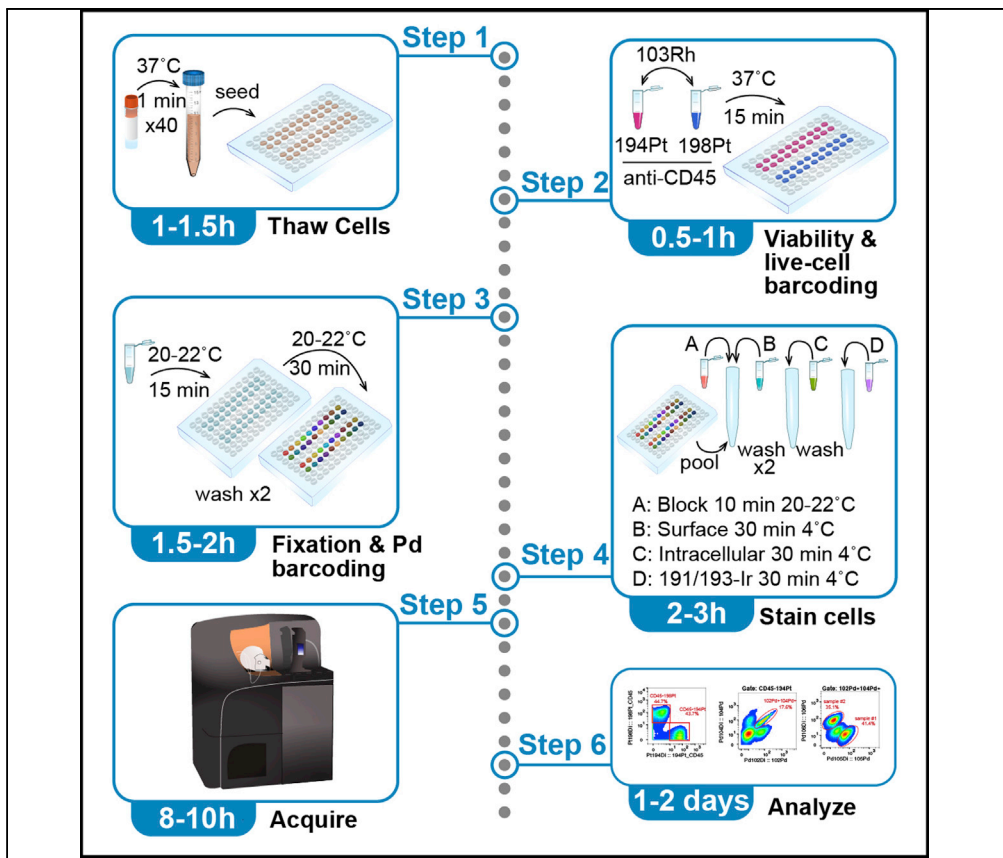# STAR Protocols

## Protocol
# Mass cytometry immunostaining protocol for multiplexing clinical samples

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## Highlights

Mass cytometry immunostaining for 40+ samples to reduce batch-to-batch variation

Barcoding samples before immunostaining ensures consistency across all samples

Reduction in antibody volume consumption and data acquisition time

This is a cytometry by time-of-flight (CyTOF) staining protocol for hematopoietic-derived cells, that leverages live-cell barcoding using receptor-type tyrosine-protein phosphatase C (CD45) antibodies conjugated to metal isotopes in combination with DNA-based palladium barcoding to multiplex up to 40 samples. In this protocol, DNA-based barcoding is performed before surface and intracellular immunostaining, which reduces the batch effects that result from day-to-day variations in staining and instrument sensitivity. This protocol also reduces antibody consumption and eliminates the need for repeated instrument adjustment.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

# STAR Protocols

**Protocol**

# Mass cytometry immunostaining protocol for multiplexing clinical samples

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## SUMMARY

This is a cytometry by time-of-flight (CyTOF) staining protocol for hematopoietic-derived cells, that leverages live-cell barcoding using receptor-type tyrosine-protein phosphatase C (CD45) antibodies conjugated to metal isotopes in combination with DNA-based palladium barcoding to multiplex up to 40 samples. In this protocol, DNA-based barcoding is performed before surface and intracellular immunostaining, which reduces the batch effects that result from day-to-day variations in staining and instrument sensitivity. This protocol also reduces antibody consumption and eliminates the need for repeated instrument adjustment.

## BEFORE YOU BEGIN

This CyTOF staining protocol describes the steps for barcoding and staining human immune cells, including peripheral blood mononuclear cells (PBMCs) and single-cell suspensions isolated from human tissues and fluid samples, using titrated metal-tagged antibodies.

### Institutional approvals

Ensure that all institutional approvals have been obtained prior to working with human specimens. This protocol includes examples using data generated from a human peripheral blood leukopak (institutional approval #56878).

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| CD16 | Fluidigm | 3209002B |
| CD80 | BD Biosciences | 55568 |
| CD45RA | BioLegend | 304143 |
| Helios | BioLegend | 137202 |
| Ki67 | BioLegend | 350523 |
| T-bet | Fluidigm | 3161014B |
| CD45 | Fluidigm | 3089003B |
| CD45 | Fluidigm | 3198001B |
| CD28 | BioLegend | 302937 |
| CD137 | BioLegend | 309802 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| CD86 | BioLegend | 555655 |
| TIM3 | BioLegend | 345019 |
| PKR | Novus Biologicals | Custom-made |
| CD73 | Fluidigm | 3168015B |
| CTLA4 | Thermo Fisher Scientific | 14-1529-82 |
| CD39 | BioLegend | 328221 |
| CD3 | BioLegend | 300443 |
| CD303 | BioLegend | 354215 |
| CD14 | BioLegend | 301802 |
| CD27 | BioLegend | 302839 |
| CD25 | BioLegend | 356102 |
| PD1 | Fluidigm | 3165039D |
| IL-10 | BioLegend | 501423 |
| SOCS1 | Millipore | 04-002 |
| PDL1 | Fluidigm | 3175008B |
| HLA-DR | Fluidigm | 307651 |
| ICOS | BD Biosciences | 557801 |
| CD163 | BioLegend | 333602 |
| CD8 | BioLegend | 301053 |
| CD15 | BioLegend | 323002 |
| CD103 | Thermo Fisher Scientific | 14-1038-82 |
| CD4 | BioLegend | 300541 |
| FOXP3 | Thermo Fisher Scientific | 14-4777-82 |
| CD11c | BioLegend | 337221 |
| CD69 | BioLegend | 310939 |
| CD19 | BioLegend | 302202 |
| TCRgd | Thermo Fisher Scientific | TCR1061 |
| Granzyme B | Thermo Fisher Scientific | MA1-80734 |
| CD127 | Thermo Fisher Scientific | 14-1278-82 |
| NKp46 | BioLegend | 331902 |
| TCF-1 | BD Biosciences | Custom-made |
| LAG3 | BioLegend | 369202 |
| CD45RO | BioLegend | 304239 |
| **Biological samples** | | |
| Human Peripheral Blood Leukopak, Fresh | STEMCELL Technologies | 70500 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Maxpar® 10× Barcode Perm Buffer | Fluidigm | 201057 |
| Maxpar® PBS | Fluidigm | 201058 |
| RPMI 1640 Medium | Gibco | 11875093 |
| Cell-ID™ Intercalator-103Rh 500 μM | Fluidigm | 201103A |
| Cisplatin | BioVision | 1550-1000 |
| Cell-IDTM Intercalator-Ir-125 μM | Fluidigm | 201192A |
| eBioscience FOXP3/Transcription Factor staining buffer kit | Thermo Fisher Scientific | 00-5523-00 |
| Maxpar® Fix and Perm Buffer—100 mL | Fluidigm | 201067 |
| PBS, Ca2+ and Mg2+-free, pH 7.2 | Gibco | 20012-050 |
| 16% Formaldehyde, methanol-free, Ultrapure | Analychem Corporation | 18814-20 |
| Albumin bovine serum, BSA | Sigma-Aldrich | 12657-25GM |
| Fc Receptor Binding Inhibitor Polyclonal Antibody, Fc blocker | Invitrogen | 16-9161-73 |
| **Critical commercial assays** | | |
| Cell-IDTM 20-plex Pd Barcoding Kit | Fluidigm | 201060 |
| FlowJo v10 | FlowJo | https://www.flowjo.com/solutions/flowjo/downloads |
| Cytobank | Cytobank Inc. | https://premium.cytobank.org |

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***Continued***

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| **Other** | | |
| 0.1 µm pore size, hydrophilic PVDF, 0.5 mL volume | MilliporeSigma | UFC30VV25 |
| 12 × 75 mm Tubes, Polystyrene | Diamed | STK 8550 |
| Falcon® 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap | Corning | 352235 |
| 96 Well Conical V-Bottom Plate | Thermo Fisher Scientific | 277143 |
| Sterile Multichannel Disposable Solution Basins | | |
| Centrifuge | | |
| Recently calibrated P10, P20, P200, and P1000, and a P200 multichannel pipette | | |
| CO2 incubator | | |
| Hemocytometer | | |
| Biological safety cabinet | | |
| Vacuum aspiration system | | |
| Fluidigm Helios™ Mass Cytometer | Fluidigm | |

## MATERIALS AND EQUIPMENT

| CyTOF staining media (CSM) | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| FBS | 2% | 10 mL |
| PBS | N/A | 490 mL |
| Sodium azide* | 0.5% | 0.1 g |
| Total | N/A | 500 mL |

Store at 4°C.

*Sodium azide is highly toxic if ingested or inhaled, and is a skin irritant. Use personal protective equipment when dispensing and only handle powder under a fume hood.

> *Alternatives:* Naturally abundant cis-platin can be used as an alternative to Cell-ID intercalator-Rh to label dead cells. However if used, isotopically pure platinum conjugated antibodies cannot be included. In addition, cis-platin is a known carcinogen/mutagen and if used should be handled with care.

| Alternative CyTOF Staining Media (CSM) | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| BSA | 1% | 150 g |
| Sodium azide* | 0.5% | 25 g |
| PBS | N/A | 500 mL |
| Total | N/A | 500 mL |

*Sodium azide is highly toxic if ingested or inhaled and is a skin irritant. Use personal protective equipment when dispensing and only handle powder under a fume hood.

| Alternative DNA staining buffer | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Cell-ID™ intercalator-Ir (500 µM) | 100 nM | 1 µL |
| 16% formaldehyde (frozen aliquots) | 1.6% | 0.5 mL |
| 0.3% saponin (filtered 1.5 g in 500 mL PBS) | N/A | 4.5 mL |
| Total | N/A | 5 mL |

Prepare fresh on day of staining.

Once opened, 16% formaldehyde should be stored at −20°C in 0.5 mL or 1 mL aliquots. Thaw aliquots as much as needed for the experiment. Thawed aliquots or leftover should not be refrozen and used.

## STEP-BY-STEP METHOD DETAILS

### The day before staining

We recommend performing experiments over two consecutive days, as the protocol is lengthy and could be exhausting. In the first day, samples, buffers, regents, and labels are prepared as follows.

⏲ Timing: 2–3 h

Collect sample vials in a separate box for quick retrieval on the day of staining. Keep samples in liquid nitrogen storage until ready to begin the protocol. Cells were cryopreserved as described by Bull et al. (2007).

Prepare tubes, labels, buffers, and antibody mixtures as described below.

Fill pre-labeled 15 mL tubes with 5 mL of pre-warmed thawing media – prepared as described below. This is for resuspending the thawed samples. The number of tubes depends on the number of samples to be stained (up to 40).

Prepare antibody master mixes as described in step 2 below and store them at 4°C.

Prepare the barcode table, where each sample will be assigned to one CD45- Pt (either 194Pt or 198Pt) and one palladium code from the Cell-ID™ 20-plex Pd barcoding kit, as shown in Table 1.

1. Preparing buffers and reagents.
   a. CyTOF staining media (CSM): sterile PBS supplemented with 2% FBS and 0.5% sodium azide. Store at 4°C.
   b. Thawing media: RPMI containing 5% FBS. Incubate in 37°C water bath.
2. Preparing antibody master mixes.

*Prior to preparing staining master mixes, subtract the total volume of antibodies that will be added from the desired final volume of master mix to determine the amount of diluent (CSM or permeabilization buffer) to use.
   a. Surface stain – <u>master mix</u> 1: Prepare a 2× mix in CSM including all the pre-titrated, pre-conjugated extracellular antibodies in Table 2.
      i. Prepare a 1.5 mL Eppendorf tube with the volume of CSM necessary to achieve a 2× antibody concentration. Place the individual antibody vials and the Eppendorf on ice. Arrange the vials in the order of the antibody list.
      ii. Add the first antibody to the Eppendorf tube to reach the desired concentration. A final volume of 50 μL is sufficient for staining up to 10 × 10$^6$ PBMCs. For highly multiplexed samples, a master mix volume of 150 μL is recommended. Prepare 25 μL of extra master mix to account for any pipettor error.

      For example, to make a 2× anti-CD3 that has an optimal dilution of 1:50 in a final volume of 175 μL, add 7 μL of conjugated anti-CD3 antibody to 40.25 μL of CSM. Add the rest of the antibodies similarly. See Table 2.
      iii. Once added, transfer the vial to another ice bucket and check it off of the antibody list. This helps keep track of the antibodies that have been added and prevents errors in repetition or omission.
      iv. Repeat steps 2.1.2 and 2.1.3 until all surface antibodies have been added.
      v. Store master mix 1 at 4°C and vortex before use.
   b. Intracellular stain – <u>master mix 2</u>: Prepare a 1× mix in 1× permeabilization buffer according to the pre-determined optimal dilutions for each of the intracellular antibodies in Table 3.

**Table 1. Barcodes table**

| Sample# | CD45 | Barcode ID | Sample ID |
|---|---|---|---|
| 1 | 194-Pt | Barcode 1 | |
| 2 | 194-Pt | Barcode 2 | |
| 3 | 194-Pt | Barcode 3 | |
| 4 | 194-Pt | Barcode 4 | |
| 5 | 194-Pt | Barcode 5 | |
| 6 | 194-Pt | Barcode 6 | |
| 7 | 194-Pt | Barcode 7 | |
| 8 | 194-Pt | Barcode 8 | |
| 9 | 194-Pt | Barcode 9 | |
| 10 | 194-Pt | Barcode 10 | |
| 11 | 194-Pt | Barcode 11 | |
| 12 | 194-Pt | Barcode 12 | |
| 13 | 194-Pt | Barcode 13 | |
| 14 | 194-Pt | Barcode 14 | |
| 15 | 194-Pt | Barcode 15 | |
| 16 | 194-Pt | Barcode 16 | |
| 17 | 194-Pt | Barcode 17 | |
| 18 | 194-Pt | Barcode 18 | |
| 19 | 194-Pt | Barcode 19 | |
| 20 | 194-Pt | Barcode 20 | |
| 21 | 198-Pt | Barcode 1 | |
| 22 | 198-Pt | Barcode 2 | |
| 23 | 198-Pt | Barcode 3 | |
| 24 | 198-Pt | Barcode 4 | |
| 25 | 198-Pt | Barcode 5 | |
| 26 | 198-Pt | Barcode 6 | |
| 27 | 198-Pt | Barcode 7 | |
| 28 | 198-Pt | Barcode 8 | |
| 29 | 198-Pt | Barcode 9 | |
| 30 | 198-Pt | Barcode 10 | |
| 31 | 198-Pt | Barcode 11 | |
| 32 | 198-Pt | Barcode 12 | |
| 33 | 198-Pt | Barcode 13 | |
| 34 | 198-Pt | Barcode 14 | |
| 35 | 198-Pt | Barcode 15 | |
| 36 | 198-Pt | Barcode 16 | |
| 37 | 198-Pt | Barcode 17 | |
| 38 | 198-Pt | Barcode 18 | |
| 39 | 198-Pt | Barcode 19 | |
| 40 | 198-Pt | Barcode 20 | |

i. A final volume of 100 μL is sufficient for staining up to $10 \times 10^6$ PBMCs. For highly multiplexed samples, a master mix volume of 300 μL is recommended. Prepare 25 μL of extra master mix to account for any pipettor error.

ii. Prepare a 1.5 mL Eppendorf tube as in 2.1.1 with the volume of permeabilization buffer needed to achieve a 1× concentration.

iii. Add antibodies as in 2.1.2–2.1.4.

iv. Store master mix 2 at 4°C and vortex before use.

*Note:* A- The optimal dilutions of all the metal-tagged antibodies used in these protocol were determined through serial dilutions testing as described by van Vreden et al. (2019).

**Table 2. Dilution of extracellular antibodies**

| Tag | Marker | 2× dilution | Antibody volume (ul) |
|---|---|---|---|
| 141 Pr | CD45RA | 200 | 1.75 |
| 112 Cd | CD45RO | 200 | 1.75 |
| 152 Sm | CD11c | 200 | 1.75 |
| 158 Gd | CD27 | 200 | 1.75 |
| 159 Tb | CD19 | 200 | 1.75 |
| 162 Dy | CD28 | 200 | 1.75 |
| 172 Yb | CD127 | 200 | 1.75 |
| 142 Nd | HLA-DR | 100 | 3.5 |
| 144 Nd | CD163 | 100 | 3.5 |
| 146 Nd | CD8 | 100 | 3.5 |
| 147 Sm | CD4 | 100 | 3.5 |
| 151 Eu | CD39 | 100 | 3.5 |
| 154 Sm | CD69 | 100 | 3.5 |
| 156 Gd | CD14 | 100 | 3.5 |
| 160 Gd | CD25 | 100 | 3.5 |
| 165 Ho | PD1 | 100 | 3.5 |
| 168 Er | CD73 | 100 | 3.5 |
| 173 Yb | NKp46 | 100 | 3.5 |
| 175 Lu | PDL1 | 100 | 3.5 |
| 166 Er | TIM3 | 100 | 3.5 |
| 153 Eu | CD3 | 50 | 7 |
| 111 Cd | CD80 | 50 | 7 |
| 143 Nd | ICOS | 50 | 7 |
| 148 Nd | CD15 | 50 | 7 |
| 150 Nd | CD103 | 50 | 7 |
| 155 Gd | CD303 | 50 | 7 |
| 163 Dy | CD137 | 50 | 7 |
| 164 Dy | CD86 | 50 | 7 |
| 169 Tm | TCRgd | 50 | 7 |
| 176 Yb | LAG3 | 50 | 7 |
| 209 Bi | CD16 | 50 | 7 |
| | Total volume of Abs | | 134.75 |
| | CSM volume | | 40.25 |

B- We highly recommend that the performance of the panel should be examined first on control samples prior to proceeding to valuable clinical samples.

### On day of staining

The major steps on the second day are staining cells with viability marker, surface and intracellular markers to be able to identify cell types and protein expression.

⏱ Timing: 6 h

3. Prepare the following buffers. Store at 4°C and vortex before use.
   a. 1× Maxpar perm barcode buffer: dilute 10× Maxpar barcode buffer using Maxpar PBS.
   b. Fixation/permealization buffer: add fixation/permeabilization concentrate from eBioscience FOXP3/transcription factor staining buffer kit to the fixation/permeabilization diluent at dilution 1:3 (e.g., 100 µL of the fixation concentrate to 300 µL of the dilutent).
   c. Fc blocker: add 50 µL of Fc receptor binding inhibitor to 450 µL CSM.
   d. Viability buffer: add 8 µL Cell-ID™ intercalator – 103Rh to 4 mL of RPMI.
   e. 1× Permeabilizaiton buffer: add 1 mL of 10× Permeabilization Buffer from eBioscience FOXP3/transcription Factor Staining Buffer Kit to 9 mL of ddH2O.

**Table 3. Dilution of Intracellular antibodies**

| Tag | Marker | 2× dilution | Antibody volume (ul) |
|---|---|---|---|
| 113 In | IL-10 | 200 | 1.625 |
| 116 Cd | Ki67 | 200 | 1.625 |
| 170 Er | CTLA4 | 200 | 1.625 |
| 171 Yb | Granzyme B | 100 | 3.25 |
| 114 Cd | Helios | 50 | 6.5 |
| 115 In | SOCS1 | 50 | 6.5 |
| 149 Sm | FOXP3 | 50 | 6.5 |
| 161 Dy | T-bet | 50 | 6.5 |
| 167 Er | PKR | 50 | 6.5 |
| 174 Yb | TCF-1 | 50 | 6.5 |
| | Total volume of Abs | | 47.125 |
| | Permeabilization buffer | | 277.875 |

    f. <u>DNA staining buffer:</u> prepare 1 mL of iridium-DNA intercalation solution by adding 1 μLCell-ID™ intercalator-Ir (125 μM) to 1 mL of Maxpar fix and perm buffer.

4. Antibody master mix filtration:
   a. Add each antibody master mix into a separate 0.1 μm Millipore centrifuge tube.
   b. Spin the centrifuge tubes with a fixed-angle centrifuge rotor at 12,000 × g, 4°C for 2 min.
   c. Discard the Millipore filter and store the tubes at 4°C.
5. Thawing cells:
   a. Bring a bucket filled with dry ice and another bucket filled with regular ice to the 37°C water bath.
   b. Place sample vials on the dry ice and place the pre-labeled 15 mL tubes in the ice bucket.
   c. Thaw samples one at a time by placing vial on a rack in the 37°C water bath for approximately 1 min.
   d. Quickly transfer the contents of the freshly thawed vial into the appropriate pre-labeled tube filled with 5 mL thawing media and leave it on ice.
   e. Centrifuge the sample tubes at 600 × g, 4°C for 5 min.
   f. Aspirate the supernatant.
   g. If the samples cell count were not previously performed and recorded prior to cryopreservation, count each sample manually using a hemocytometer and record the viable cell counts.
   h. Resuspend cell pellets in 100 μL thawing media again and transfer it to a 96-well V-bottom plate*.

*Notes:

A- Ideally, reserve up to 2 million cells from each sample for staining. Expect ~30%–50% cell loss during the staining process. Note, we have stained as few as 10,000 cells from a single sample (Gadalla et al., 2019).

B- Arrange samples in the plate so that there are 10 samples in each row, and each barcode set is separated from the other to avoid mixing up staining (Figure 1A).

    i. Centrifuge the plate at 600 × g, 4°C for 3 min. Discard supernatant.
6. Viability stain and live-cell barcoding:
   a. For 40 samples experiment, split the 4 mL of 103Rh viability buffer in two 2 mL Eppendorf tubes.
   b. Add 40 μL of CD45-194Pt to one of the 103Rh-viability buffer tubes (dilution 1:50), and 40 μL of CD45-198Pt to the other 103Rh-viability buffer tube. These antibodies were pretitrated for optimal performance.
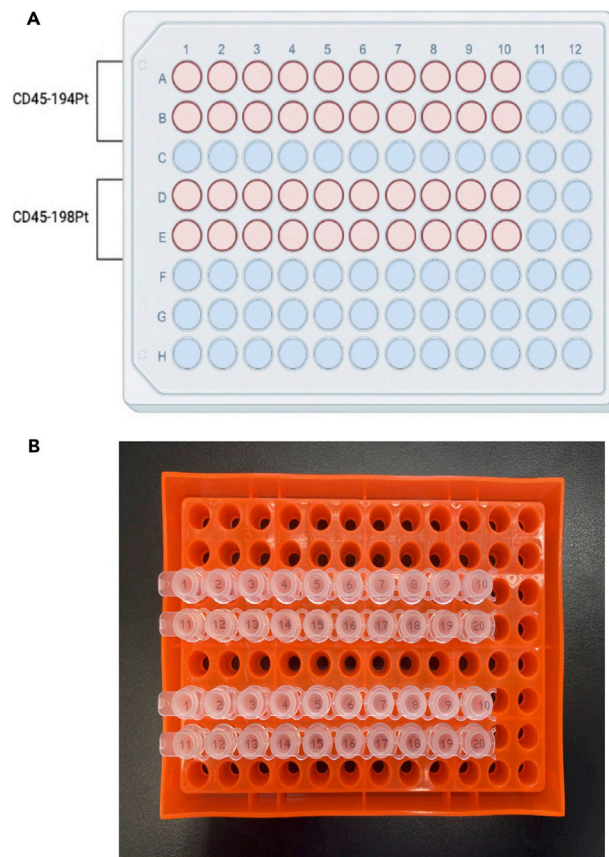
**Figure 1. Sample and Maxpar barcoding kit vial layouts**
(A) Figure shows the layout of samples in a 96-well V-bottom plate.
(B) Picture shows Maxpar barcoding kit minivial strips.

> *Note:* Other metal-tags for CD45 antibodies could be used. 194Pt and 198Pt here are just examples. This need to be tailored according to each panel and the available channels.

    c. Using multichannel pipette, add 100 μL of the CD45-194Pt-103Rh-RPMI mix to the first 20 wells of the 96-well V-bottom plate containing your samples, and mix by gentle pipetting.

    d. Add 100 μL of the CD45-198Pt-103Rh-RPMI mix to the remaining 20 wells of the 96-well V-bottom plate containing the rest of your samples, and mix by gentle pipetting.

    e. Put the cover lid on the 96-well V-bottom.

    f. Incubate the plate for 15 min at 37°C.

    g. Add 100 μL of PBS to each well.

    h. Centrifuge at 600 × *g*, 4°C for 3 min.

    i. Flick the plate to discard the supernatant.

    j. Blot the plate gently on paper towel to get rid of any residual supernatant.

7. Fixation and permeabilization:

    a. Add 5 mL of the previously prepared fixation/permeabilization buffer to a multichannel pipette solution basin.

    b. Using a multichannel pipette, add 100 μL of fixation/permeabilization buffer to each well and resuspend cells by gentle pipetting.

    c. Incubate for 15 min at room temperature.

    d. Remove 2 sets of barcode vials from the −20°C freezer (each set has 20 mini-vials labeled from 1 to 20 – each 10 barcodes are attached in one single strip).

e. Place the 4 mini-vial strips in rack as shown in Figure 1B and let them thaw at room temperature for the next step.

f. Add 100 μL of 1× Maxpar perm barcode buffer to each well of the plate containing the sample using multichannel pipette and solution basin.

g. Centrifuge the plate at 900 × $g$, 4°C for 3 min.

h. Flick the plate to discard the supernatant.

i. Blot the plate gently on paper towel to get rid of any residual supernatant.

8. Barcoding:

a. Add 100 μL of 1× Maxpar barcode buffer to each barcode mini-vial, and mix well by gentle pipetting.

b. Transfer 100 μL from each barcode vial to its respective sample well in the 96-well V-bottom plate according to the barcode map (Table 1). Mix well by gentle pipetting.

c. Incubate for 30 min at room temperature.

d. Add 100 μL of Maxpar CSM to each well.

e. Centrifuge at 900 × $g$, 4°C for 3 min.

f. Flick the plate to discard the supernatant.

g. Blot the plate gently on paper towel to get rid of any residual supernatant.

h. Add 200 μL of Maxpar CSM to each well. Repeat steps 7.5 and 7.6.

*Washing the cells twice ensures that no leftover barcodes remain in any wells, and prevents mixing of barcodes when the samples are pooled in the next step.

i. Add 100 μL Maxpar CSM to each well.

j. Pool samples by transferring the content of each well to a 4 mL polystyrene conical "FACS" tube.

k. Centrifuge at 900 × $g$, 4°C for 5 min.

l. Aspirate and discard the supernatant.

9. Cell staining:

a. Fc blocking step:

i. For up to 80 × $10^6$ cells, resuspend cells in 150 μL of Fc blocker and mix by gentle pippetting.

ii. Incubate for 10 min at room temperature.

b. Surface staining:

i. Add 150 μL of 2× surface stain (master mix 1) directly to the sample (suspended in same volume of Fc blocking buffer) to make final 1× concentration of antibodies cocktail. Mix by gentle pipetting.

ii. Incubate for 30 min at 4°C.

iii. Add 2 mL of CSM to each well, and mix by gentle pipetting.

iv. Centrifuge at 900 × $g$, 4°C for 5 min.

v. Add 2 mL 1× permeabilization buffer and resuspend cell pellet by gentle pipetting.

vi. Centrifuge at 900 × $g$, 4°C for 5 min.

vii. Aspirate and discard the supernatant.

c. Intracellular staining:

i. Add 300 μL of 1× intracellular stain (master mix 2); mix by pipetting up and down 3 times and incubate for 30 min at 4°C.

ii. Add 2 mL of PBS.

iii. Centrifuge at 900 × $g$, 4°C for 5 min.

iv. Aspirate and discard the supernatant.

d. Iridium labeling of cellular DNA:

i. Suspend cell pellet in 300 μL of DNA staining buffer.

ii. Incubate for 30 min 4°C.

iii. Add 2 mL PBS.

iv. Centrifuge at 900 × $g$, 4°C for 5 min.

    v. Aspirate and discard the supernatant.
10. Storing stained cells:
    a. Samples can be stored in 200 μL of 1.6% FA/PBS for up to 1 week at 4°C.

*Note:* *For long-term storage, cryopreservation in 10% DMSO 90% FBS has been shown to be effective in preserving the signal quality for up to 4 weeks (Sumatoh et al., 2017).

11. Preparing stained cells for acquisition.
    a. On the day of acquisition, add 2 mL of PBS to the stained and barcoded cells.
    b. Centrifuge at 900 × *g*, 4°C for 5 min.
    c. Aspirate the supernatant and resuspend the cells in 1 mL PBS.
    d. Count cells manually using a hemocytometer and record the cell count.

*Note:* Obtaining the total cell count is necessary for CyTOF acquisition, as the CyTOF operator uses it to determine the volume of the acquisition buffer mixed with normalization beads to add to the sample (Lee and Rahman, 2019).

    e. Filter the cells using the 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap.
    f. Centrifuge at 900 × *g*, 4°C for 5 min.
    g. Discard supernatant and keep the tube containing the cell pellet on ice.

*Note:* More than 40 samples can be barcoded and stained by using more than two CD45 antibodies e.g., 60 samples can be barcoded by using 3 separate CD45 antibodies (Thrash et al., 2020).

## EXPECTED OUTCOMES

This protocol details all of the necessary steps prior to sample acquisition on the Helios mass cytometer. Acquired data are saved as FCS files. The data are exported in a bead-normalized FCS file format that is ready for downstream analysis, including data preparation, and debarcoding to separate individual samples. Figure 2 shows the use of Gaussian parameters to exclude unwanted events/noise from the data (Bagwell et al., 2020). Figure 3 shows the use of biaxial plotting to debarcode the data based on combination of the platinum and palladium signals Individual cells are assigned to specific barcodes based on the absent and presence of platinum and palladium signals (Mei et al., 2015).

Typically, high-dimensional data analysis tools are employed with CyTOF data, such as phenograph-based clustering (Levine et al., 2015), t-Distributed Stochastic Neighbor Embedding (Amir et al., 2013), and Uniform Manifold Approximation and Projection for dimension reduction (UMAP) (Becht et al., 2018) to identify and visualize immune populations in different experimental groups and/or samples (Mathew et al., 2020; Lheureux et al., 2022).

## LIMITATIONS

This protocol is designed for the analysis of CD45-expressing human immune cells. Therefore, only barcoded immune cell data will be acquired from any samples that also include non-immune cells, such as single-cell suspensions of tumor biopsies or ascites/pleural effusion fluids from cancer patients. If the non- immune components of the samples are of interest, then the same protocol can be used with DNA-based barcoding alone – without the use of multiple CD45 antibodies – and thus limiting the sample number to 20 per batch (Zunder et al., 2015; Lheureux et al., 2022).
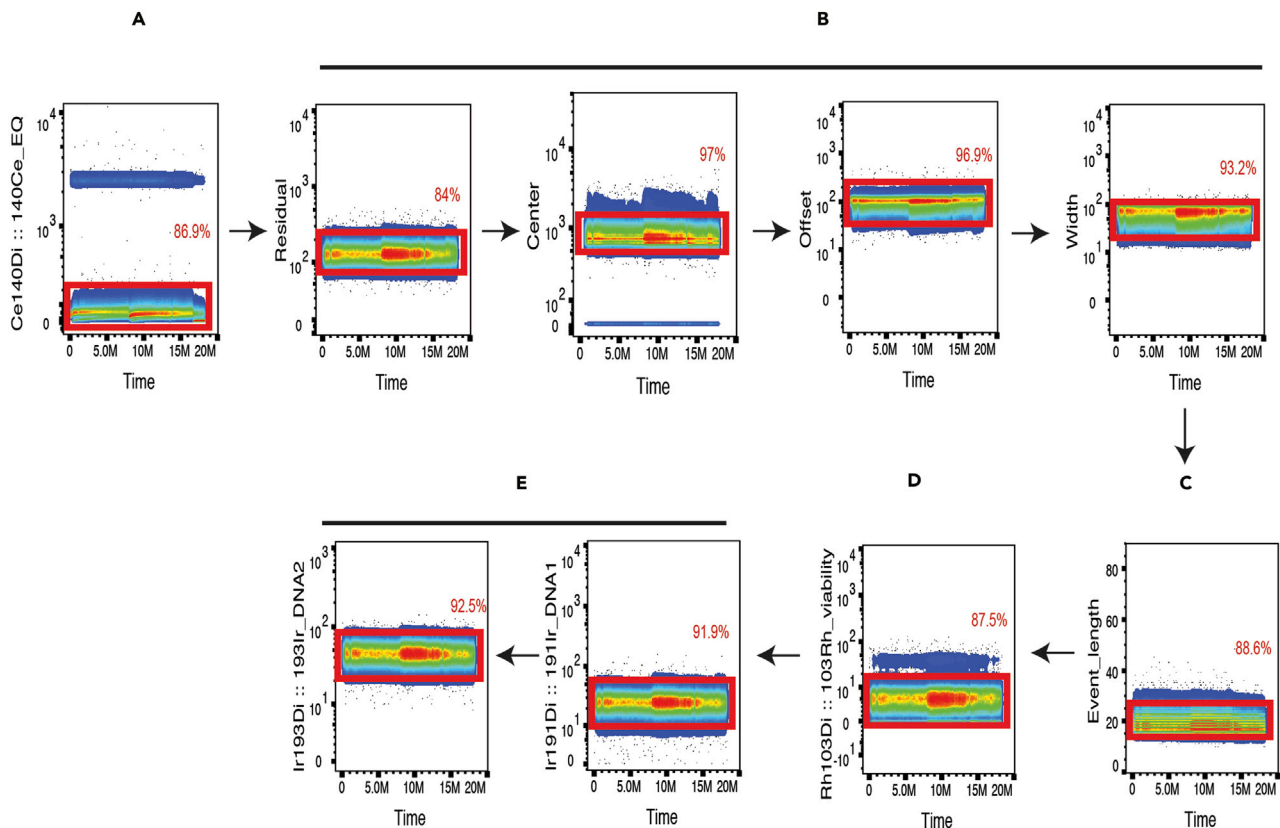
**Figure 2. Standard CyTOF data preparation steps**

(A) Normalization beads added during acquisition are excluded by plotting time vs Ce-140.

(B) Series of biaxial plots of time vs Gaussian parameters (residual, center, offset, width) used to eliminate non- specific signals.

(C) Plot shows time vs event length, which is used to eliminate signals from cell aggregates.

(D) Plot shows time vs 103Rh to distinguish live and dead cells.

(E) Plot shows time vs DNA-Ir191 and Ir193 to eliminate cell debris and to gate on intact cells. Using time as a common parameter in all plots is important to monitor signal fluctuations during acquisition. These steps can be performed using any software that is compatible with FCS-based cytometry data e.g., FlowJo, CytoBank.

Cell/event loss occurs at several points in the protocol: during repeated wash steps, cell filtering steps, cell acquisition, as well as during debarcoding. This should be taken into consideration when designing the experiment and choosing the number of cells to stain and acquire. Reducing the volume in the washing steps helps with reducing cell loss.

CyTOF data acquisition: the average and optimal rate of acquisition on the Helios mass cytometer is 300 cells/s (approximately 1 million cells/h). Higher acquisition rates could lead to clogging, increased numbers of doublets and aggregate events as a result of ion clouds overlapping. When barcoding up to 40 samples, and depending on the abundance of the immune population(s) of interest, long acquisition times (10–12 h) are expected.

Costs and panel design: there are many possible combinations of antibodies that can be used to create 40+ marker CyTOF panels, but commercially available ready-to-use antibodies are limited. Custom antibody-metal conjugations are an additional cost to consider.

Cells are not recoverable after acquisition:unlike traditional flow cytometry, where sorted population(s) of interest can be viably isolated for subsequent downstream applications (e.g., functional assays, RNA-sequencing), cells acquired by the CyTOF are vaporized.
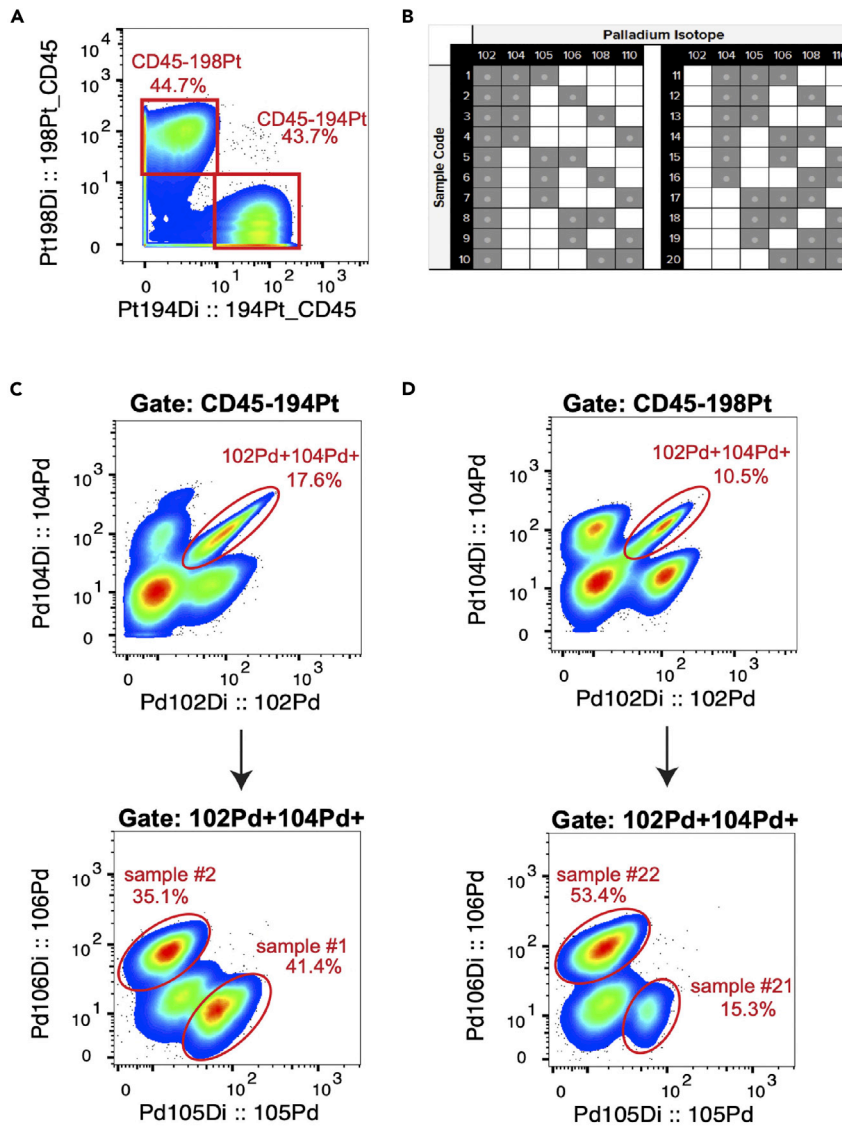
**Figure 3. Manual debarcoding strategy**

Manual debarcoding through a series of gates on biaxial plots aims to separate the individual samples by sequentially deconvoluting the CD45 barcodes and DNA-palladium barcodes.

(A) Biaxial plot with gates on samples stained with each of the CD45 antibodies.

(B) Barcode map from Cell-ID™ 20-plex Pd barcoding kit, where rows represent the barcode (sample number) and columns represent the associated palladium isotope mass number.

(C and D) (C) Plots show examples of debarcoding gates for samples number 1 and 2, and (D) samples number 21 and 22. Note: This figure is not generated from single-experiment data. It was put together to show the workflow.

Traces of platinum in biospecimens from patients who have been subjected to any form of platinum-based therapy, will interfere with the platinum isotope signal. In this case, other metal-tags, can be used for CD45-barcoding.

## TROUBLESHOOTING

### Problem 1

High level of 138Ba signal during acquisition.

### Potential solution

Ambient barium is a known contaminant of CyTOF staining and can be avoided by keeping samples closed/covered, working in a laminar flow hood and changing out older buffers.

### Problem 2

High background staining and/or high number of events recorded that do not correspond to cells.

### Potential solution

This problem could indicate insufficient washing, poor antibody filtration, incorrect antibody concentration or an expired antibody. Ensure no wash steps are skipped. Always filter antibody mixes on the day of staining (not before). Re-titrate antibodies if necessary. Check antibody stock for black flecks (metal precipitates) that could indicate the antibody is no longer usable.

### Problem 3

Low fraction of events corresponding to cells (191-Ir/103-Ir double positive). Potential solution:

See troubleshooting tips for Potential problem 2. Another possibility is suboptimal DNA staining. An alternative DNA staining buffer can be used with FA and 0.3% saponin (See materials and equipment setup). Alternatively, longer incubation periods and staining at room temperature can increase the fraction of labeled cells.

### Problem 4

Signal spill-over from the platinum tags and/or high background in the platinum channels.

### Potential solution

Normally, there is no signal spill-over from the platinum tags, if monoisotopic platinum is used to conjugate CD45. However, if this problem occurred, it could be a result of long incubation time or high antibody concentration. The combination of time and incubation will then have to be determined through testing various incubation times and antibody concentrations.

### Problem 5

Time-consuming debarcoding.

### Potential solution

Manual debarcoding consumes a lot of time. CyTOF software provided by Fluidigm has a debarcoding functionality, but it is dedicated to palladium barcodes only. So, users can debarcode the CD45 platinum tag and then input two separate files into Fluidigm software to debarcode the palladium tags. Alternatively, R package *premessa* allows users to define the barcodes in a graphical user interface. However, implementation of these methods requires users to set global parameters/thresholds that are not always accurate to all samples, which results in significant cell loss.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Ben Wang; ben.wang@uhnresearch.ca.

### Materials availability

No newly generated materials or reagents are associated with this protocol.

### Data and code availability

No new datasets are associated with this protocol.

## AUTHOR CONTRIBUTIONS

R.G., G.B., D.B., and B.W. developed and optimized the protocol. R.G. and G.B. wrote the manuscript. D.B. and B.W. edited and reviewed the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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