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Standardised flow cytometric protocol for the detection of immune cells subsets in breast cancer patients. V.2

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We use this protocol and it's working

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Abstract

The tumour microenvironment supports tumour growth in several ways including manipulating the immune response to cancer cells. Developing accurate and reproducible assays to detect immune and characterize immune cells in cancer patients is important for understanding their impact on tumour growth and development. Multicolour flow cytometry allows for characterization of many cell populations in a single sample. In this protocol, we optimized detecting several cytotoxic and suppressive immune cell subsets that may impact on patient outcomes. In addition, this protocol can be used to monitor changes in immune cells during and after chemotherapy.



Materials

For one sample, the following is required:

1 DURAClone IM phenotyping basic kit tube (Beckman Coulter, California, USA. B53309-25 tests)

1 DURAClone IM phenotyping Treg kit tube 1 (Beckman Coulter, California, USA. B53346-25 tests)

1 DURAClone IM phenotyping Treg kit tube 2 (Beckman Coulter, California, USA. B53346-25 tests)

🧴 5 µL CD127-AF700 liquid antibody (BioLegend, California, USA. 351344)

🧴 5 µL CD206-PB liquid antibody (BioLegend, California, USA. B36119)

🧴 5 µL HLA-DR-BV785 liquid antibody (BioLegend, California, USA. 307642)

🧴 30 µL Brilliant Violet stain buffer (BD Biosciences, New Jersey, USA. 563794)

🧴 9.5 mL 1 x PBS

🧴 50 µL Heat-inactivated FBS (ThermoFisher Scientific, Massachusetts, USA. 16140071)

🧴 5 µL PerFix-nc kit buffer 1 (Beckman Coulter, California, USA. B31168)

🧴 400 µL PerFix-nc kit buffer 2 (Beckman Coulter, California, USA. B31168)

🧴 3.5 mL PerFix-nc kit buffer 3 (diluted) (Beckman Coulter, California, USA. B31168)

🧴 2 mL VersaLyse solution (Beckman Coulter, California, USA. A09777)

Additional requirements:

CytoFLEX Flow Cytometer (Beckman Coulter, California, USA)

CytExpert (Beckman Coulter, California, USA)

Centrifuge

Vortex

Pipettes

Clean 5 ml flow cytometry tubes

VersaComp Compensation beads (Beckman Coulter, USA)

FlowSet Pro Standardisation beads (Beckman Coulter, USA)

Ready-to-use Daily Quality Control beads (Beckman Coulter, USA)

CytoFlex Sheath Fluid (Beckman Coulter, USA)

Deionised water

FlowClean cleaning agent (Beckman Coulter, USA)

Contrad 70 cleaning solution (Beckman Coulter, USA)

FlowJo™ analysis software (Beckton Dickinson, USA)



Before start

30 minutes before running a sample, allow the following to come to room temperature:

- 1x Phosphate Buffered Saline (PBS)
- Fetal Bovine Serum (FBS)
- Brilliant Violet stain buffer (BD Biosciences, New Jersey, USA)

Note:

- Samples must be centrifuged with the brake on and at room temperature.
- Samples must be incubated at room temperature.
- The reagent tubes, liquid antibodies, and other light-sensitive reagents must be kept in the dark as much as possible.

Flow cytometer start-up

- 1 Refill the sheath tank, empty the waste tank, and check that there is enough cleaning solution in the internal compartment of the flow cytometer.
- 2 Turn on the CytoFLEX flow cytometer and the PC and allow both to start up. Log in to the PC and open the CytExpert software.
- 3 Initiate the CytoFLEX daily start-up program and load a tube of deionised water when prompted.

Compensation set-up

- 4 Add two drops of positive and negative VersaComp beads into a clean 5 ml tube and to each compensation tube contained within the Basic and Treg immunophenotyping kits (Beckman Coulter, USA). If using a compensation product other than VersaComp beads, follow the product information booklet.
- 5 Add 10 μ l of CD206, HLA-DR and CD127 liquid antibodies into separate 5 ml tubes and add two drops of the negative and positive compensation beads into each.
- 6 Vortex each tube and incubate in the dark for 15 minutes at room temperature (RT).
- 7 Add 3 ml of 1x phosphate buffered saline (PBS) and centrifuge the tubes at 500 relative centrifugal force (RCF) for 5 minutes (brake off).
- 8 Discard the supernatant and resuspend the pellet in 600 μ l 1x PBS.
- 9 On the CytExpert software, select 'New Compensation' under 'File' and save. Select the channels you wish to run compensation for, select sample type and uncheck tubes not needed for the experiment. Select 'Ok'. The CytExpert software will create a list of matching empty tubes in the 'Tube' side panel as well as all required plots.
- 10 Load the correct unstained or single color fluorospheres as prompted and select 'Run' in the 'Acquisition' panel. Adjust the scatter gate if necessary. Use the slider scales so that the positive and negative peaks are suitably positioned, and adjust the positive and negative gates accordingly. Select 'Record'. Repeat for all tubes.

- 11 After running all tubes, generate the compensation matrix by selecting 'Compensation Calculation' from the toolbox or settings menu. Select 'Save As' to export the matrix as a .comp file and to save the values to the compensation library, select 'Save to Compensation Library' . Select 'Ok' and 'Close'.
- 12 To save or overwrite the compensation matrix to the library, select 'Open compensation experiment' and run the compensation controls. Once completed, calculate compensation. Then save the compensation matrix to the compensation library.
- 13 Run a 3 minute Flow Clean cleaning solution 3 minute deionised water daily clean before proceeding.

Daily quality control

- 14 To run daily Quality Control (QC), make sure that the lot-specific target value file for the CytoFLEX Ready to Use Daily QC fluorophores are loaded onto the CytExpert software.
- 15 Open the QC/Standardisation tab and select the CytoFLEX Ready to Use Daily QC fluorophores lot number from the relevant drop-down menu. Vortex the bottle of QC fluorophores for 10 seconds before adding 10 drops into a clean 5 ml tube or 34 drops into one well of a 96-well plate. Put the tube/plate into the sample loader and initialise the instrument, then select start.
- 16 If the QC step fails, try re-vortexing the QC fluorophores and re-running the QC/standardisation step. If it fails again, run a daily clean, prime and re-run. If it fails again, contact Beckman Coulter.
- 17 Do a 3 minute FlowClean cleaning solution 3 minute deionised water Daily Clean before proceeding.

Daily standardisation

- 18 To set up the daily standardisation experiment, follow the instructions in your standardisation beads' product information booklet. This protocol utilises Flow-Set Pro fluorospheres from Beckman Coulter (California, USA).
- 19 Vortex the beads and add 10 drops into a sterile tube. Open the Standardisation tab on CytExpert, select the relevant standardisation experiment, and acquire the tube.
- 20 Import the results into the gain acquisition settings for the basic and Treg experiment files.

- 21 Plot the high voltage and/or gain values for each parameter on its respective Levey-Jennings graph.
Note: 95% of values should fall within 2 standard deviations of the high voltage and/or gain ranges for each parameter. If values move outside of this range, conduct troubleshooting.
- 22 Do a 3 minute FlowClean cleaning solution 3 minute deionised water Daily Clean before proceeding.

Test procedure for Treg immunophenotyping panel

- 23 Label a DURAClone IM Phenotyping Treg kit tube 1, and add  5 µL CD127-AF700 liquid antibody to the tube.
- 24 Add  50 µL whole blood to reagent tube 1, and vortex for 10 seconds. Incubate the tube in the dark for 15 minutes at room temperature (RT).
- 25 Add  3 mL Phosphate Buffered Saline (PBS) to reagent tube 1 and centrifuge the tube at 500 RCF for 5 minutes.
- 26 Discard the supernatant, and gently vortex the cell pellet for 8 seconds.
- 27 Resuspend the cell pellet in  50 µL 100% heat-inactivated Fetal Bovine Serum (FBS)
- 28 Add  5 µL PerFix-nc buffer 1 and vortex for 8 seconds. Incubate the tube in the dark for 15 minutes at RT.
- 29 Add  400 µL PerFix-nc buffer 2 and vortex for 8 seconds.
- 30 Label a DURAClone IM Phenotypic Treg kit Tube 2, and transfer the contents of reagent tube 1 into reagent tube 2 and vortex for 10 seconds.
- 31 Incubate the tube in the dark for 60 minutes at RT.



- 32 Add  3 mL PBS and incubate in the dark for 15 minutes at RT.
- 33 Centrifuge the tube at 500 RCF for 5 minutes. Discard the supernatant and vortex the pellet for 8 seconds.
- 34 Resuspend the cell pellet in  3 mL DILUTED PerFix-nc buffer 3 and centrifuge the tube at 500 RCF for 5 minutes. Discard the supernatant and vortex the pellet for 8 seconds.
- 35 Resuspend the pellet in  500 μ L DILUTED PerFix-nc buffer 3 and acquire the results on the flow cytometer. Run a daily clean before running any DURAClone IM Phenotyping basic kit tubes.

Test procedure for basic immunophenotyping panel

- 36 Label a DURAClone IM Phenotyping Basic kit reagent tube, add the following, and then vortex for 8 seconds:
 -  5 μ L CD206-PB liquid antibody
 -  5 μ L HLA-DR-BV785 liquid antibody
- 37 Add  30 μ L Brilliant Violet Stain buffer then  100 μ L whole blood into the reagent tube, and vortex for 10 seconds. Incubate the tube in the dark for 15 minutes at RT.
- 38 Add  2 mL VersaLyse solution and vortex for 3 seconds. Incubate the tube in the dark for 15 minutes at RT.
- 39 Centrifuge the tube at 200 RCF for 5 minutes. Discard the supernatant, and resuspend the cell pellet in  3 mL PBS .
- 40 Centrifuge the tube at 200 RCF for 5 minutes, discard the supernatant, and resuspend the cell pellet in  500 μ L PBS . Acquire the results on the flow cytometer.

Post-acquisition

- 41 Save the Flow Cytometry Standard (FCS) files onto an external storage device.
- 42 Run a 3 minute FlowClean cleaning solution 3 minute deionised water and then shut down the Flow Cytometer and PC.



- 43 Import the FCS files into an analysis software such as FlowJo™ (Becton Dickinson, USA) and follow the gating strategies outlined in the sections below.

Gating strategy for the Treg immunophenotyping panel

- 44 Create a Forward Scatter height (FSC-H) vs. Forward Scatter Area (FSC-A) plot and gate singlet events (Fig. 1 A). Next, create a CD45 vs. Side Scatter (SSC) dot plot and gate the CD45+ lymphocytes (Fig. 1 B).
- 45 Create a CD4 vs. CD3 dot plot and gate the Helper T-cells (CD3+CD4+) (Fig. 1 C).
- 46 Using the gated Helper T-cell (CD3+CD4+) events, create the following dot plots:
- A CD4 vs. CD25 dot plot. Gate the CD4+CD25+ and CD4+CD25dim populations (Fig. 1 D).
 - A CD4 vs. CD39 dot plot. Gate the CD4+CD39+ population (Fig. 1 E).
 - A CD4 vs. CD45RA dot plot. Gate the CD4+CD45RA+ population (Fig. 1 F).
 - A CD4 vs. FoxP3 dot plot. Gate the CD4+FoxP3+ population (Fig. 1 G).
 - A CD4 vs. Helios dot plot. Gate the CD4+Helios+ population (Fig. 1 H).
 - A FoxP3 vs. Helios dot plot. Gate the FoxP3+Helios+ population (Fig. 1 I)

47

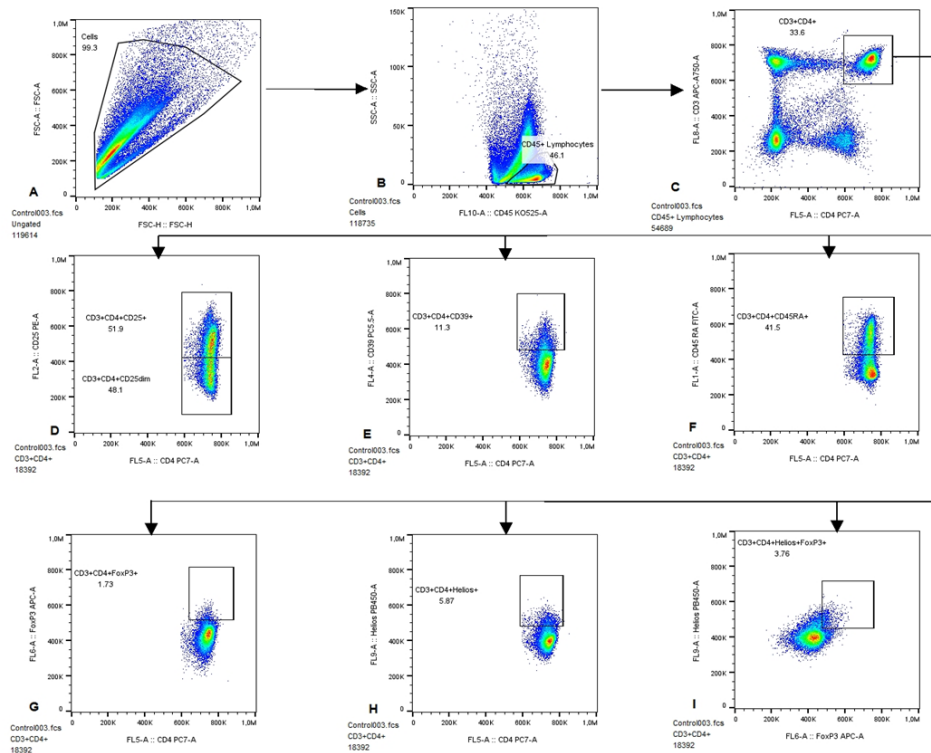


Figure 1: Gating strategy used to identify CD4+ T-cell subsets in study participant whole blood.

48 To identify five CD25+ and CD25dim CD4+ T-cell subsets, create the following plots for each:

- A CD4 vs. CD39 dot plot. Gate the CD39+ population (Fig. 1 J and O).
- A CD4 vs. CD45RA dot plot. Gate the CD45RA+ population (Fig. 1 K and P).
- A CD4 vs. FoxP3 dot plot. Gate the FoxP3+ population (Fig. 1 L and Q).
- A CD4 vs. Helios dot plot. Gate the Helios+ population (Fig. 1 M and R).
- A FoxP3 vs Helios dot plot. Gate the FoxP3+Helios+ population (Fig. 1 N and S).

49 CCD25+ and CD25dim

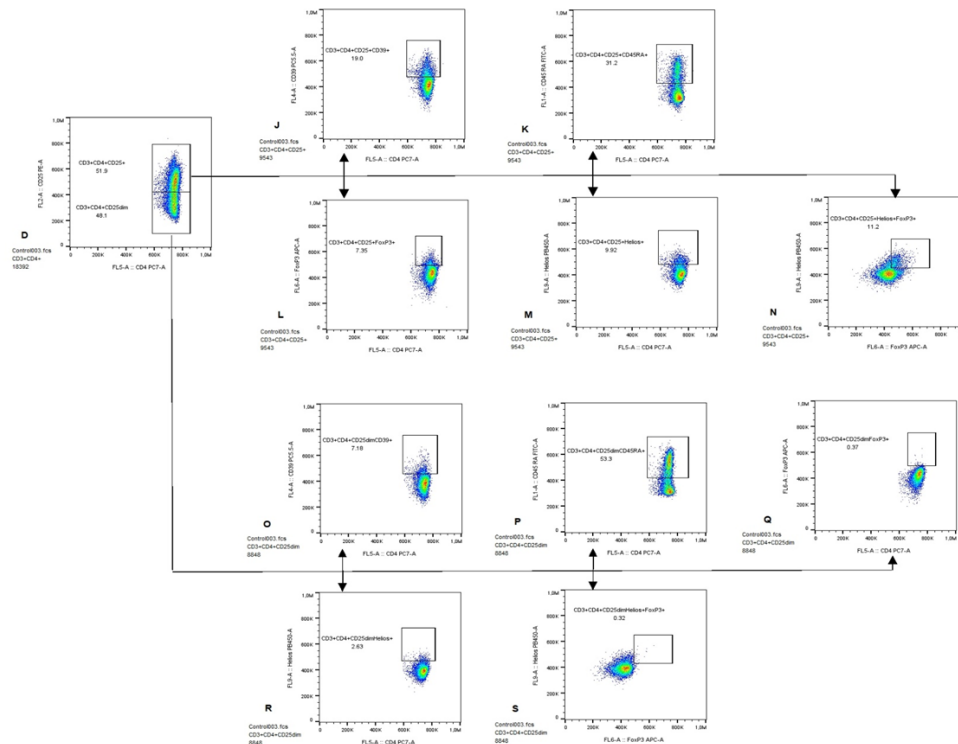


Figure 1 continued: Gating strategy used to identify CD25+ and CD25dim CD4+ T-cell subsets in study participant whole blood.

- 50 To further improve the characterisation of cells detected using this panel, determine the MFI of CD127 for all populations.

Gating strategy for the basic immunophenotyping panel

- 51 Create a Forward Scatter Height (FSC-H) vs. Forward Scatter Area (FSC-A) plot and gate singlet events (Fig. 2 A). Next, create a CD45 vs. side scatter (SSC) dot plot and gate the CD45+ leukocytes (Fig. 2 B).

52

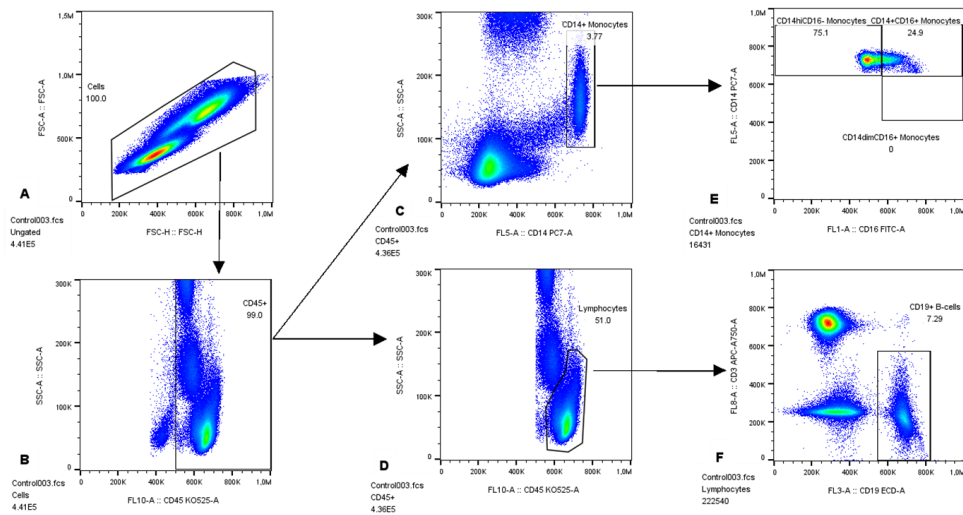


Figure 2: Gating strategy used to identify T-cell, B-cell, NK cell, and monocyte subsets in study participant whole blood.

- 53 Create a CD14 vs. SSC-A dot plot and gate the CD14+ cells (monocytes) (Fig. 2 C). Next, create a CD45 vs. SSC-A dot plot and gate the CD45+ lymphocytes (Fig. 2 D).
- 54 To identify classical (CD14+CD16-), intermediate (CD14+CD16+), and non-classical (CD14dimCD16+) monocytes, create a CD16 vs. CD14 dot plot, apply the gated CD14+ events, and gate the three subsets as shown in figure 2 plot E.
- 55 To identify B-lymphocytes, create a CD19 vs. CD3 dot plot, apply the CD45+ lymphocytes gate, and gate CD19+CD3- events (Fig. 2 F).
- 56

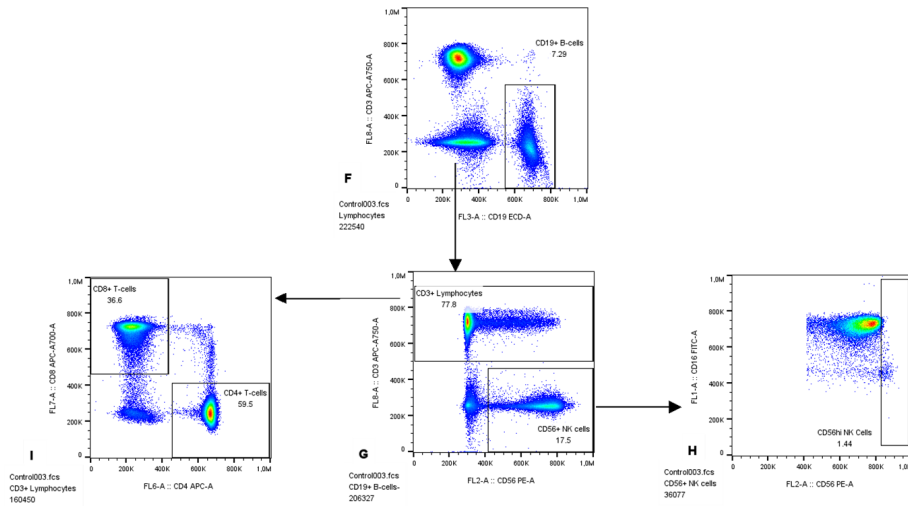


Figure 2 continued: gating strategy for the identification of T-cell, B-cell, NK cell, and monocyte subsets in participant peripheral blood.

- 57 Use a Boolean gate to exclude B-cells from the rest of the gating strategy, then create a CD56 vs. CD3 dot plot and gate CD3+ Lymphocytes and CD56+ NK cells (Fig. 2 G).
- 58 Using the gated CD3+ lymphocytes, create a CD4 vs. CD8 dot plot and identify the cytotoxic (CD4-CD8+) and helper (CD4+CD8-) T-cells (Fig. 2 I).
- 59 To identify a cytokine-producing NK subset, create a CD56 vs. CD16 dot plot, apply the CD56+ NK cell population, and gate the CD56high NK cells (Fig. 2 H).
- 60 Record the mean fluorescence intensity (MFI) of CD206 on B-cells; CD56, CD206, and HLA-DR on monocytes; and CD16 and HLA-DR on NK cells.

Protocol references

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