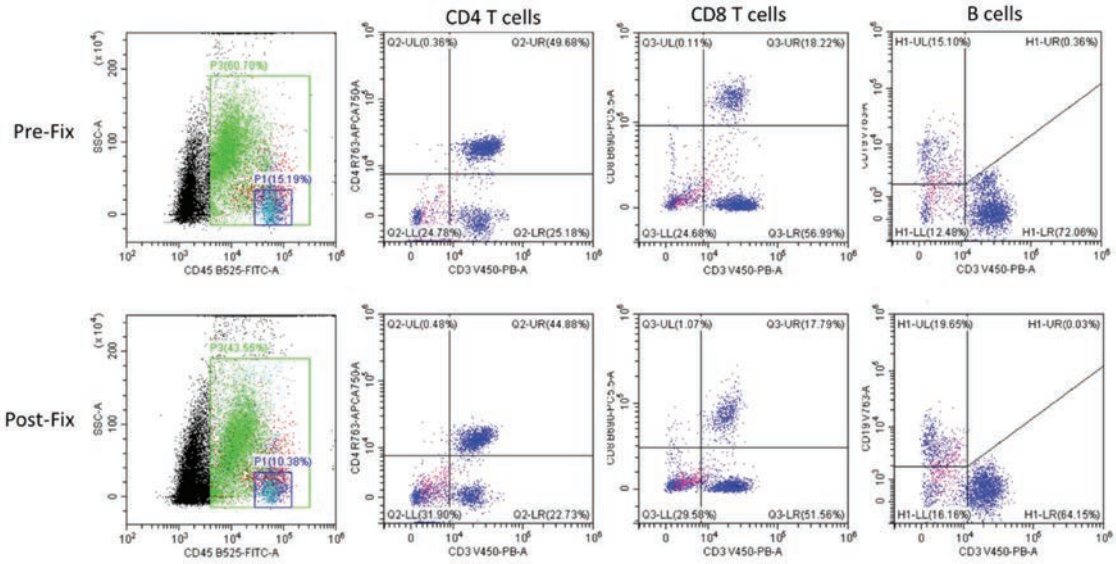


Figure 5: Lymphocyte populations as analyzed by flow cytometry. The lymphocyte population is gated on P1.

Conclusion

This application note presents several different protocols for staining of TruCulture cell samples prior to flow cytometry that can be adapted at collection sites. Due to the potential changes due to fixation that may alter the binding of antibodies to cell surface proteins, it is strongly recommended that flow antibody panels are stained on non-fixed fresh TruCulture cell samples.

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