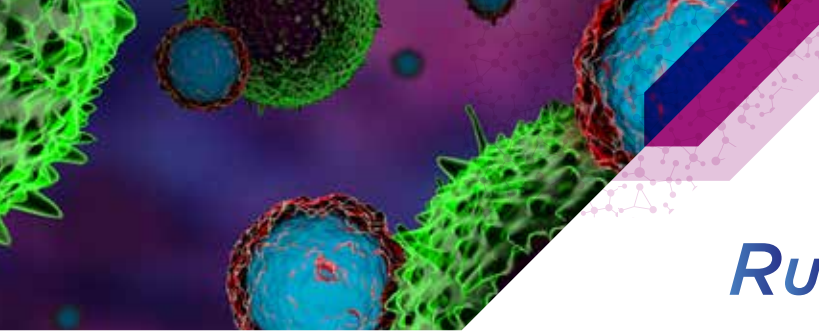

RULES BASED MEDICINE
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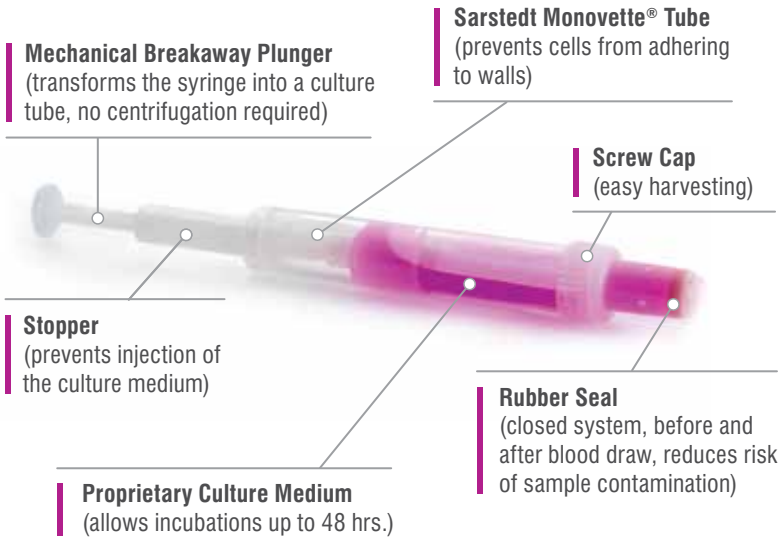
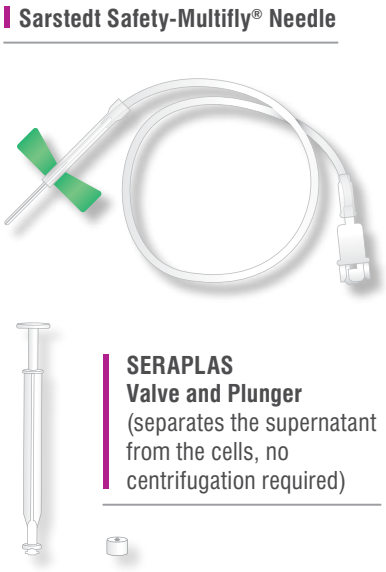
# Flow Cytometry

Standard flow cytometry protocol for analysis of surface markers for TruCulture®

## Introduction

Analysis of pharmacodynamic immune responses from clinical samples often includes protein measurements from serum or plasma and flow cytometry analysis of cell populations. TruCulture is a whole blood collection and culturing system that is easily implemented at the clinical trial site and provides access to both supernatants and cell samples. After a stationary incubation at 37°C, supernatants and cells can be separated and individually analyzed for secreted protein and cell marker investigation. This application note describes a protocol for analyzing TruCulture cell samples for surface marker expression by multi-color flow cytometry immediately after supernatant separation.

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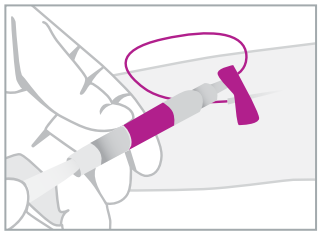
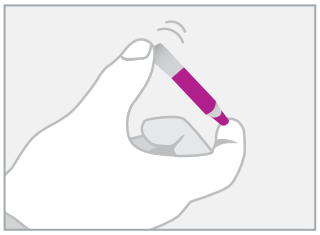


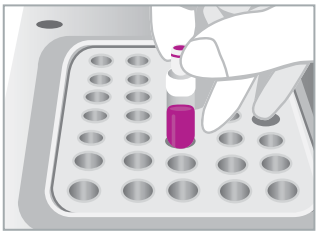
### 1. COLLECT

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



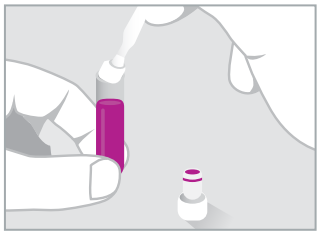
### 2. MIX

Gently invert tube to mix 3 to 5 times.



### 3. INCUBATE

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



### 4. SEPARATE

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

## Protocol

This section describes a workflow for collecting cells from TruCulture tubes for analysis with the Beckman Coulter CytoFLEX LX. Note that modifications may be necessary depending on deviations in marker panel and the use of other instruments. Step-by-step instructions for use (IFU) for the TruCulture system can be found online at https://rbm.q2labsolutions.com/products-services/truculture/.

### Protocol for flow staining of TruCulture cell samples

1. Following incubation, the supernatant was separated from the cell layer using the Seraplas valve.
2. Remove supernatant.
3. Remove the Seraplas valve.
4. Entire cell layer was collected and transferred to a 15 mL conical tube containing 5mL of ACK buffer (VWR: 118-156-101) and incubated in an ice bucket filled with chilled Lab Armor® beads and two ice packs (1-8˚C) for 20 minutes.
5. Samples were washed with 5mL 1xDPBS (VWR: 119-068-131).
6. Samples stained for viability using 1µL/sample Zombie Red (BioLegend: 77475) in 1xDPBS (100µL/sample) at room temperature for 15 minutes.
7. Samples washed with 1mL/sample 1xDPBS with 1% BSA (Sigma: A7030)
8. Samples blocked with 5µL/sample Human TruStain FcXTM (BioLegend: 422302)
9. Samples stained with 2.5µL/sample of the following antibodies (BioLegend) on cold Lab Armor® beads for 30 minutes. (Alternately: Incubate at room temperature for 15 minutes.)
   a. CD45-FITC (368508)
   b. CD3-Pacific Blue (300431)
   c. CD4-APC/FIRETM 750 (300560)
   d. CD8-PerCP/Cy5.5 (344710)
   e. CD19-Brilliant Violet 785TM (302240)
   f. CD11b-Brilliant Violet 650TM (301336)
   g. CD66b-APC (305118)
   h. CD161-PE (339904)
   i. HLA-DR-PE/Cy7 (361612)
10. Samples washed (1mL/sample 1xDPBS with 1% BSA) and fixed with 4% paraformaldehyde (250µL/sample) for 15 minutes at 1-8˚C.
11. Samples stored in 1xDPBS with 1% BSA (500µL/sample) at 4˚C until analysis.

NOTE: All centrifuge speed is at 400RCF.

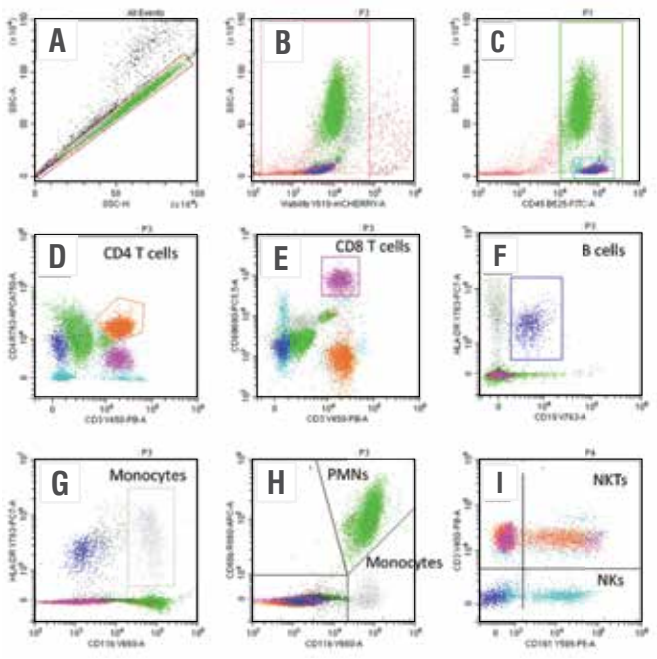
For this study, whole blood was collected into null (Cat# 782-001086), LPS (Cat# 782-001087), SEB (Cat# 782-001124), and CD3/CD28 (Cat# 782-01125) TruCulture tubes. For each subject, blood was collected into duplicate tubes of each type. Samples were incubated for 24 or 48 hours. TruCulture supernatants were analyzed with the OptiMAP immunoassay panel (13 analytes: ENA-78, GM-CSF, IFNγ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17, IL-23, TNFα) and the cells were analyzed by flow cytometry.

## Results

*Figure 1* demonstrates the general gating strategy for these targets.

**Figure 1: Flow gating strategy for TruCulture cell samples**

Samples were read on the Beckman Coulter CytoFLEX LX flow cytometer. P2 is the single cell gate (A), P1 is the viability gate (B), P3 is CD45+ cells (C), and P4 is gated on lymphocytes (C). Figure D to H were all gated on CD45+ cells. Figure I was gated on lymphocytes.




By using a combination of common surface markers, the major leukocyte populations from TruCulture samples can be clearly separated.

*Figure 2* illustrates that leukocyte populations exhibit obvious changes under different TruCulture stimulating conditions. Whole blood cultured without any stimulant demonstrated normal distribution of $CD45^+$ cells when plotted against side scatter (SSC). The LPS stimulant is known to target monocytes which expresses TLR-4, and a noticeable shift in the monocyte population can be observed following activation. With SEB stimulation, a shift in monocyte activation was observed, consistent with the mechanism of action targeting the antigen presentation molecule on the monocyte and the T cell receptor on the T cell.

Anti-CD3/CD28 antibodies directly engage the first and second signals for T cell activation and are potent stimulators of all T cell populations. In the TruCulture tubes, anti-CD3 and anti-CD28 antibodies are not bound but are in suspension in the medium. Due to a well-documented polymorphism in the FcγR1 (Michelle Stein, et al. *Gene & Immunity* 2018) within the human population, certain individuals do not respond to anti-CD3/CD28 stimulation when the antibodies are in suspension. In the 10 healthy donors examined, 5 subjects were selected based on their non-responsiveness to anti-CD3/CD28. For the responders, there is noticeable shift of the lymphocyte population, indicating morphological changes accompanying T cell activation. The observed shift in the monocyte population is most likely as a result of bystander activation. For the non-responders, the cell populations were comparable to whole blood cultured under Null conditions, without any stimulants.

**Figure 2: CD45 population distribution changes under different stimulating conditions**

Whole blood was collected and incubated in Null, LPS, SEB, or CD3/CD28 TruCulture tubes. After staining, samples were read on the Beckman Coulter CytoFLEX LX flow cytometer and gated on live cells. The density plots are generated for CD45+ vs SSC: Lymphocytes are in purple, monocytes are in dark green, and granulocytes are in blue.

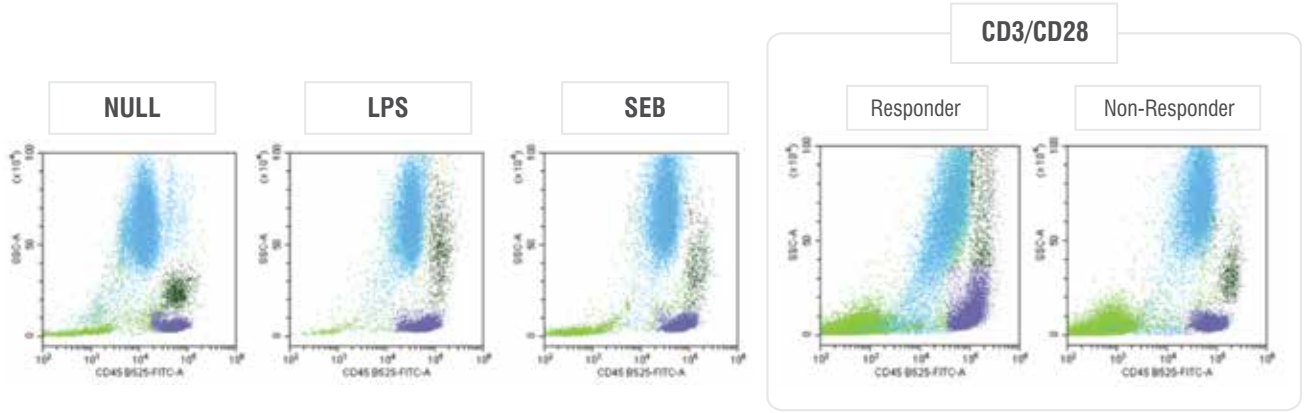
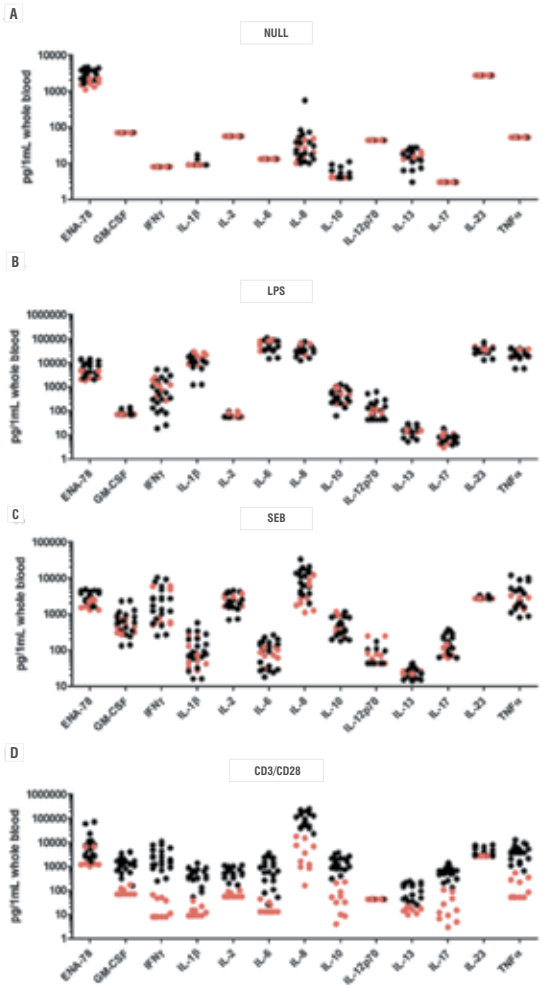


*Figure 3* shows the OptiMAP profiles from TruCulture supernatants from 10 healthy donors cultured under common stimulants in duplicate. As expected, most of the analytes examined from whole blood cultured in the TruCulture null tube (with no stimulant) were below the LLOQ of the assay (GM-CSF, IFNγ, IL-1β, IL-2, IL-6, IL-12p70, IL-17, IL-23, and TNFα). Of the 4 measurable analytes only the two neutrophil chemokines, IL-8 and ENA-78, were produced in significant concentrations. Whole blood cultured in LPS, SEB, or CD3/CD28 TruCulture tubes demonstrated expected immune responses to these stimulants. The CD3/CD28 non-responders are denoted in pink. Consistent with other studies, the inability to respond to CD3/CD28 stimulation did not affect individual responses to LPS or SEB stimulantion.

**Figure 3: OptiMAP profile of whole blood cultured using TruCulture tubes**




Whole blood was collected in duplicate tubes and incubated in Null, LPS, SEB, or CD3/CD28 TruCulture tubes. Supernatants were collected at 24 hours for Null, LPS and SEB and at 48 hours for CD3/CD28. Data is plotted per tube (there 2 data points per subject) for each analyte indicated on the X-axis. Red dots indicate subjects with low or no response to CD3/CD28 as measured by IFNγ. LLOQ of each analyte is indicated as follows:

| Analyte | LLOQ |
|---|---|
| ENA-78 | 0.010 ng/mL |
| GM-CSF | 70 pg/mL |
| IFN-γ | 8.5 pg/mL |
| IL-1β | 9.9 pg/mL |
| IL-2 | 57 pg/mL |
| IL-6 | 14 pg/mL |

## Conclusion

This application note illustrates that TruCulture cell samples can be analyzed using standardized flow cytometry methods. The use of TruCulture stimulations in clinical trials for pharmacodynamic monitoring and other applications can be accomplished through a variety of analytical methods and can be optimized based on the choice of stimulant.

## *Contact us*

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