# Harnessing violet and ultraviolet fluorophores for flexibility in T cell panel design

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## Abstract

The cytotoxic capabilities of T cells are being leveraged for immunotherapeutic approaches in the fight against cancer. These treatments have demonstrated significant promise thus far and warrant refinement to increase efficacy, reduce unwanted effects, and expand their capabilities to more diverse tumor types. As such, continuing to broaden our understanding of T cell biology at a basic research level is critical for developing the next generation of T cell immunotherapeutics.

Spectral flow cytometry enables recognition of the unique spectral signature of individual fluorophores and facilitates the addition of more antibodies by accommodating fluorophore combinations which are incompatible on conventional cytometers. An increased availability of fluorophore-conjugated antibodies for surface and intracellular antigens in the ultraviolet and violet laser range enable researchers to deepen their understanding of T cell activation and function with more flexibility in adding markers of interest. Here, we developed an optimized panel for the investigation of human T cell differentiation, activation, proliferation, and function. This panel demonstrates consistent and robust characterization of *in vitro*-activated human T cells, while leaving most channels on the blue, yellow-green, and red lasers available for additional markers of interest. This approach lends itself additional customization, facilitating deeper insights from a single, limited sample enabled by the broad availability of fluorophore-conjugated antibodies supporting these laser lines.


## Introduction

Immune checkpoint blockade targets the inhibitory signals that occur during T cell activation, allowing a patient's T cells to overcome the regulatory mechanisms that promote immune tolerance and mount an anti-tumor response. These therapies have revolutionized the treatment of various cancers, with treatments against a broad range of tumor types approved for use in patients and the expansion of these applications likely. While these therapies have demonstrated significant promise, they are often associated with significant immune-related adverse effects, as the targeted pathways are necessary for host protection against autoimmunity. Further research is critical to promote a deeper understanding of the mechanisms behind these approaches in order to reduce unwanted effects and increase the overall efficacy of these treatments, making the continued study of T cell biology an essential field in immunology.

Our aim in designing this panel was to enable T cell research through robust detection of key differentiation, activation, and proliferation markers. The surface antigens CD197 (CCR7) and CD45RA were used to identify four maturation subsets of T cells: CCR7+CD45RA+ naïve cells (TN), CCR7+CD45RA- central memory cells (TCM), CCR7-CD45RA- effector memory cells (TEM), and a population of effector memory cells that regains CD45RA expression, CCR7-CD45RA+ TEMRA. The latter two populations were further divided based on expression of CD127 and KLRG1, with CD127-KLRG1+ subsets indicating terminally differentiated cells. The antigens CD25, CD69, Ki-67, CD154 (CD40L), CD223 (LAG-3), and CD152 (CTLA-4) were included as markers of proliferation and activation. Lastly, the transcription factor, HELIOS, was added as an identifying marker of T regulatory cells.

In order to provide flexibility for future modifications and expansions of this panel, we heavily leveraged fluorophores on the ultraviolet and violet channels, leaving many channels open on the yellow-green, blue, and red laser lines available to customization.

| Approx. nm | UV | Approx. nm | Violet | Approx. nm | Blue | Approx. nm | Yellow Green | Approx. nm | Red |
|---|---|---|---|---|---|---|---|---|---|
| UV1 (372) | | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| UV2 (387) | CD45 BUV395 | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| UV3 (427) | | V1 (428) | CD127 BV421 | ----- | ----- | ----- | ----- | ----- | ----- |
| UV4 (443) | | V2 (443) | | ----- | ----- | ----- | ----- | ----- | ----- |
| UV5 (458) | | V3 (458) | | ----- | ----- | ----- | ----- | ----- | ----- |
| UV6 (473) | | V4 (473) | HELIOS BV480 | ----- | ----- | ----- | ----- | ----- | ----- |
| UV7 (514) | CD3 BUV496 | V5 (508) | | B1 (508) | | ----- | ----- | ----- | ----- |
| ----- | ----- | V6 (525) | | B2 (525) | CD45RA FITC | ----- | ----- | ----- | ----- |
| UV8 (542) | CD223 BUV563 | V7 (542) | | B3 (542) | Live/Dead Olive | ----- | ----- | ----- | ----- |
| UV9 (581) | | V8 (581) | | B4 (581) | | YG1 (577) | CD25 PE | ----- | ----- |
| ----- | ----- | V9 (598) | | B5 (598) | | YG2 (598) | | ----- | ----- |
| UV10 (612) | | V10 (615) | | B6 (615) | | YG3 (615) | | ----- | ----- |
| UV11 (664) | | V11 (664) | CD69 BV650 | B7 (660) | | YG4 (660) | | R1 (660) | KLRG1 APC |
| ----- | ----- | ----- | ----- | B8 (678) | | YG5 (678) | | R2 (678) | |
| UV12 (691) | | V12 (692) | | B9 (697) | | YG6 (697) | | R3 (697) | |
| UV13 (720) | | V13 (720) | CD4 SB702 | B10 (717) | | YG7 (720) | CD197 PE-Cy5.5 | R4 (717) | |
| ----- | ----- | ----- | ----- | B11 (738) | | ----- | ----- | R5 (738) | |
| UV14 (750) | | V14 (750) | | B12 (760) | | YG8 (750) | | R6 (760) | |
| UV15 (780) | Ki-67 BUV737 | V15 (780) | | B13 (783) | | YG9 (780) | | R7 (783) | CD154 APC-eF780 |
| UV16 (812) | CD152 BUV805 | V16 (812) | CD8 BV786 | B14 (812) | | YG10 (812) | | R8 (812) | |

## Materials and methods

**Antibodies and buffers:** All antibodies and buffers used are products of Thermo Fisher Scientific and were used according to the manufacturer's instructions at optimal concentration (5 µL) with the exception of CD25 and CCR7, which were titrated to determine optimal (see: Table 1 for list of antibodies). Surface staining and washes were performed in Invitrogen™ eBioscience™ Flow Cytometry Staining Buffer (00-4222-26, Thermo Fisher Scientific). Intracellular staining was performed using the Invitrogen™ eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (00-5523-00, Thermo Fisher Scientific). Antibody staining was performed in the presence of Invitrogen™ Brilliant Stain Buffer (00-4409-42, Thermo Fisher Scientific).

**Cell culture:** Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood from normal donors and cultured 72 hours in complete RPMI medium either unstimulated or with a T cell receptor (TCR) stimulation. TCR stimulation was performed with 3 µg/mL CD3 Monoclonal Antibody (clone OKT3; 16-0037-81, Thermo Fisher Scientific) and 5 µg/mL CD28 Monoclonal Antibody (clone CD28.6; 16-0288-81, Thermo Fisher Scientific).

**Flow cytometry:** Following culture, cells were harvested and stained with Invitrogen™ LIVE/DEAD™ Fixable Olive (557 nm) Viability Kit (L34977, Thermo Fisher Scientific), then surface stained with CD45, CD3, CD4, CD8, CCR7, CD45RA, CD25, CD127, CD154, CD223, CD69, KLRG1 in the presence of Brilliant Stain Buffer. Following surface staining and washing in Flow Cytometry Staining Buffer, cells were fixed, permeabilized, and stained intracellularly with Ki-67, HELIOS, and CD152, again in the presence of Brilliant Stain Buffer. Single-color controls and fluorescence minus one (FMO) controls were included for all fluorochromes in this panel. Data were acquired on a Cytek® Aurora 5-laser spectral flow cytometer and analyzed using SpectroFlo® software.

## Results

**Initial gating (data not shown):** Two singlet gates, one based on Forward Scatter (FSC-A vs FSC-H) and one based on Side Scatter (SSC-A vs SSC-H) were applied to exclude any coincident events from further analysis. Lymphocytes were then identified based on FSC-A vs SSC-A profile, followed by gating of viable lymphocytes using viability dye vs CD45. T cells were identified based on CD3 staining, then further subdivided based on CD4 and CD8 staining. All populations percentages were within published ranges for normal donors.

**Figure 1. HELIOS transcription factor allows for reliable characterization of T regulatory population in cultured cells**
Unstimulated (left) or stimulated (right) CD4+ cells were analyzed for expression of CD25 and CD127 in order to identify the T regulatory population, defined as CD4+CD25+CD127low. As CD25 was observed to be upregulated upon cell stimulation, a majority of the CD4+ cells met the criteria for Treg subsets as described above. The Treg transcription factor HELIOS was included as an additional discriminating marker, and the HELIOS+ populations below are thought to more accurately represent the suppressive CD4+ T cell population in this experiment.

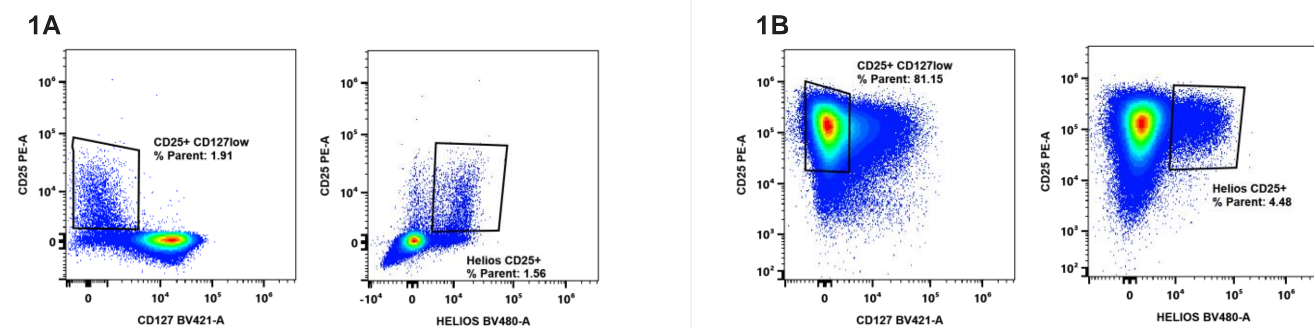
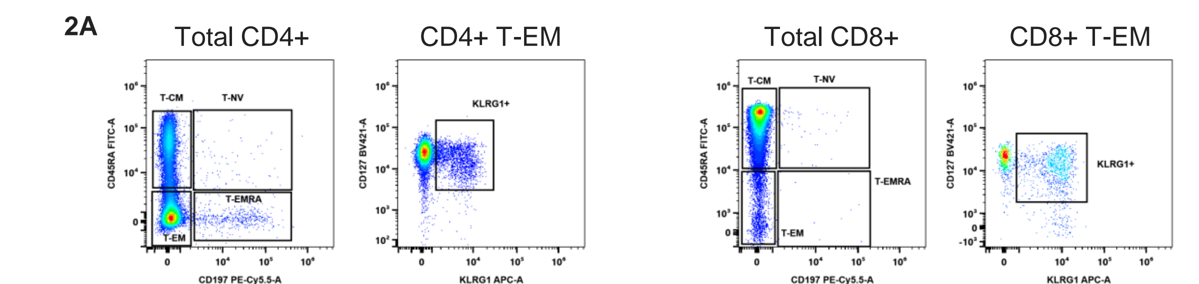



**Figure 1A. Unstimulated CD4+ T cells:** note similar percentages of CD25+CD127low and CD25+HELIOS+ populations in cells that have not upregulated CD25 due to stimulation

**Figure 1B. TCR-stimulated CD4+ T cells:** upregulation of CD25 upon stimulation results in difficulty characterizing Treg based on CD25 and CD127 expression. In this case, HELIOS is likely to be a more accurate representation of the Treg subset.

**Figure 2. Unstimulated cells downregulated CCR7 expression upon culture, while significant upregulation of CCR7 was observed in TCR-stimulated cells2**

**Figure 2A.** Unstimulated CD4+ and CD8+ T cells were analyzed for expression of CD45RA and CCR7 maturation markers and gated to indicate CD45RA+CCR7+ TN, CCR7+CD45RA- TCM, CCR7-CD45RA- TEM, and CCR7-CD45RA+ TEMRA. Note loss of CCR7 expression in unstimulated cells. TEM population was further divided based on CD127 and KLRG1 expression, with KLRG1 staining indicating terminally differentiated cells. There were too few events in the TEMRA subsets to further investigate KLRG1 staining.



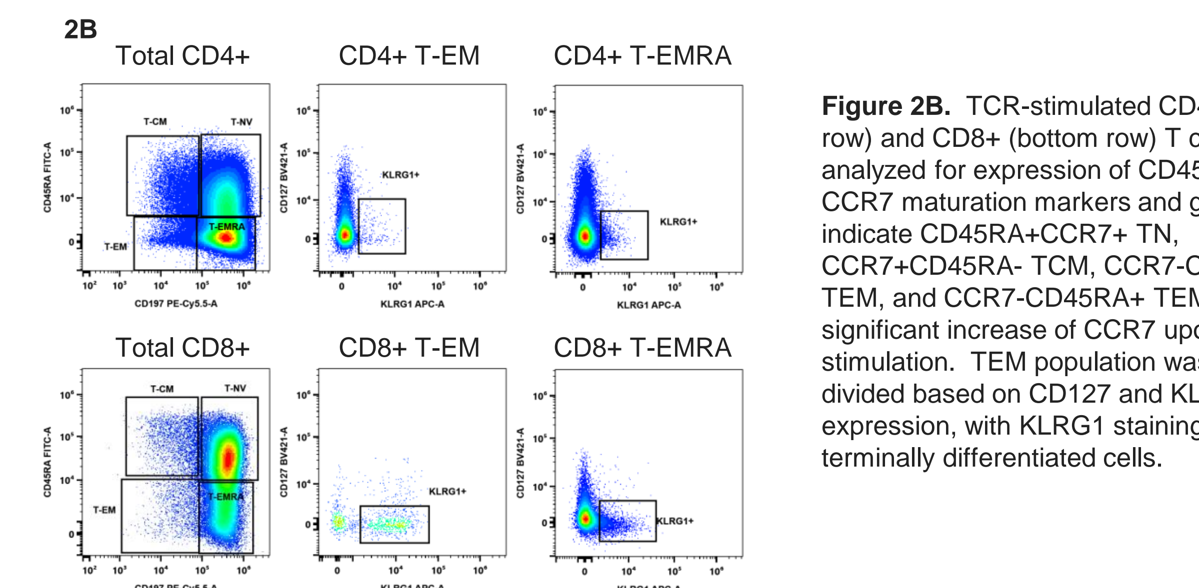




**Figure 2B.** TCR-stimulated CD4+ (top row) and CD8+ (bottom row) T cells were analyzed for expression of CD45RA and CCR7 maturation markers and gated to indicate CD45RA+CCR7+ TN, CCR7+CD45RA- TCM, CCR7-CD45RA-TEM, and CCR7-CD45RA+ TEMRA. Note significant increase of CCR7 upon stimulation. TEM population was further divided based on CD127 and KLRG1 expression, with KLRG1 staining indicating terminally differentiated cells.

**Figure 3. TCR stimulation results in upregulation of activation and proliferation markers in cultured T cells**

**Figure 3A.** CD25, CD69, CD223, Ki-67, CD152, and CD154 expression in unstimulated (top) and TCR-stimulated (bottom) CD4+ T cells. All gates were drawn using FMO controls (not shown).

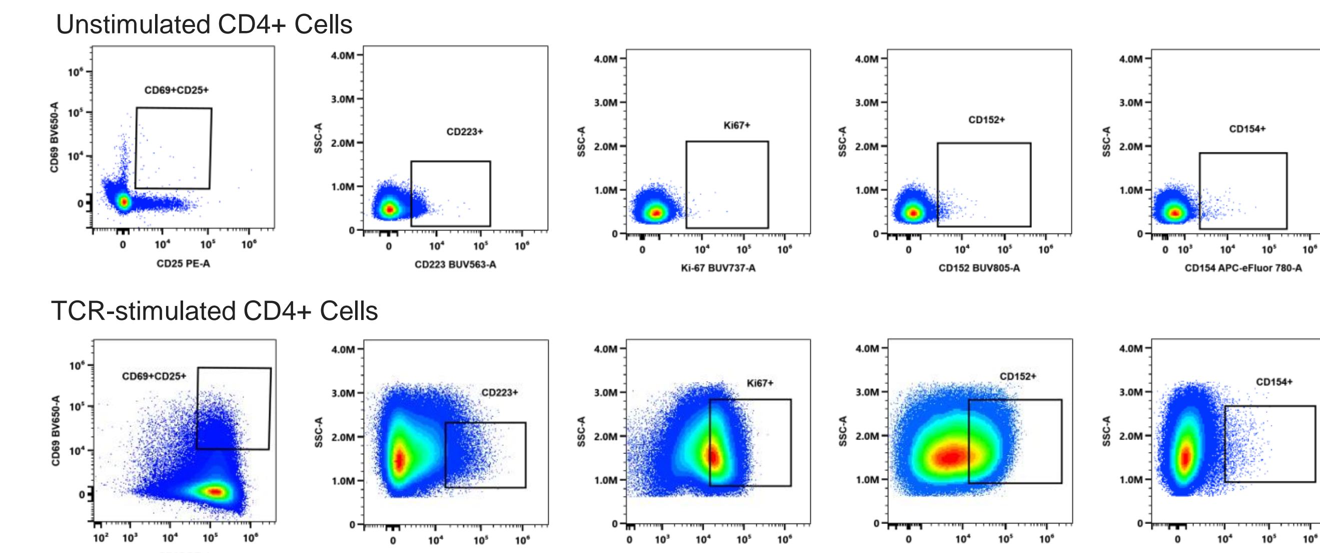



**Figure 3B.** CD25, CD69, CD223, Ki-67, CD152, and CD154 expression in unstimulated (top) and TCR-stimulated (bottom) CD8+ T cells. All gates were drawn using FMO controls (not shown).

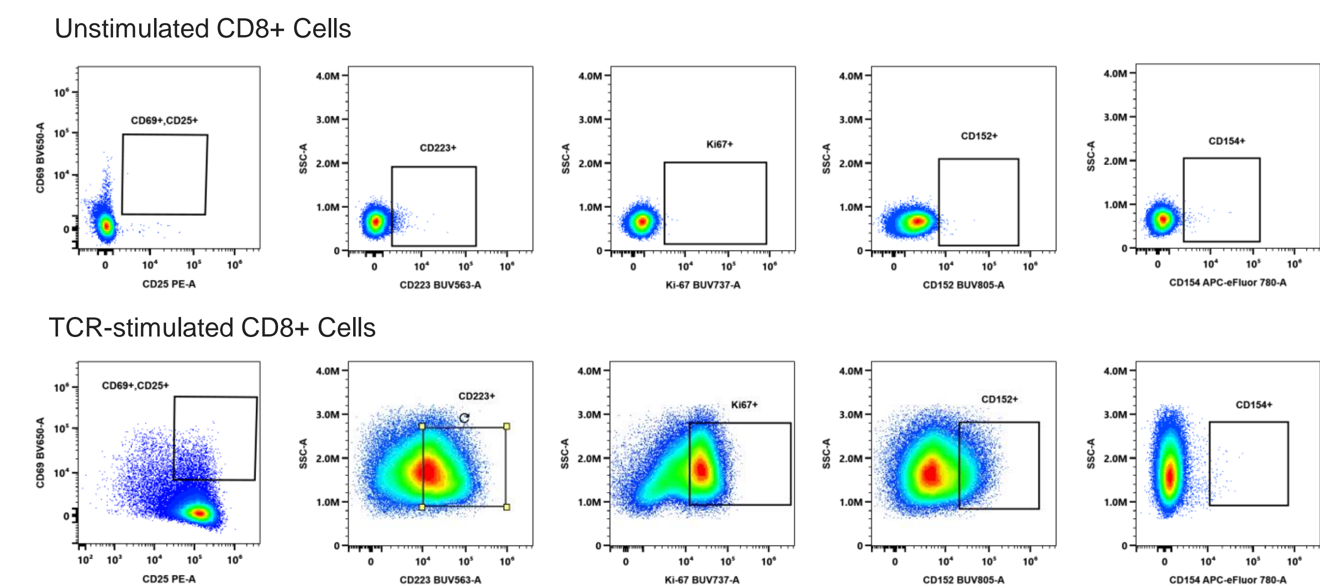
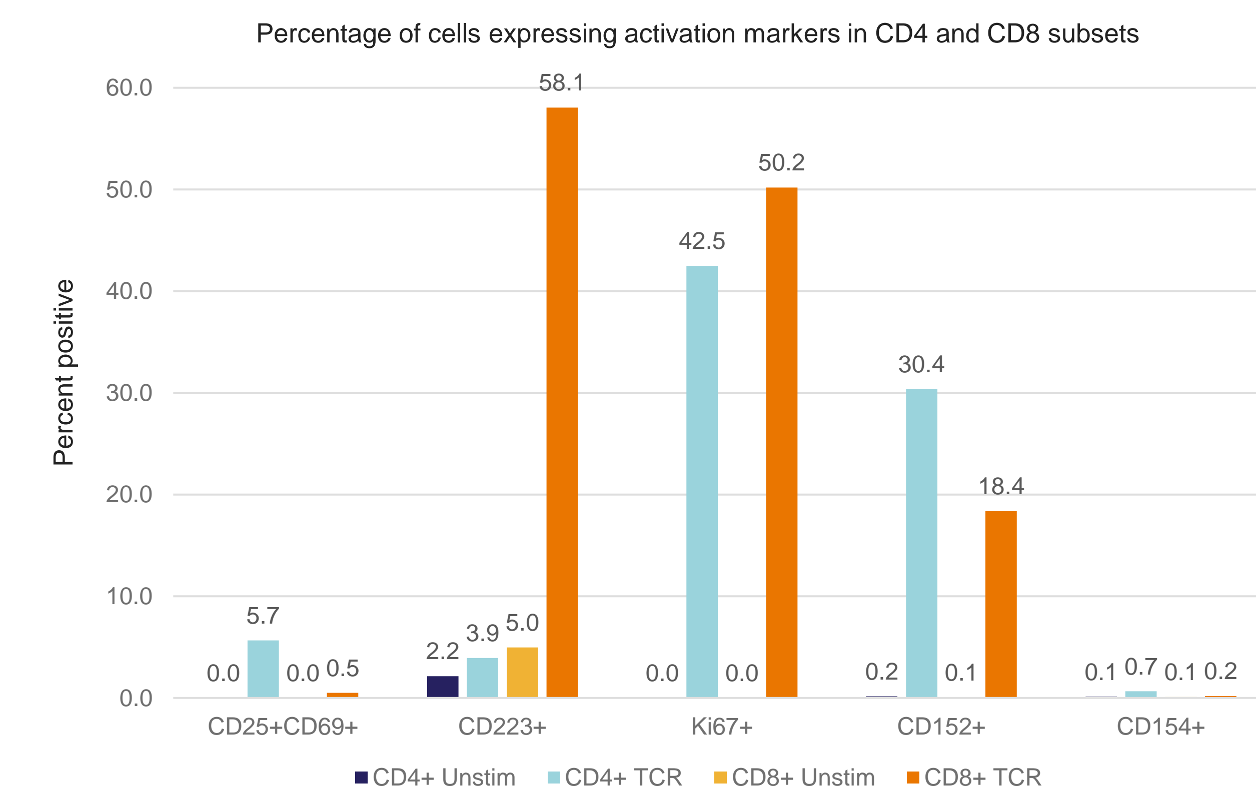



**Figure 3C.** Percentage of unstimulated and TCR-stimulated cells expressing activation and proliferation markers among both CD4+ and CD8+ subsets. Note upregulation of most markers upon stimulation in both T cell subsets, with exception of CD154.




## Conclusions

Some key takeaways from this panel and opportunities for further optimization are listed below.

- Unsurprisingly, TCR stimulation significantly upregulated CD25 expression on most CD4+ T cells, leading to difficulty in identification of T cells using the CD25+CD127low expression profile. The resulting majority of CD4+ cells that met this criteria were not likely to be suppressive cells, and expression of HELIOS is instead thought to be a more accurate representation of the Treg subset in this context. This is supported by the observation that CD25+HELIOS+ and CD25+CD127low percentages are similar in the unstimulated cells that have not upregulated CD25. The transcription factor Foxp3 can be included in future iterations of this panel for further confirmation of Treg characterization.
- We observed unexpectedly low-to-absent levels of CCR7 expression in the unstimulated cultured cells, and very high levels in the TCR-stimulated cells, making it difficult to identify some of the differentiation subsets based on CCR7 and CD45RA expression. This can be further investigated by staining uncultured PBMC in parallel to the unstimulated and TCR-stimulated cells in order to assess levels of CCR7 expression *ex vivo* and compare those to cultured cells during analysis. Additionally, CCR7 PE-Cyanine 5.5 single-color controls were observed to express higher levels of this antigen than the fully-stained samples, indicating a possible inhibition of CCR7 staining in the presence of other antibodies (data not shown). This can be mitigated by pre-staining CCR7 prior to the other surface antigens in future attempts to repeat.
- TCR stimulation resulted in upregulation of most activation and proliferation markers in this panel, with a minimal increase in CD154. This panel can also be attempted using cells that have undergone stimulation with peptides or other physiologically relevant treatments to assess activation under those conditions.

Overall, this panel allows for robust identification of surface and intracellular antigens critical for identification of T cells within a mixed lymphocyte population and for assessing their maturation and activation status, while also allowing for customization with additional markers relevant to users' individual research. The Brilliant™ dyes in our portfolio of reagents facilitate panel building in both spectral and conventional flow cytometry by providing a broad range of options across the ultraviolet and violet spectrum.

## References


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**Table 1.** Antibody panel

| Specificity | Fluorophore | Catalog No. |
|---|---|---|
| CD45 | Brilliant™ UV 395 | 363-0459-42 |
| CD3 | Brilliant™ UV 496 | 364-0038-42 |
| CD4 | Super Bright™ 702 | 67-0049-42 |
| CD8 | Super Bright™ 780 | 78-0088-42 |
| CD25 | PE | 12-0259-42 |
| HELIOS | Brilliant™ Violet 480 | 414-9883-42 |
| CD127 | Brilliant™ Violet 421 | 404-1278-42 |
| CD69 | Brilliant™ Violet 650 | 416-0699-42 |
| CD197/CCR7 | PE-Cyanine 5.5 | 35-1979-42 |
| CD45RA | FITC | 11-0458-42 |
| CD223/LAG-3 | Brilliant™ UV 563 | 365-2239-42 |
| Ki-67 | Brilliant™ UV 737 | 367-5699-42 |
| CD152/CTLA4 | Brilliant™ UV 805 | 368-1529-42 |
| CD154/CD40L | APC-eFluor™ 780 | 47-1548-42 |
| KLRG1 | APC | 17-5893-42 |

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