Flow Cytometry Panel Design Sheet

9-Colour Treg / Tconv Immunophenotyping Panel

Apex Immunology Core Facility ▪ Nexa Life Sciences Campus ▪ Panel ID: FCM-PNL-0142 ▪ v2.1

1 Panel Overview

Study Context

Study: Project Meridian-7 — Regulatory T-cell frequency and functional-marker characterisation in peripheral blood, longitudinal cohort.

Rationale: Nine fluorescence parameters plus a viability dye resolve CD4/CD8 lineage, Treg-defining markers (CD25^{hi}, CD127^{lo}, FoxP3), and CCR7/CD45RA memory subsets in a single tube, minimising sample consumption on limited-volume paediatric draws.

Sample type: Cryopreserved PBMC, 1×10^6 cells/tube, surface stain followed by fix/permeabilisation for intracellular FoxP3.

Run Metadata

Panel ID	FCM-PNL-0142
Version	2.1
Designed by	Dr. Elena Marsh
Facility	Apex Immunology Core
Instrument	Nimbus CytoSense 5L
Configuration	UV/V/B/YG/R, 5 laser
Parameters	9 colour + viability

1.1. Instrument Configuration

The Nimbus CytoSense 5L analyser is configured with five spatially separated lasers — ultraviolet (355 nm), violet (405 nm), blue (488 nm), yellow-green (561 nm), and red (640 nm) — feeding eighteen photomultiplier detectors. Panel design below assigns each fluorophore to a single primary detector and lists secondary laser excitation only where it materially affects compensation.

Laser	Wavelength	Detectors used	Fluorophores on this laser
UV	355 nm	UV3	BUV737
Violet	405 nm	V1, V4, V6	BV421, BV605, BV711
Blue	488 nm	B1	FITC
Yellow-Green	561 nm	YG1, YG5	PE, PE-Cy7
Red	640 nm	R1	APC
UV	355 nm	UV1	Fixable Viability Dye (UV)

2 Fluorophore / Detector Matrix

Assignment follows the standard rule of thumb: dim, low-density antigens receive the brightest available fluorophores; bright, high-density lineage markers absorb the dimmer reagents. Relative brightness tier is taken from in-house staining-index benchmarking on this instrument configuration, not from vendor catalogue values, which do not transfer across PMT voltages.

Marker	Fluorophore	Laser	Detector	Clone	Brightness	Biological role
Viability	Zombie-UV Fixable	UV	UV1 450/50	—	Bright	Dead-cell exclusion, dump gate
CD3	BUV737	UV	UV3 740/35	UCHT1	Medium	Pan-T-cell backbone, high density
CD4	BV421	Violet	V1 450/50	SK3	Bright	Helper lineage, high density
CD8	BV605	Violet	V4 610/20	SK1	Medium	Cytotoxic lineage, mid-high density
CD45RA	FITC	Blue	B1 525/50	HI100	Medium	Naive / memory discriminator
CD25	PE	Yellow-Green	YG1 575/26	BC96	Bright	IL-2Rα, low-density activation marker
CD127	PE-Cy7	Yellow-Green	YG5 780/60	HIL-7R-M21	Bright	IL-7Rα, low density, Treg exclusionary
CCR7	BV711	Violet	V6 710/50	G043H7	Bright	Chemokine receptor, low-mid density
FoxP3	APC	Red	R1 670/30	259D/C7	Bright	Intracellular, low abundance transcription factor

⚠ Tandem-Dye Handling

PE-Cy7 and BV711 are tandem conjugates and degrade under prolonged light exposure or repeated freeze–thaw. Store protected from light at 4°C, titrate a fresh vial lot-to-lot, and re-run single-stain controls whenever a new lot of either reagent enters service — degraded tandem produces a false compensation value, not a visible staining failure.

3 Spillover Considerations

Fluorophore emission spectra overlap into neighbouring detectors; the compensation matrix corrects for this mathematically, but panel layout should still minimise the largest spreads before compensation is even calculated. The pairs below are the material spillover interactions for this configuration, ranked by the spreading error they introduce into the recipient channel.

Source	Spills into	Severity	Mitigation
BV421	BV605 (V4)	Medium	Adjacent violet cascade; confirm with BV421-only control at assay voltage.
BV605	BV711 (V6)	Medium	Same cascade, opposite direction; re-check after any PMT voltage change.
FITC	PE (YG1)	Low	Minor blue-into-yellow-green bleed; stable across lots.
PE	PE-Cy7 (YG5)	High	Tandem degradation products fluoresce in the parent PE channel; largest single source of error in this panel.
BUV737	APC (R1)	Low	Small UV-into-red tail; negligible after standard compensation.
Zombie-UV	BUV737 (UV3)	Medium	Both on the UV laser; viability dye must be titrated down to the lowest concentration that still resolves dead cells cleanly.

⚠ Highest-Risk Pair: PE → PE-Cy7

CD25 (PE) and CD127 (PE-Cy7) are the two markers that define the Treg gate. A spreading error between them directly inflates or deflates the reported Treg frequency. Compensate from freshly stained single-colour controls run *the same day* as the experiment, not from a stored compensation matrix, and confirm the correction visually on an FMO-CD127 sample before accepting any run.

4 Compensation Controls

Single-Stain Controls (beads)

- ☐ BUV737 only
- ☐ BV421 only
- ☐ BV605 only
- ☐ BV711 only
- ☐ FITC only
- ☐ PE only
- ☐ PE-Cy7 only
- ☐ APC only
- ☐ Unstained (blank) beads

Cell-Based Controls

- ☐ Viability dye single stain (heat-killed + live cell mix, *not* beads — amine dye needs protein)
- ☐ Unstained PBMC (autofluorescence baseline)
- ☐ Fully stained reference PBMC, same donor lot every run
- ☐ Isotype control — FoxP3 intracellular step only

4.1. Fluorescence-Minus-One (FMO) Controls

FMOs are run for every marker used to draw a gate boundary rather than a simple positive/negative split. They are not optional here: without them the Treg gate boundary is drawn on an assumption, not a measurement.

FMO omitting	Purpose
CD25 (PE)	Sets the CD25^{hi} boundary against activated non-Treg background staining.
CD127 (PE-Cy7)	Confirms the CD127^{lo} cutoff independent of the CD25 gate.
CCR7 (BV711)	Resolves the central-memory / effector-memory boundary, which has no clean negative population.
FoxP3 (APC)	Sets the intracellular positive gate against fix/perm background, which varies by lot.

Bead vs. Cell Controls

Use compensation beads for every surface fluorophore — they give a brighter, more reproducible positive peak than cells and use far less sample. Beads do not bind the viability dye or survive fixation chemistry correctly, so the viability control and the FoxP3 FMO must be run on actual cells.

5 Gating Strategy & Instrument QC

5.1. Gating Hierarchy

1. Time gate — exclude flow instability (flag any run with a fluidics interruption before analysis).
2. Singlets — FSC-H vs. FSC-A, then SSC-H vs. SSC-A.
3. Lymphocyte scatter gate — FSC-A vs. SSC-A.
4. Live cells — Zombie-UV negative.
5. CD3^{+} T cells.
6. CD4^{+}CD8^{-} and CD4^{-}CD8^{+} lineage split.
7. Within CD4^{+}: CD25^{hi}CD127^{lo} candidate Treg gate, confirmed against the CD25 and CD127 FMOs.
8. FoxP3^{+} confirmation within the candidate Treg gate.
9. CCR7 vs. CD45RA on the CD4^{+} and CD8^{+} populations for naïve / central-memory / effector-memory / effector subsetting.

5.2. Instrument QC

Check	Frequency & acceptance
Daily performance QC beads	Every run day, before samples; all detectors within ±10% of the rolling 30-day median MFI.
PMT voltration	On any voltage change, or monthly; peak channel resolution recorded and compared to baseline.
Reference control chart	Fully stained reference PBMC run alongside every batch; frequency of each subset plotted on a Levey-Jennings chart.
Compensation re-verification	Whenever a reagent lot changes, or every 90 days, whichever comes first.

Data Handling Note

Export compensated and uncompensated FCS files together. Analysts must be able to re-derive the compensation matrix from raw data if a spreading error is suspected after the fact — exporting only the compensated file makes that impossible.

6 Design Review & Sign-Off

Role	Name / signature	Date
Panel designed by	Dr. Elena Marsh, Senior Cytometrist	
Reviewed by	Dr. Farid Osei, Core Facility Director	
Compensation verified by	Priya Anand, Cytometry Specialist	
QC approved by	Dr. Elena Marsh	

Revision history: v1.0 initial 8-colour design (2025-11); v2.0 added BV711/CCR7 for memory subsetting (2026-03); v2.1 current — BUV737 replaced APC-Cy7 on CD3 to resolve UV-laser detector conflict (2026-07).

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