Designing “backbone” multi-color panels for T-cell and Treg populations with allowance for drop-in reagents

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ABSTRACT

Introduction. Multi-parametric analysis by flow cytometry has become an essential tool to characterize complex lymphocyte populations, but is complicated by the need to optimize antibody/fluor combinations for each panel. We describe the use of a “backbone” strategy in panel design to facilitate higher-plex panel design: In this case, five Treg markers (+ viability) were combined to allow common gating across 2 panels and to allow easy drop-in of elective conjugates using commonly available fluoros.

Regulatory T cells or Tregs contribute in a range of foreign and self-antigenic immune responses that help distinguish and maintain the homeostatic relationship between immunity and tolerance. Naturally occurring regulatory T cells in humans has been defined as CD4+FOXP3+, CD4+CD25+ or CD4+CD25+FOXP3+. Two 10-color panels were developed: a backbone 6-color panel using CD3-ECD, CD4-Krome Orange, CD8-APC-Alexa 700, CD25-PC7, CD45RA-APC-Alexa 750, and 7AAD, which allowed drop-ins conjugated to FITC, PE, APC, and Pacific Blue. This approach allows modification of the panels with ease and allows a standard way to identify CD4+CD25+ Treg cells.

Results. Sample analysis with this backbone strategy allowed the use of one compensation matrix and a common gating approach for CD4+CD25+ cells. This Treg population can be further characterized into distinct functional and developmental subsets with the use of the selective drop-in reagents, such as CCR4, CCR7, CD38, CD39, CD127, and HLA-DR. Several populations can be identified in panel 1 based on CCR4 and CD39 expression when gating on CD4+CD25+CD127lo Treg cells. In addition, three classically described populations can be identified in the Tregs based on CCR7 and CD45RA expression (panel 2): central memory, effector memory and naïve. We observed an additional subset, the effector population, in non-Treg cells. Furthermore, a progression of Treg maturation can be observed using CD38 and CD39 in these subsets.

Conclusion. The implementation of the standard 5-color backbone allowed the use of a common gating strategy and a common compensation matrix for analysis. Easy drop-in conjugates for CCR4, CCR7, CD39, CD127, and HLA-DR enabled a detailed characterization of Treg subsets with little added design effort.
MATERIALS & METHODS

All dye-conjugated antibodies were from Beckman Coulter (Brea, CA), with the exceptions of: CD39- FITC (Bio-Legend, San Diego, CA), CCR4-PE and CCR7-PE (R&D Systems, Minneapolis, MN). Conjugates were titered for optimal test dosages.

100 µL of normal blood was stained with cocktails (20 minutes, room temperature), lysed with VersaLyse Lysing Solution (Beckman Coulter), washed and resuspended with 7-Aminoactinomycin D (7-AAD). Cells were fixed in 750 µL of buffer + 0.5% formaldehyde. Samples were assessed on a Gallios* cytometer standardized with Flow Check Pro (alignment) and Flow Set Pro (PMT voltage), and analyzed using Kaluza* software (Beckman Coulter).

T & Treg Panel Requirements

T cell panel – required markers:
- CD3, CD4, CD8, CD45RA, CCR7 (to identify central memory, effector memory, terminal effector, and CD4/CD8 lineage)
- CD38, HLA-DR (activation)

Treg panel
- Required markers: CD4, CD25, CD127, Viability
- Optional markers: CD8, CD45RA, CD45, CCR4, HLA-DR
Panel Design Guidance

Design Principals

• Use moderately bright fluors (organic dyes) for well-resolved gating markers
• Use PE & APC used for antigens requiring greatest resolution (dim, continuum expression)
• Tandems dyes effective for mid-density to bright antigens
• Detect co-expressed markers on different lasers or on well-separated channels
• Do not put a dim surface marker in a channel next to a bright marker
• Can place surface markers that are mutually exclusive on the adjacent channels
• Use viability discrimination (7-AAD) to gate out dead cells
• Can use Peak Integral & TOF information for doublet discrimination

Design Choices

• 2 panels with same backbone of gating markers, largely using organic dyes or moderately bright tandem dyes
• One compensation matrix that satisfies both panels
• Viability marker (7-AAD) in PC5.5 channel
• Leave space for at least 2 drop-ins in Pacific Blue and Fluorescein (fluors with high availability)
RESULTS

<table>
<thead>
<tr>
<th>405 nm</th>
<th>488 nm</th>
<th>635 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>Krome Orange</td>
<td>Pacific Blue</td>
</tr>
<tr>
<td>Treg</td>
<td>CD4: Open</td>
<td>CD28</td>
</tr>
<tr>
<td>T-cell</td>
<td>CD4: HLA-DR</td>
<td>CD28</td>
</tr>
</tbody>
</table>

Figure 1. Panel designs and compensation matrix.
Final design fulfills all marker requirements, incorporates a common backbone, and requires one compensation matrix (derived using single color controls). CD39 is placed in the FITC channel as an option to enhance finer resolution in differentiating T/T-regulatory cells.

Figure 2. Gating Strategy.
One gating strategy is applied to both panels. Live cells are gated using 7-AAD and the singlet lymphocyte population is identified. CD3 positive cells are gated and then discriminated into CD4 and CD8 subsets. In the Treg panel, CD4+ cells are further gated using CD127 and CD25 to identify. For T-cell panel, CD4 positive cells are plotted against CD25 to identify the T-regulatory and non-T-regulatory cells. A fluorescence-minus-one (FMO) stain was performed without CD25 to verify that CD25+ cells are correctly gated.

REGULATORY T-CELL ANALYSIS • BECKMAN COULTER
A comparison of bivariate density plots gated on non-Treg cells (CD4+CD25+CD127-), CD127-negative Treg cells (population 1), and CD127-low Treg cells (population 2) show very different distributions of CCR4 and CD39 expression.

CD4 subset backgating shows possible differences in scatter, CD127, and CD25 surface expression.

**Figure 3. Treg panel.**

**Figure 4. Treg panel.**
**Figure 5. Treg panel.**
The four populations identified in the CD4^+^CD25^+^ are shown in multiparameter radar plots to show their surface expression differences. The center plot overlays the four gated populations.

**Figure 6. T-cell panel.**
CD4^+^CD25^+^ and CD4^+^CD25^−^ cells are further distinguished based on expression of CCR7, CD45RA, CD38, and CD39. Four distinct subsets can be identified based on these markers: Naïve, Central memory, Effector memory, and Effector cells. It should be noted that very few Effector cells can be observed in the CD4^+^CD25^+^ as opposed to the CD4^+^CD25^−^ cells.
Figure 7. T-cell panel: Phenotypic progression of CD4^+CD25^+ Treg cells.

The three main subsets in CD4^+CD25^+ Treg cells have distinct CD39 and CD38 patterns in their phenotypic progression.

Figure 8. Phenotypic characterization of Treg cells in T-cell panel.

The four subsets within the CD4^+CD25^+ and CD4^+CD25^- populations are shown in the radar plots above to reveal their occupancy in multi-parametric dimensions.
Table 1. Gating hierarchy and statistics for the panels.

The hierarchal scheme and population percentages for donor 1 in both panels are shown in the left section. The statistical analysis of all five donors normalized to singlets is shown in the right section.
SUMMARY

- We were able to develop panels that met design requirements for T-cells / Tregs using a backbone and drop-in strategy.
- This approach allowed a common gating strategy and one compensation matrix to be used between panels.
- The panels are capable of high resolution identification of T cell populations (relatively low spreading in critical populations), including subsets of CD4⁺CD25⁺ (Treg) cells.
- The backbone approach provides a powerful way to rapidly design and execute multiple panels with related or overlapping markers.

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