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Spectral Flow Phenotyping of CD226 KO CD127$^-$ Tregs During Expansion

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DISCLAIMER

ABSTRACT

This Standard Operating Procedure provides instructions for staining surface and intracellular markers on primary cells to be used for spectral flow cytometry analysis. This SOP was designed for the purpose of staining isolated primary CD4$^+$ CD25$^+$ Tregs and Tconv cells for experiments pertaining to CRISPR KOs of CD226 on CD127$^-$ Tregs. Cells will be stained for CD4, CD25, CD45RA, CD127, CD197, CD226, FoxP3, Helios, L/D NIR, & TIGIT.

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KEYWORDS

Treg, Flow Cytometry, Extracellular Staining, Intracellular Staining, FOXP3, Helios

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GUIDELINES

Ensure all light-sensitive and temperature-sensitive materials are stored properly to prevent degradation

MATERIALS TEXT

- Microcentrifuge Tubes: 0.6 mL **Fisher Scientific Catalog #05-408-120** In 4 steps
- Falcon™ 5 mL Round-Bottom Polystyrene Test Tubes (Without Caps) **Fisher Scientific Catalog #14-959-5** Step 1
- LIVE/DEAD™ Fixable Far Red Dead Cell Stain Kit, for 633 or 635 nm excitation **Thermo Fisher Catalog #L10120** In 3 steps
- 1X PBS (Phosphate-buffered saline) **Contributed by users** In 6 steps
- Stain Buffer **In-house** In 11 steps
- Sterile deionized H2O **Contributed by users** In 2 steps
- eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set **Thermo Fisher Catalog #00-5523-00** In 4 steps
- BV510 Anti-Human CD4 OKT4 Antibody **BD Biosciences Catalog #566804** In 2 steps
- APC Anti-Human CD25 BC96 Antibody **BioLegend Catalog #302610** In 2 steps
- BV605 Anti-Human CD45RA HI100 Antibody **BioLegend Catalog #304134** In 2 steps
- PE Anti-Human CD127 (IL-7Rα) A019D5 Antibody **BioLegend Catalog #351304** In 2 steps
- APC-R700 Anti-Human CD197 (CCR7) 2-L1-A Antibody **BD Biosciences Catalog #566766** In 2 steps
- PE/Cyanine7 Anti-Human CD226 (DNAM-1) 11A8 Antibody **BioLegend Catalog #338316** In 2 steps
- PerCP-eFluor 710 Anti-Human TIGIT MBSA43 Antibody **Invitrogen Catalog #46-9500-42** In 2 steps
- Alexa Fluor 488 Anti-Human FOXP3 206D Antibody **BioLegend Catalog #320112** In 2 steps
- Alexa Fluor 488 Anti-Human FOXP3 259D Antibody **BioLegend Catalog #320212** In 2 steps
- Pacific Blue Anti-Mouse/Human Helios 22F6 Antibody **BioLegend Catalog #137220** In 2 steps

SAFETY WARNINGS

- Do NOT perform the tasks outlined in this SOP without proper universal safety precautions for handling human samples as well as personal protective equipment including but not limited to: gloves, lab coat, and arm guards
- Dispose all solutions and supplies that come in contact with cellular products in biohazardous waste containers

- Do NOT operate any centrifuges without confirming the centrifuge is balanced

DISCLAIMER:

> DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK
>
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BEFORE STARTING

Before beginning procedure, ensure that an AO/PI cell count has been conducted to determine cellular viability and concentration.

Preparing Falcon Tubes and Cells for Staining 16m

1 Obtain the appropriate number of 5 mL Falcon Tubes 2m

Falcon™ 5 mL Round-Bottom Polystyrene Test Tubes (Without Caps) **Fisher Scientific Catalog #14-959-5**

based on the cell subsets and timepoints being analyzed.

Step 1 includes a Step case.
CD226 KO Tregs (Day 0)
CD226 KO Tregs (Day 7 & Day 14)

step case

CD226 KO Tregs (Day 0)

If Day 0 Phenotyping is being conducted for experiments pertaining to the CD226 KO Tregs, 2 tubes will be required (unstained control & $CD127^-$ Tregs).

2 Transfer 100,000 Unelectroporated CD127- Tregs into the "$CD127^-$ Tregs" tube. 2m

3 Transfer 100,000 Unelectroporated $CD127^-$ Tregs into the "Unstained Control" tube. 2m

4 Centrifuge all tubes for 7 minutes at 350 g **350 x g, 23°C, 00:07:00** . 7m

5 After centrifugation, decant the supernatant and proceed to viability staining. 1m

Conducting Live/Dead NIR Staining of Tregs 15m 5s

6 Prepare **1 mL** of **5 Milimolar (mM)** Live/Dead Near IR Working Solution by diluting **0.1 µl** of reconstituted 3m

LIVE/DEAD™ Fixable Far Red Dead Cell Stain Kit, for 633 or 635 nm excitation **Thermo Fisher Catalog #L10120**

to **1 mL** of 1X PBS (Phosphate-buffered saline) **Contributed by users**

To reconstitute

LIVE/DEAD™ Fixable Far Red Dead Cell Stain Kit, for 633 or 635 nm excitation **Thermo Fisher Catalog #L10120**

, add **50 µl** of DMSO provided within the kit to a single unopened tube of lyophilized dye.

7 Add **1 mL** of **5 Milimolar (mM)** Live/Dead Near IR Working Solution to the "CD127- Tregs" tube. 1m

8 Add **1 mL** of 1X PBS (Phosphate-buffered saline) **Contributed by users** to the "Unstained Control" tube. 1m

9 Vortex all tubes for **00:00:05** to ensure complete resuspension of the cells within the dye (or PBS for the unstained control). 5s

10 After vortexing, incubate all cells for **00:05:00** at **4 °C** in the dark. 5m

11 After incubation, add **1 mL** of Stain Buffer **In-house** to each tube. 1m

12 Vortex all tubes for **00:00:05** to ensure complete dilution of the dye (or PBS for the unstained control). 5s

13 After vortexing, centrifuge all tubes for 5 minutes at 450 g **450 x g, 23°C, 00:05:00** . 5m

14 After centrifugation, decant the supernatant and proceed to extracellular staining. 1m

Conducting Extracellular Staining of Tregs 43m 15s

15 Generate the Extracellular Phenotyping Cocktail as described below in a Microcentrifuge Tubes: 0.6 mL **Fisher Scientific Catalog #05-408-120** : 5m

15.1 BV510 Anti-Human CD4 OKT4 Antibody **BD Biosciences Catalog #566804**

Add **1 µl** of

15.2 APC Anti-Human CD25 BC96

Add **1 µl** of Antibody **BioLegend Catalog #302610**

15.3 BV605 Anti-Human CD45RA HI100
Add **1 µl** of Antibody **BioLegend Catalog #304134**

15.4 Add **1 µl** of
PE Anti-Human CD127 (IL-7Rα) A019D5
Antibody **BioLegend Catalog #351304**

15.5 Add **1 µl** of
APC-R700 Anti-Human CD197 (CCR7) 2-L1-A Antibody **BD Biosciences Catalog #566766**

15.6 Add **1 µl** of
PE/Cyanine7 Anti-Human CD226 (DNAM-1) 11A8
Antibody **BioLegend Catalog #338316**

15.7 Add **1 µl** of
PerCP-eFluor 710 Anti-Human TIGIT MBSA43
Antibody **Invitrogen Catalog #46-9500-42**

16 After generating the Extracellular Phenotyping Cocktail, add all **7 µl** to the "CD127$^-$ Tregs" tube. 1m

The "Unstained Control" tube should NOT recieve any of the antibody cocktail. Optionally, **7 µl** of 1X PBS (Phosphate-buffered saline) **Contributed by users** may be added to the "Unstained Control" tube, but this is not necessary

17 Vortex all tubes for **00:00:05** to ensure complete resuspension of the cells within the antibody cocktail (or, optionally, PBS for the unstained control). 5s

18 After vortexing, incubate all cells for **00:15:00** at **4 °C** in the dark. 15m

19 After incubation, vortex all cells for **00:00:05** . 5s

20 After vortexing, incubate all cells for **00:15:00** at **Room temperature** in the dark. 15m

21 After incubation, add **1 mL** of Stain Buffer **In-house** to each tube. 1m

22 Vortex all tubes for **00:00:05** to ensure complete dilution of the antibody cocktail (or, optionally, PBS for the unstained control). 5s

23 After vortexing, centrifuge all tubes for 5 minutes at 450 g **450 x g, 23°C, 00:05:00** . 5m

24 After centrifugation, decant the supernatant and proceed to fixation/permeabilization. 1m

Conducting Fixation/Permeabilization of Tregs 1h 28m 30s

25 Generate **400 µl** 1x Fixation/Permeabilization Buffer by combining **100 µl** of 4x Fixation/Permeabilization Concentrate and **300 µl** of Fixation/Permeabilization Diluent provided within the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set **Thermo Fisher Catalog #00-5523-00** . 5m

The 1x Fixation/Permeabilization Buffer should be stored at **4 °C** when not actively in use.

26 Add **100 µl** of 1x Fixation/Permeabilization Buffer to all tubes. 1m

27 Vortex all tubes for **00:00:05** to ensure complete resuspension of the cells within the 1x Fixation/Permeabilization Buffer. 5s

28 After vortexing, incubate all cells for **00:20:00** at **Room temperature** in the dark. 20m

29 After incubation, add **1 mL** of Stain Buffer **In-house** to all tubes. 1m

30 Vortex all tubes for **00:00:05** to ensure complete dilution of the 1x Fixation/Permeabilization Buffer. 5s

31 After vortexing, centrifuge all tubes for 5 minutes at 450 g **450 x g, 23°C, 00:05:00** . 5m

32 After centrifugation, decant the supernatant from all tubes. 1m

33 Conduct a second 1x Fixation/Permeabilization Buffer wash. **go to step #26** 27m 10s

34 Generate **2 mL** 1x Permeabilization Buffer by combining **0.2 mL** of 10x Permeabilization Concentrate provided within the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set **Thermo Fisher Catalog #00-5523-00** and **1.8 mL** of Sterile deionized H2O **Contributed by users** . 5m

The 1x Permeabilization Buffer should be stored at **4 °C** when not actively in use.

35 Add **1 mL** of 1x Fixation/Permeabilization Buffer to all tubes. 1m

36 Vortex all tubes for **00:00:05** to ensure complete resuspension of the cells within the 1x Permeabilization Buffer. 5s

37 After vortexing, incubate all cells for **00:15:00** at **Room temperature** in the dark. 15m

38 After incubation, add **1 mL** of Stain Buffer **In-house** to all tubes. 1m

39 Vortex all tubes for **00:00:05** to ensure complete dilution of the 1x Permeabilization Buffer. 5s

40 After vortexing, centrifuge all tubes for 5 minutes at 450 g **450 x g, 23°C, 00:05:00** . 5m

41 After centrifugation, decant the supernatant from all tubes and proceed to intracellular staining. 1m

Conducting Intracellular Staining of Tregs 43m 15s

42 Generate the Intracellular Phenotyping Cocktail as described below in a Microcentrifuge Tubes: 0.6 mL **Fisher Scientific Catalog #05-408-120** : 5m

42.1 Add **1.25 µl** of

Alexa Fluor 488 Anti-Human FOXP3 206D Antibody **BioLegend Catalog #320112**

42.2 Add **1.25 µl** of Alexa Fluor 488 Anti-Human FOXP3 259D Antibody **BioLegend Catalog #320212**

42.3 Add **2.5 µl** of Pacific Blue Anti-Mouse/Human Helios 22F6 Antibody **BioLegend Catalog #137220**

43 After generating the Intracellular Phenotyping Cocktail, add all **5 µl** to the "CD127⁻ Tregs" tube. 1m

The "Unstained Control" tube should NOT recieve any of the antibody cocktail. Optionally, **5 µl** of 1X PBS (Phosphate-buffered saline) **Contributed by users** may be added to the "Unstained Control" tube, but this is not necessary

44 Vortex all tubes for **00:00:05** to ensure complete resuspension of the cells within the antibody cocktail (or, optionally, PBS for the unstained control). 5s

45 After vortexing, incubate all cells for **00:15:00** at **4 °C** in the dark. 15m

46 After incubation, vortex all cells for **00:00:05** . 5s

47 After vortexing, incubate all cells for **00:15:00** at **Room temperature** in the dark. 15m

48 After incubation, add **1 mL** of Stain Buffer **In-house** to each tube. 1m

49 Vortex all tubes for **00:00:05** to ensure complete dilution of the antibody cocktail (or, optionally, PBS for the unstained control). 5s

50 After vortexing, centrifuge all tubes for 5 minutes at 450 g **450 x g, 23°C, 00:05:00** . 5m

51 After centrifugation, decant the supernatant and add **50 µl** of Stain Buffer **In-house** to all tubes, then proceed to collect data using the 1m

Aurora - 5L Configuration
Spectral Flow Cytometer
Cytek Aurora **N/A**
5L Configuration

Cells may be stored at **4 °C** until acquisition, however, acquisition must be completed within 48 hours of fixation to ensure sufficient data quality.

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