# Standardised flow cytometric protocol for the detection of immune cells subsets in breast cancer patients. V.1

Nicole Chicken[1], Pieter Meyer[2], Ronald Anderson[3], Catherine Bernardo Rapoport[4], Worsley[2]

[1]Department of Medical Immunology, University of Pretoria;
[2]Department of Medical Immunology, University of Pretoria, National Health Laboratory Service;
[3]Department of Medical Immunology, University of Pretoria.;
[4]Department of Medical Immunology, University of Pretoria, Medical Oncology Centre of Rosebank.

Nicole Chicken

VERSION 1

JAN 17, 2024

OPEN ACCESS

**DOI:** dx.doi.org/10.17504/protocols.io.14egn379ml5d/v1

**Protocol Citation:** Nicole Chicken, Pieter Meyer, Ronald Anderson, Bernardo Rapoport, Catherine Worsley 2024. Standardised flow cytometric protocol for the detection of immune cells subsets in breast cancer patients. . **protocols.io** https://dx.doi.org/10.17504/protocols.io.14egn379ml5d/v1



**Protocol status:** Working
We use this protocol and it's working

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.

**Created:** Jul 25, 2023

**Last Modified:** Jan 17, 2024

**PROTOCOL integer ID:** 85451

**Keywords:** Breast cancer, Flow cytometry, Tumour microenvironment, Tregs, Monocytes, NK cells, B-cells

**Funders Acknowledgement:**
National Research Foundation Thuthuka 2023
Grant ID: TTK2203291110

## MATERIALS

**For one sample, the following is required:**

2 EDTA Vacutainer® tubes (BD Biosciences, New Jersey, USA)
1 sterile Pasteur pipette (Merck, Darmstadt, Germany)
1 15mL Corning® centrifuge tube with conical bottom (Merck, Darmstadt, Germany)
2 sterile 1.5mL tubes (Merck, Darmstadt, Germany)
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 and CytoBank software (Beckman Coulter, California, USA)
Ready-to-use Daily Quality Control beads (Beckman Coulter, California, USA. C65719)

BEFORE START INSTRUCTIONS

**30 minutes before running a sample, allow the following to come to room temperature:**
-1x Phosphate Buffered Saline (PBS)
-Fetal Bovine Serum (FBS) (ThermoFisher Scientific, Massachussetts, USA)
-Brilliant Violet stain buffer (BD Biosciences, New Jersey, USA.563794)

**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 and daily quality control[1]

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.

4 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.

5 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 5mL 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.

6 If the QC step fails, try vortexing the aliqoted QC fluorphores and re-running the QC/standardisation step. If it fails again, run a daily clean and prime and re-run. If it fails again, contact Beckman Coulter.

7 Do a 3 minute FlowClean 3 minute deionised water Daily Clean before proceeding.

## Compensation set-up[1]

8 First prepare the required unstained and single color controls using compensation beads or cells. Then 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 panel as well as all required plots.

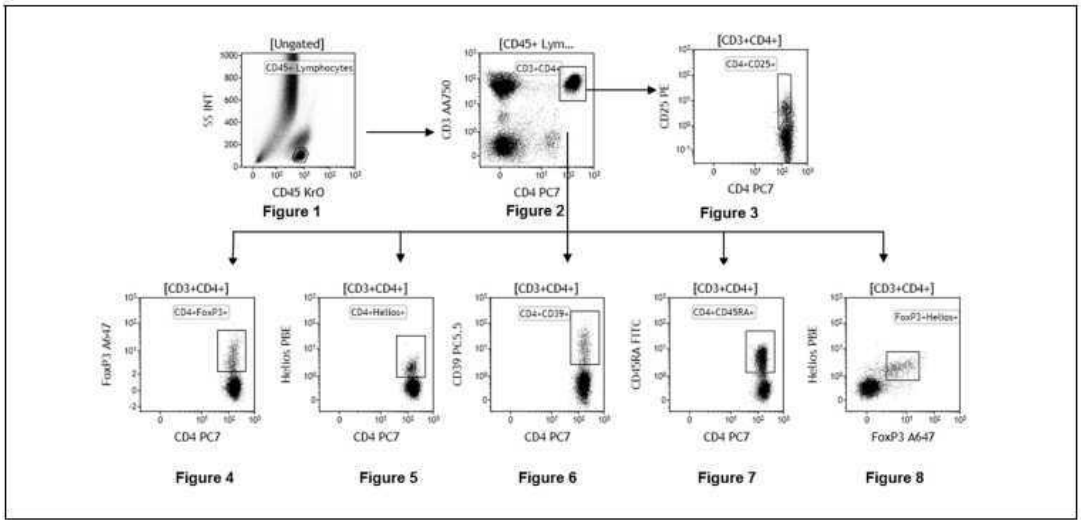
9 Load the correct unstained or single color sample fluorospheres or cells and select 'Run' in the Acquisition panel. Adjust the scatter gate if necessary. Use the slider scales so that the positive and negative peaks are suitably positioned, and adjust the positive and negative gates accordingly. Select 'Record'. Repeat for all tubes.

10 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'.

11 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. Run a 3 minute Flow Clean 3 minute deionised water daily clean before proceeding.

## Setting up the gating strategy for the Treg kit[2]

12 Create an analysis protocol on the CytExpert software and insert a series of dual parameter plots. Make sure to set the discriminator on the forward scatter (FS) parameter/axis low enough to prevent the exclusion of any lymphocytes from the data acquisition.




The gating strategy for the DuraClone IM Treg (Beckman Coulter, California, USA) kit. Image taken from reference [2] .

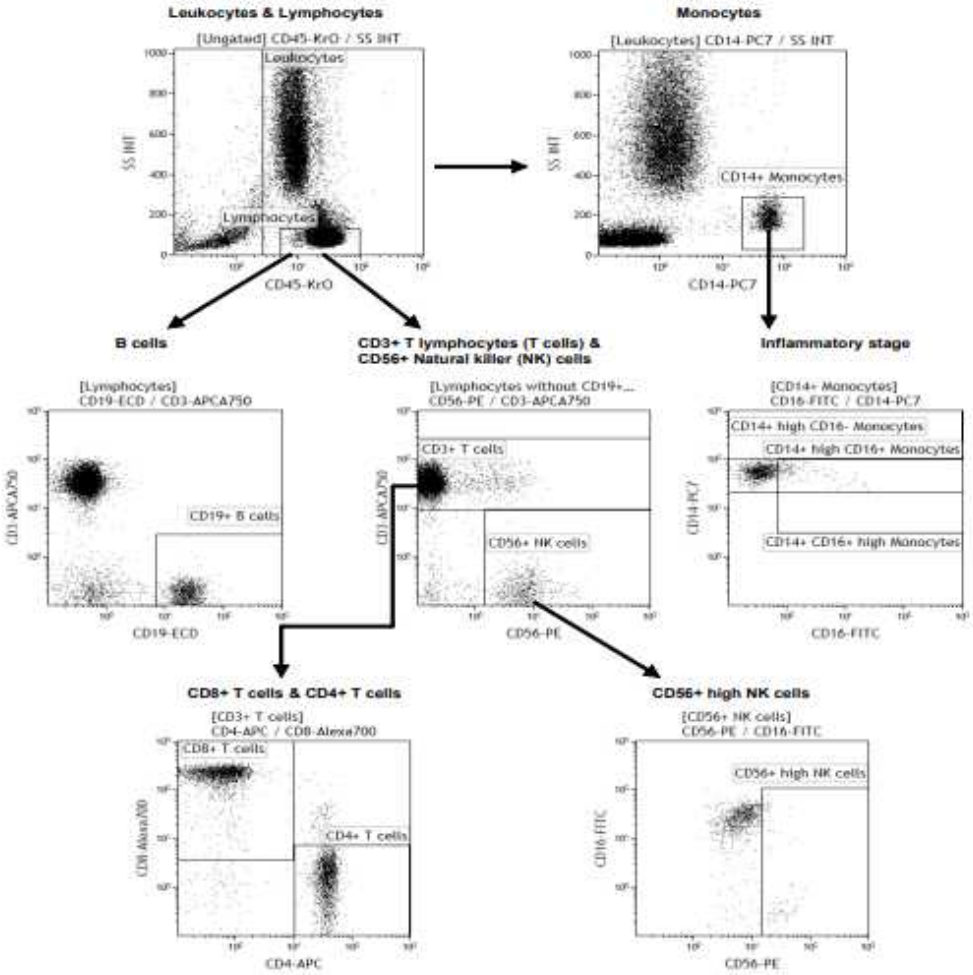
13 Create a CD45-Kr0 vs. Side scatter (SSC) dot plot and apply the leukocyte gate. Next, create a gate around the CD45+ lymphocytes. (Figure 1)

14 Create a CD4-PC7 vs. CD3-AA750 dot plot and gate the CD3+CD4+T-cells. (Figure 2)

15 Create the following dot plots, and apply the CD3+CD4+ gate to each:
- A CD4-PC7 vs. CD25-PE dot plot. Gate the CD4+CD25+ population. (Figure 3)
-A CD4-PC7 vs. FoxP3-A647 dot plot. Gate the CD4+FoxP3+ population. (Figure 4)
-A CD4-PC7 vs. Helios-PBE dot plot. Gate the CD4+Helios+ population. (Figure 5)
-A CD4-PC7 vs. CD39-PC5.5 dot plot. Gate the CD4+CD39+ population. (Figure 6)
-A CD4-PC7 vs. CD45RA-FITC dot plot. Gate the CD4+CD45RA+ population. (Figure 7)
-A FoxP3-A647 vs. Helios-PBE dot plot. Gate the FoxP3+Helios+ population. (Figure 8)

## Setting up the gating strategy for the Basic kit [3]

16 Create an analysis protocol on the CytExpert software and insert a series of dual parameter plots. Make sure to set the discriminator on the forward scatter (FS) parameter/axis low enough to prevent the exclusion of any lymphocytes from the data acquisition.




The gating strategy for the DURAClone IM basic (Beckman Coulter, California, USA) kit. ube. Image taken from reference [3] .

**17** Create a CD45-KrO vs. side scatter (SSC) dot plot and create a gate around the CD45+ lymphocytes.

**18** Create three plots as outlined below:
- A CD14-PC7 vs. SSC dot plot. Gate the CD14+ cells (monocytes).
-A CD19-ECD vs. D3-APC-A750 dot plot. Gate the CD19+ cells (B-cells).
-A CD56-PE vs. CD3-APC-A750 dot plot. Create and apply a Boolean gate "Lymphocytes AND (NOT CD19+)". Gate the CD56+ (Natural Killer (NK) cell) and CD3+ (CD3+ T-cells) cell populations.

**19** Create a CD56-PE vs. CD16-FITC dot plot. Apply the gate from the CD56-PE vs. CD3-APC-A750 dot plot. Gate the CD56+ high NK cells.

**20** Create a CD16-FITC vs. CD14-PC7 dot plot. Apply the CD14+ gate to this plot, and create separate gates

around each of the following populations:
- CD14+ high CD16- monocytes
-CD14+ high CD16+ monocytes
-CD14+CD16+ high monocytes

21 Create a CD4-APC vs. CD8-A700 dot plot. Apply the CD3+ T-cells gate from the CD56-PE vs. CD3-AA750 dot plot. Gate the CD4+ T-cells, and then the CD8+ T-cells.

22 Record the mean fluorescence intensity (MFI) of all gated populations.

## DURAClone IM Phenotyping Treg kit test protocol for 1 sample[2]

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 xg 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 xg 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 xg for 5 minutes. Discard the supernatant and cortex the pellet for 8 seconds.

35 Resuspend the pellet in 500 µL 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.

## DURAClone IM Phenotyping Basic kit protocol for 1 sample[3]

36 Label a DURAClone IM Phenotyping Basic kit reagent tube, add the following, and then vortex for 8 seconds:

- 5 µL CD206-PB Liquid antibody
- 5 µL HLA-DR-BV785 liquid antibody

37 Add 30 µL Brilliant Violet Stain buffer then 100 µL 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 xg for 5 minutes. Discard the supernatant, and resuspend the cell pellet in 3 mL PBS .

40 Centrifuge the tube at 200 xg for 5 minutes, discard the supernatant, and resuspend the cell pellet in 500 µL PBS . Acquire the results on the flow cytometer.

## Post-acquisition

41 Once all events have been acquired, copy all work to an external storage device by selecting 'Export FCS files' from the 'File' menu, choosing the tubes you want to save, selecting your desired file path and selecting 'Ok'.

42 Run a daily clean, close CytExpert, and shut down the PC and flow cytometer.