Simplified mass cytometry protocol for in-plate staining, barcoding, and cryopreservation of human PBMC samples in clinical trials

With the increasing use of mass cytometry in clinical research, a simplified and standardized protocol for immunophenotyping human peripheral blood mononuclear cells (PBMCs) in clinical trials is needed. We present a simplified in-plate staining protocol for up to 80 samples, for laboratories of all mass cytometry expertise levels, aimed to generate reproducible datasets for large clinical cohorts. In this protocol, we provide details on the requirements to obtain meaningful results, spanning from sample quality, barcoding, and batch-freezing of stained samples.

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Highlights
Mass cytometry protocol to stain, barcode, and freeze up to 80 ready-to-acquire samples

Freezing stained, barcoded samples allows for better management of machine acquisition

Insights on gating strategy and multiparameter data analysis

Useful for all expertise levels to generate datasets with reduced batch effects

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Simplified mass cytometry protocol for in-plate staining, barcoding, and cryopreservation of human PBMC samples in clinical trials

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SUMMARY
With the increasing use of mass cytometry in clinical research, a simplified and standardized protocol for immunophenotyping human peripheral blood mononuclear cells (PBMCs) in clinical trials is needed. We present a simplified in-plate staining protocol for up to 80 samples, for laboratories of all mass cytometry expertise levels, aimed to generate reproducible datasets for large clinical cohorts. In this protocol, we provide details on the requirements to obtain meaningful results, spanning from sample quality, barcoding, and batch-freezing of stained samples.

BEFORE YOU BEGIN
Generation of peripheral blood mononuclear cells (PBMCs) from whole blood clinical samples

© Timing: 30 min

Whole blood samples were obtained per the blood collection protocol approved by the Institutional Review Board of Brigham and Women’s Hospital. All participants gave written informed consent prior to the blood draw. To isolate PBMCs from whole blood, standard density gradient centrifugation was performed using Ficoll-Paque™ Plus.

1. Add 12 mL of Ficoll-Paque™ Plus per 50 mL conical tube.
2. Evenly distribute 25 mL of whole blood into necessary number of 50 mL conical tubes.
3. Dilute each 50 mL conical containing 25 mL of whole blood with 5 mL of PBS.
4. Slowly and gently layer the diluted whole blood sample on top of the Ficoll-Paque™ with a maximum volume of 35 mL per conical tube.

CRITICAL: Do not let the Ficoll-Paque™ Plus and blood mix together, as this will affect the yield and purity of isolated PBMCs.

5. Centrifuge conical tubes at room temperature (RT), (800 g, 20 min), with the brake off.
6. Carefully collect PBMC ‘buffy’ layer and transfer to a new 50 mL conical tube and fill it up to 50 mL with room temperature (17°C–21°C) PBS, mixing well.

**Optional:** If cell viability loss is observed, FBS can be added to the room temperature PBS in step 6 (PBS with 1% FBS).

7. Count cells using AO/PI (1:1 dilution; 20 µL sample in 20 µL AO/PI) with the Cellometer® Auto 2000 Cell Viability Counter (or alternative method).
   a. Gently invert the conical tubes to ensure accurate cell counts.
   b. Add 20 µL of the AO/PI-diluted sample to a Cellometer® SD100 Cell Counting Chamber.
   c. Cells should be >90% viable.

8. Centrifuge conical tubes from step 6 containing PBMCs in PBS (400 g, 5 min, RT).

△ CRITICAL: Be careful to not touch the Ficoll-Paque™ layer with the pipette tip when collecting the PBMCs clouds, as this contributes to iodine contamination of the samples and can negatively affect quality and analysis.

**Freezing of PBMCs**

△ Timing: 10 min

The PBMCs are frozen in freezing media to preserve the cells until needed for mass cytometry staining.

9. After centrifugation, carefully aspirate and discard supernatant.

10. According to cell count, resuspend PBMC pellet at a concentration of 1 × 10^7 in freezing media (85% FBS, 15% DMSO).

11. Aliquot 500 µL of resuspended pellet into cryovials for a final concentration 5 × 10^6 cells per cryovial.

12. Place vials in controlled-rate freezing container (for example CoolCell™) and transfer to −80°C overnight.

13. Transfer vials the following day to liquid nitrogen for storage until mass cytometry staining.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-Human CD19 (HIB19)-142Nd—100 Tests (1:100) | Fluidigm | Cat#3142001B |
| Anti-Human CD45RA (H1000)-143Nd—100 Tests (1:100) | Fluidigm | Cat#3134006B |
| Anti-Human CD69 (FNS0)-144Nd—100 Tests (1:100) | Fluidigm | Cat#3144018B |
| Anti-Human CD4 (RPA-T4)-145Nd—100 Tests (1:100) | Fluidigm | Cat#3145001B |
| Anti-Human CD8 (RPA-T8)-146Nd—100 Tests (1:100) | Fluidigm | Cat#3146001B |
| Anti-Human CD11c (Bu15)-147Sm—100 Tests (1:100) | Fluidigm | Cat#3147008B |
| Anti-CD278/ICOS (C398.4A)-148Nd—100 Tests (1:100) | Fluidigm | Cat#3148019B |
| Anti-Human CD56/NCAM (NCAM16.2)-149Sm—100 Tests (1:100) | Fluidigm | Cat#3149021B |
| Anti-Human CD223/LAG-3 (11C3C65)-150Nd—100 Tests (1:100) | Fluidigm | Cat#3150030B |
| Anti-Human CD123/IL-3R (6H6)-151Eu—100 Tests (1:100) | Fluidigm | Cat#3151001B |
| Anti-Human TC Rdg (11F2)-152Sm—100 Tests (1:100) | Fluidigm | Cat#3152008B |
| Anti-Human CD185/CXCR5 (RF8B2)-153Eu—100 Tests (1:100) | Fluidigm | Cat#3153020B |
| Anti-Human CD3 (UCHT1)-154Sm—100 Tests (1:200) | Fluidigm | Cat#3154003B |
| Anti-Human CD27 (L128)-155Gd—100 Tests (1:100) | Fluidigm | Cat#3155001B |
| Anti-Human CD14 (HCD14)-156Gd—100 Tests (1:100) | Fluidigm | Cat#3156019B |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Anti-Human CD33 (WM53)-158Gd—100 Tests (1:100) | Fluidigm | Cat#3158001B |
| Anti-Human TIGIT (MBSA43)-159Tb—100 Tests (1:100) | Fluidigm | Cat#31590388 |
| Anti-Human/Mouse MIP1beta (D21-1351)-160Gd—100 Tests (1:100) | Fluidigm | Cat#3160013B |
| Anti-Human/Mouse Tbet (4B10)-161Dy—50 Tests (1:100) | Fluidigm | Cat#3161014B |
| Anti-Human FoxP3 (259D/C7)-162Dy—50 Tests (1:100) | Fluidigm | Cat#3162002B |
| Anti-Human CD57 (HCD57)-163Dy—100 Tests (1:200) | Fluidigm | Cat#3163002B |
| Anti-Human IL-17A (N49-653)-164Dy—100 Tests (1:100) | Fluidigm | Cat#3164002B |
| Anti-Human IFNg (B27)-165Ho—100 Tests (1:100) | Fluidigm | Cat#3165002B |
| Anti-Human CD314/NKG2D (ON72)-166Er—100 Tests (1:100) | Fluidigm | Cat#3166016B |
| Anti-Human CD197/CCR7 (G043H7)-167Er—50 Tests (1:100) | Fluidigm | Cat#3167009A |
| Anti-Ki-67 (B56)-168Er—100 Tests (1:100) | Fluidigm | Cat#3168007B |
| Anti-Human CD25 (2A3)-169Tm—100 Tests (1:100) | Fluidigm | Cat#3169003B |
| Anti-Human CD152/CTLA-4 (14D3)-170Er—100 Tests (1:100) | Fluidigm | Cat#3170005B |
| Anti-Human CD195/CCR5 (NP-6G4)-171Yb—50 Tests (1:50) | Fluidigm | Cat#3171017A |
| Anti-Human CD38 (HIT2)-172Yb—100 Tests (1:100) | Fluidigm | Cat#3173005B |
| Anti-Human HLA-DR (L243)-173Yb—100 Tests (1:200) | Fluidigm | Cat#3174002B |
| Anti-Human CD194/CCR4 (L291H4)-175Lu—50 Tests (1:50) | Fluidigm | Cat#3175035B |
| Anti-Human CD127/IL-7Ra (A019D5)-176Er—100 Tests (1:100) | Fluidigm | Cat#3176004B |
| Anti-Human CD16 (3G8)-209Bi—100 Tests (1:100) | Fluidigm | Cat#3209002B |

### Chemicals, peptides, and recombinant proteins

| Chemicals, peptides, and recombinant proteins | SOURCE | IDENTIFIER |
|-----------------------------------------------|--------|------------|
| Ficoll-Paque™ Plus | Sigma-Aldrich | Cat#17144003 |
| Dimethyl Sulfoxide (DMSO) | Fisher BioReagents | Cat#BP231-100 |
| Gibco™ Phosphate-Buffered Saline (PBS) | Thermo Fisher Scientific | Cat#10010023 |
| Antibiotic-Antimycotic (100x) | Gibco | Cat#15240096 |
| Bovine Serum Albumin (BSA), 30% ± 2% in 0.85% NaCl | Sigma-Aldrich | Cat#A7284 |
| Sodium Azide, 10% (w/v) solution in Ultra-Pure H2O | Teknova | Cat#50209 |
| RPMI Medium 1640 (+) L-glutamine | Thermo Fisher Scientific | Cat#11875093 |
| Gibco™ Premium Plus Fetal Bovine Serum, heat inactivated, qualified (FBS) | Thermo Fisher Scientific | Cat#10438026 |
| UltraPure™ DNAse/RNase-Free Distilled Water | Invitrogen | Cat#10977023 |
| ViaStain™ APOI Staining Solution | Nexcelom Biosciences | Cat#CS2-0106-5mL |
| Cell-ID™ Intercalator-103Rh—500 µM | Fluidigm | Cat#2010103A |
| Cell-ID™ Intercalator-191/193Ir—125 µM | Fluidigm | Cat#201192B |
| Maxpar® Cell Acquisition Solution | Fluidigm | Cat#201237 |
| Maxpar® Cell Staining Buffer | Fluidigm | Cat#201068 |
| Maxpar® 10X Barcode Perm Buffer | Fluidigm | Cat#201057 |
| EQ™ Four Element Calibration Beads | Fluidigm | Cat#201078 |
| eBioscience™ Permeabilization Buffer (10X) | Invitrogen | Cat#00833356 |
| eBioscience™ Fixation/Permeabilization Concentrate | Invitrogen | Cat#00512343 |
| eBioscience™ Fixation/Permeabilization Diluent | Invitrogen | Cat#00522356 |
| Human TruStain FcX™ (FcR Blocking Solution) | BioLegend | Cat#422302 |
| Pierce™ 16% Formaldehyde Solution (w/v), Methanol-free | Thermo Fisher Scientific | Cat#28906 |
| CyTOF® Tuning Solution, 250 mL | Fluidigm | Cat#201072 |

### Critical commercial assays

| Critical commercial assays | SOURCE | IDENTIFIER |
|----------------------------|--------|------------|
| Cell-ID™ 20-Plex Pd Barcoding Kit | Fluidigm | Cat#201060 |

### Biological samples

| Biological samples | SOURCE | IDENTIFIER |
|--------------------|--------|------------|
| Human PBMCs isolated from whole blood clinical samples (sex, age as required per study) | Any Supplier | N/A |

### Software and algorithms

| Software and algorithms | SOURCE | IDENTIFIER |
|-------------------------|--------|------------|
| FlowJo v.10 | Becton Dickinson & Company | https://www.flowjo.com/solutions/flowjo/downloads/ |
| GraphPad Prism v9.2 | GraphPad Software, LLC | https://www.graphpad.com/scientific-software/prism/ |
| CyTOF Software v7.0.8493 | Fluidigm | https://www.fluidigm.com/software |

(Continued on next page)
**Materials and Equipment**

## Complete RPMI

| Reagent                          | Final concentration | Amount   |
|----------------------------------|---------------------|----------|
| RPMI Medium 1640 (1X)            | n/a                 | 500 mL   |
| FBS                              | 10%                 | 50 mL    |
| Antibiotic-Antimycotic (100x)    | 1%                  | 5 mL     |
| **Total**                        | n/a                 | 555 mL   |

Store at 4°C for up to 2 weeks.

## CyFACS

| Reagent                   | Final concentration | Amount   |
|---------------------------|---------------------|----------|
| PBS                       | n/a                 | 500 mL   |
| 30% BSA                   | 0.5%                | 8.3 mL   |
| 5% Sodium Azide           | 0.02%               | 2 mL     |
| **Total**                 | n/a                 | 510.3 mL |

Store at 4°C for up to 6 weeks.

## FoxP3 Fixation/Permeabilization Buffer

| Reagent                               | Final concentration | Amount   |
|---------------------------------------|---------------------|----------|
| Fixation/Permeabilization Concentrate | 25%                 | 8 mL     |
| Fixation/Permeabilization Diluent     | 75%                 | 32 mL    |
| **Total**                             | n/a                 | 40 mL    |

Store at 4°C during the day of experiment, prepare fresh every time.
Table 1. Surface antibody master mix, titrated for staining of up to 3 M cells per condition

| Marker       | Metal | Clone   | Volume per sample (μL) |
|--------------|-------|---------|------------------------|
| CD19         | 142Nd | HI19    | 1.00                   |
| CD45RA       | 143Nd | HI100   | 1.00                   |
| CD69         | 144Nd | FN50    | 1.00                   |
| CD4          | 145Nd | RPA-T4  | 1.00                   |
| CD8          | 146Nd | RPA-T8  | 1.00                   |
| CD11c        | 147Sm | Bu1S    | 1.00                   |
| CD278/ICOS   | 148Nd | C398.4A | 1.00                   |
| CD56/NCAM    | 149Sm | NCAM16.2| 1.00                   |
| CD223/LAG3   | 150Nd | 11C3C65 | 1.00                   |
| CD123/IL-3R  | 151Eu | 6H6     | 1.00                   |
| TC1Rgd       | 152Sm | 11F2    | 1.00                   |
| CD185/CXCR5  | 153Eu | RF8B2   | 1.00                   |
| CD3          | 154Sm | UCHT1   | 0.50                   |
| CD27         | 155Gd | L128    | 1.00                   |
| CD14         | 156Gd | HCD14   | 1.00                   |
| CD33         | 158Gd | WM53    | 1.00                   |
| TIGIT        | 159Tb | MBSA43  | 1.00                   |
| CD57         | 163Dy | HCD57   | 0.50                   |
| CD314/NKG2D  | 166Er | ON72    | 1.00                   |
| CD197/CCR7   | 167Er | G043H7  | 1.00                   |
| CD25         | 169Tm | 2A3     | 1.00                   |
| CD195/CCR5   | 171Yb | NP6G4   | 2.00                   |
| CD38         | 172Yb | HIT2    | 1.00                   |
| HLA-DR       | 173Yb | L243    | 0.50                   |
| CD279/PD-1   | 174Yb | EH12.2H7| 1.00                   |
| CD194/CCR4   | 175Lu | L291H4  | 2.00                   |
| CD127/IL-7Ra | 176Yb | A019D5  | 1.00                   |
| CD16         | 209Bi | 3G8     | 1.00                   |
| CyFACS       | n/a   | n/a     | 21.50 μL              |
| Total        | n/a   | n/a     | 50.00 μL              |

Δ CRITICAL: Sodium Azide is carcinogenic and toxic if swallowed, inhaled, or if in contact with skin. Handle in a fume hood using personal protective equipment.

Note: The timing and reagent consumption in this protocol is relative to the handling of 80 samples.

STEP-BY-STEP METHOD DETAILS
PBMC thawing, cell counting, and viability staining

© Timing: 2 h

To prepare the cryopreserved PBMC samples for mass cytometry staining, the samples must first be thawed and counted.

Δ CRITICAL: This step assumes the availability of two technicians to facilitate the quick and efficient thawing and counting of samples.

1. Before staining, annotate and fill the appropriate number of 15-mL conical tubes (one per sample) with 9 mL of warmed to 37°C RPMI +10% FBS +1% Antibiotic-Antimycotic (named Complete RPMI from now on), and prepare necessary counting slides.
2. Prepare the cell surface antibody master mix in CyFACS (Table 1).
3. Thaw the samples by placing the rack with 10 frozen vials directly into the 37°C circulating water bath until content is thawed, approximately 45 s, few small ice crystals might remain in the vial.
4. Immediately transfer the totality of thawed cell suspensions into the 15 mL conical tubes containing 9 mL of 37°C complete RPMI.
5. Repeat step 3 and 4 for remaining batches of frozen vials.
6. Filter the samples using 70 µM cell pre-wetted strainers to obtain a single cell suspension.
7. Count cells using AO/PI (1:1 dilution; 20 µL sample in 20 µL AO/PI) with the Cellometer® Auto 2000 Cell Viability Counter (or alternative cell counter).
   a. Gently invert the conical tube containing the freshly thawed cells to ensure cells are fully resuspended.
   b. Add 20 µL of the AO/PI-diluted sample to a Cellometer® SD100 Cell Counting Chamber.
   c. Cells should be >90% viable.
8. According to the cell count, adjust volume of cell suspension to a final concentration of 3 x 10⁶ cells per condition in complete RPMI.
   
   **Note:** To prepare samples for staining, prepare a total of 3 x 10⁶ cells for each condition. If more cells will be stained, this will require careful titration of antibodies.

9. Centrifuge samples (400 g, 5 min, RT).
   a. While the samples are spinning down, prepare the 103Rh Viability Stain (1:500 dilution of 103Rh Cell-ID™ Intercalator-Rh in 37°C warm complete RPMI).
10. After the sample is spun down, carefully aspirate and discard the supernatant.
11. Resuspend the sample in 1 mL of warm, Complete RPMI+103Rh Viability Stain.
12. Incubate the samples for 15 min at room temperature (17°C–25°C).
   a. During incubation, aliquot 50 µL of surface stain antibody cocktail per well into designated v-bottomed wells of the 96-well polystyrene plate.
13. Once incubation is complete, fill conical tube up to 10 mL with complete RPMI and spin down (400 g, 5 min, RT).
14. Carefully aspirate and discard supernatant without disturbing the cell pellet.
15. Resuspend each cell pellet in 50 µL CyFACS and proceed to Fc-block and surface staining.

   **Optional:** The cell surface antibody master mix can be prepared during the viability stain incubation, but we recommend preparing the cocktail prior to the thawing of samples.

**Fc-block and surface staining**

© Timing: 1 h

This portion of the protocol stains the sample with pre-selected metal-tagged antibodies to be acquired and used in downstream analysis. Prior to antibody staining, the samples are Fc-blocked to prevent non-specific staining of antibodies.

16. Fc-block:
   a. Add 5 µL undiluted Fc-block to each sample.
   b. Incubate the samples on ice for 10 min.
17. Once the incubation is complete, distribute the totality of Fc-blocked samples into the appropriate wells (already containing the antibody cocktail) according to the plate map.
18. Incubate the staining plate for 30 min at room temperature.
   a. During incubation, prepare the eBioScience™ FoxP3 Fixation/Permeabilization Buffer as per manufacturer protocol (www.thermofisher.com) (briefly, mix 1 part fix/perm concentrate (00-5123-43) + 3 parts fix/perm diluent (00-5223-56)).
   b. During incubation, prepare the eBioScience™ Perm/Wash solution by diluting 5 mL of 10× Permeabilization Buffer stock into 45 mL of Ultra-Pure H₂O.
19. After incubation is complete, centrifuge the plate to pellet cells (400 g, 5 min, RT).

20. Carefully aspirate and discard supernatant from each staining well using the multi-channel pipette, set for approximately 20 μL less than the volume in the well to avoid aspirating the pellet. For best results, tilt the plate at a 45° angle and rest the pipette tip on the edge of the well, right before the well funnels down into the v-bottom. This applies for every aspiration step in this protocol.

21. Add 200 μL CyFACs/well using multichannel pipette and centrifuge the plate (400 g, 5 min, RT).

Optional: The surface antibody master mix can be added individually to each sample well after the Fc-blocked cells have been plated, but this increases risk of technical error and experimental time.

Note: The FoxP3 Fixation/Permeabilization Buffer is prepared in excess and will be needed for the DNA stain at the end of the protocol.

Permeabilization and intracellular staining

Θ Timing: 1.5 h

This portion of the protocol describes staining the samples with pre-selected metal-tagged antibodies that target intracellular markers.

22. Carefully aspirate and discard supernatant from each staining well using multichannel pipette (as in step 20).

23. Resuspend samples thoroughly in 200 μL of FoxP3 Fixation/Permeabilization Buffer per well, using multichannel pipette.

24. Incubate the plate for 30 min at room temperature.
   a. During incubation, prepare the intracellular antibody master mix (Table 2).

Δ CRITICAL: Intracellular antibody master mix is made in Perm/Wash.

25. Centrifuge plate (800 g, 5 min, RT) and wash samples once with 200 μL of Perm/Wash per well using multichannel pipette.

26. Carefully aspirate and discard supernatant (as in step 20).

27. Resuspend each sample in 50 μL of Perm/Wash per well using multichannel pipette.

28. Add 50 μL of intracellular master mix to each well.

29. Incubate the plate for 30 min at room temperature.

30. After incubation is complete, add 100 μL of Perm/Wash solution per well using multichannel pipette and centrifuge plate (800 g, 5 min, RT).

---

Table 2. Intracellular antibody master mix

| Marker       | Metal | Clone    | Volume per sample (μL) |
|--------------|-------|----------|------------------------|
| MIP1beta     | 160Gd | D21-1351 | 1.00                   |
| Tbet         | 161Dy | 4B10     | 1.00                   |
| FoxP3        | 162Oy | 259D/C7  | 1.00                   |
| IL-17a       | 164Oy | N49-653  | 1.00                   |
| IFNγ         | 165Ho | B27      | 1.00                   |
| Ki-67        | 168Er | B56      | 1.00                   |
| CD152/CTLA-4 | 170Er | 14D3     | 1.00                   |
| Perm/Wash    | n/a   | n/a      | 43.00 μL               |
| Total        | n/a   | n/a      | 50.00 μL               |
31. Wash samples with 200 μL of Perm/Wash solution per well using multichannel pipette, centrifuge the plate (800 g, 5 min, RT), and carefully aspirate the supernatant as in step 20. 
   a. During spin, prepare fresh 4% PFA solution from 16% stock PFA in Ultra-Pure H2O.
32. Add 200 μL of freshly prepared 4% PFA per well using multichannel pipette.
33. Centrifuge the plate (800 g, 10 min, RT), aspirate the supernatant (as in step 20), and proceed to barcoding.
   a. During spin, prepare the 191/193 DNA Intercalator at a 1:5,000 dilution of 191/193 Cell-ID™ Intercalator-Ir to FoxP3 Fixation/Permeabilization Buffer.
   b. During spin, prepare Barcode Perm Buffer according to manufacturer’s protocol (www.fluidigm.com) by diluting 1 part 10x Barcode Perm Buffer in 9 parts PBS.

Δ CRITICAL: Spin time was increased to 10 min to improve cell recovery post-fixation of cells.

**Palladium-based barcoding**

**© Timing: 45 min**

This portion of the protocol applies a post stain 6-choose-3 palladium based barcoding system to mitigate potential procedural variations while retaining staining efficiency. We applied a post-stain barcoding scheme to preserve fixation sensitive staining while maintaining the acquisition consistency of running pooled samples. This Palladium-based Barcoding Strategy is adapted from the protocol “Cell-ID 20-Plex Pd Barcoding Kit” on www.fluidigm.com.

34. Remove barcode tubes from –20°C, thaw, and briefly spin down barcodes in mini centrifuge.
35. Resuspend barcodes in 100 μL Barcode Perm Buffer.
36. Resuspend freshly fixed cells from step 33 in 100 μL Barcode Perm Buffer per well using multichannel pipette.
37. Transfer the totality (100 μL) of resuspended barcodes from step 35 to respective wells containing samples using multichannel pipette.
38. Incubate the plate for 30 min at room temperature.
39. Centrifuge plate (800 g, 5 min, RT) and wash samples once with 200 μL of Barcode Perm Buffer using multichannel pipette.
40. Resuspended each well with 200 μL of Barcode Perm Buffer using multichannel pipette and combine all samples from a single barcode batch (up to 20 samples) into a 15 mL conical tube.
41. Wash once with 5 mLBarcode Perm Buffer, pellet the cells by centrifugation (800 g, 5 min, RT), and proceed to DNA staining.

**Optional:** We recommend including a healthy donor sample in each barcoding batch (i.e., 19 clinical samples, one healthy donor sample). If running multiple full barcodes, the same normal donor should be used throughout batches to assess data quality.

**Optional:** If running a smaller cohort of patients (less than 20 samples), barcoding may be omitted. In this case, proceed from step 33 to step 42 and adjust all the buffer/wash volumes to 200 μL/well. Samples can be frozen directly in plate: using multichannel pipette add 100 μL of freezing medium per well to the spun-down cells from step 47.

**DNA staining and plate freezing**

**© Timing: 1 h**

The final steps stain the cell nuclei to allow for single-cell detection and discrimination by the Helios™ mass cytometer. After completing the DNA stain, the samples are prepared for short-term
storage until acquisition. Prior to acquisition the samples are thawed and prepared accordingly for optimal Helios™ acquisition.

42. Per full barcode (20 samples) add 5 mL of a 1:5,000 dilution of 191/193 DNA Intercalator in FoxP3 Fixation/Permeabilization Buffer.
43. Incubate samples 1 h at 4°C.

- Pause point: Samples can be incubated in 191/193 DNA Intercalator staining solution up to 16 h (overnight) at 4°C instead of 1 h incubation.

44. After the incubation is complete, pellet cells by plate centrifugation (800 g, 5 min, RT).
45. Carefully aspirate and discard supernatant.
46. Wash once with 2 mL of Cell Staining Buffer.
47. Centrifuge the tube(s) (800 g, 5 min, RT) and carefully aspirate the supernatant.
48. Resuspend the pellet(s) in 500 μL of freezing media (85% FBS, 15% DMSO) and immediately transfer to −20°C for storage.

- CRITICAL: This protocol was optimized and validated for short-term storage of stained and frozen samples (up to 1 month).

Pre-acquisition thawing and wash

- Timing: 45 min

49. On the acquisition day, thaw samples with 500 μL of warm Complete RPMI per tube(s) (100 μL per well if no barcoding was applied).
50. Centrifuge the thawed samples (800 g, 5 min, RT).
51. Wash once with 2 mL of Cell Acquisition Solution (CAS).
52. Count the total number of cells with Cellometer® Auto 2000 Cell Viability Counter, and pellet cells by centrifugation (800 g, 5 min).
   a. Load 20 μL of sample directly into Cellometer® SD100 Cell Counting Chamber to count total cell concentration (in absence of stain).
   b. Count the cells using the Cellometer® Auto 2000 Cell Viability Counter during spin and record final cell count.
53. Prepare a 1:5 dilution of MaxPar EQ Calibration beads in CAS (CAS/EQ).

- CRITICAL: Vortex the EQ Calibration beads for 1 min before preparing EQ beads/CAS solution.

54. Resuspend the samples at a concentration of 1 × 10⁶ cells per 1 mL in EQ beads/CAS solution.
55. Filter resuspended sample through a 35 μm pre-wetted cell strainer cap into a labeled, polypropylene tube in 2 mL aliquots.
56. Proceed to Helios™ mass cytometer acquisition (Fluidigm).

EXPECTED OUTCOMES

Our panel presents a comprehensive scope of the innate and adaptive immune system within one mass cytometry panel, consisting of 35 markers (Figure 1A) with the ability to define multiple immune phenotypes (Figure 1B).

At the end of this protocol, FCS files are generated for every sample acquired on Helios™ and normalized using the FCS Processing tab of the Fluidigm CyTOF Software 7.0.8493.
This panel covers the major immune cell subsets, including activation, checkpoint, maturity, and memory markers. The data was cleaned following Gaussian parameters (Figure 2A). The myeloid subset determines monocyte subpopulations: classical (CD33^+CD16^-CD14^+), intermediate (CD33^+CD16^+CD14^+), and nonclassical (CD33^+CD16^+CD14^-) (Figure 2B). The lymphocyte subset distinguishes Natural Killer cells (CD33^-CD19^-CD3^-CD56^+), Natural Killer T cells (CD33^-CD19^-CD3^+CD56^+) amongst other CD56^+ T cells, and B cells (Figure 2B). The lymphocyte subset also distinguishes CD4 T cells and T regulatory cells (Figure 2C), and CD8 T cells (Figure 2D).

**LIMITATIONS**

Low quality sample (for example sample with low viability) can jeopardize debarcoding efficiency and analysis of all samples in a barcoding batch. Therefore, we suggest that low-quality samples are in a separate barcode batch from high quality samples.

**TROUBLESHOOTING**

**Problem 1**

High level of 127-Iodine contamination in samples (step 4 and data analysis).

**Potential solution**

127 Iodine contamination most frequently comes from Ficoll-Paque, as it contains sodium diatrizoate. If the Ficoll-Paque layer was disturbed while collecting the immune cell layer during PBMC isolation, the isolated cells can have trace amounts of Iodine contamination. This can potentially be resolved by adding supplementary washes after thawing.

**Problem 2**

Shift in lower mass channels expression level in barcoded samples (step 42 & data analysis).

Although sample phenotypes were not significantly affected by barcoding it is important to note that there is potential for signal shift in the lower mass channels for barcoded samples. As shown in Figure 3, the signal shift is absent in individually acquired samples (non-barcoded and single barcode) and does not occur in the full barcode sample until approximately 5.0 million acquired events. The signal shift appears during acquisition of full barcode samples due to the high number of cells when multiple samples are combined, and therefore requiring extended acquisition time. For example, the signal continually degraded over acquisition time, causing the 145Nd CD4 positive...
and negative populations to eventually merge, leaving no clear separation. Interestingly in our experience change of fixation method did not improve the outcomes.

Potential solution

To mitigate signal shift, samples should be acquired on the machine in aliquots of $1 \times 10^6$ cells per 1 mL in EQ beads/CAS solution and that newly developed panels be tested with a fully barcoded set of donor samples to ensure vital populations remain discernable before applying the panel to clinical samples. Before a clinical experiment, the barcode condition should be determined and applied to all samples in the same cohort.

Problem 3

Significant machine time use required to acquire all cellular events of 80 samples (step 56).

Although barcoding samples aids in the reduction of batch effects by allowing for combined acquisition, the length of acquisition time might become problematic due to high cell volume (Sumatoh et al., 2017). With an optimal acquisition rate of 500 events per second, the mass cytometer can only effectively analyze 2 million cells per hour. Assuming an eight-hour workday for one operator on a single machine, the threshold is around 20 million cells per day of acquisition. In addition to the limitation of machine availability, we found lengthy acquisition time to lead to signal shifts in the lower mass channels, as described above (Figure 3). This forces multiple acquisition days for a single cohort.

Potential solution

This protocol was developed to allow for successful staining of 80 samples during one workday, and subsequent acquisition of 1 batch of 20 barcoded samples per machine day. We recommend

![Figure 2. Gating strategy of innate and adaptive immune cell subsets](image)

(A) Clean-up gating strategy used for identifying of viable single cell population, using gaussian parameters and 191Ir/193Ir biaxial plots.
(B) Cleaned gating strategy to identify monocyte, B cell, NKT cell, and NK cell populations.
(C) Cleaned CD4+ T cell populations and marker expression.
(D) Cleaned CD8+ T cell populations and marker expression.

![Figure 3. Signal shift in individual sample acquisition compared to combined sample acquisition](image)

Biaxial time plots of CD4, CD8, and CD3 marker expression in individually acquired samples (non-barcoded and single barcode) compared to combined sample acquisition (full barcode).
thawing every barcoded sample batch on its day of acquisition. For best results, the stained clinical samples should be thawed and acquired within a one-week time frame to achieve best instrument consistency (Gonder et al., 2020). We recommend adding the healthy donor sample to each bar-code batch, to allow for batch normalization if needed.

Problem 4
Low Cell Count for specific populations.

Our streamlined protocol takes into consideration the importance of obtaining a high cell count. Initial poor cell viability will result in unacceptably low yield of single, live cells (Olsen et al., 2019). Through experimental exploration, we established a threshold for an acceptable cell count to confidently produce accurate cell population quantifications. Cell loss is a result of improper sample handling, poor cryopreservation methods, inadequate fixation, and prolonged staining protocols, specifically the washing steps (Maecker and Harari, 2015). Using our described protocol, we obtained an average staining cell recovery rate of 50%, or approximately 1–1.5 million cells per sample at the end of staining protocol. In addition to cell loss while staining, mass cytometers have a typical machine cell recovery rate of 30%–50% (Maecker and Harari, 2015). After routine clean up gating using Gaussian parameters, we had an end point cell count of at least 200,000 CD45+103Rh- alive single cells per patient available to analyze (Figure 4.). For an in-depth immune phenotyping encompassing small cellular populations, samples need to have a minimum of 100,000 alive single cells analyzed per condition (Marsh-Wakefield et al., 2021) (Maecker and Harari, 2015).

Potential solution
Do not increase the number of washes, as those were optimized for best cell recovery. Do not use Eppendorf tubes at any point of staining, as there is a huge loss of cells during centrifugation in this type of tubes. The use of 96-well plates was optimal for the best cell recovery, thus always use plate or 15 mL conical tubes at precise steps. Do not decrease time of or modify fixation methods recommended in this protocol.
Problem 5
103Rh Viability weak staining intensity (step 11 and data analysis).

Staining 3 × 10⁶ cells with a concentration of 1:500 103Rh viability stain should produce two distinct populations of 103Rh negative live cells and 103Rh positive dead cells. If there is no 103Rh positive populations or the positive populations forms a smear rather than a distinct population, downstream analysis could be negatively affected.

Potential solution
We recommend thawing a frozen aliquot of 103Rh no earlier than the morning of staining. Additionally, we do not recommend using frozen aliquots that are over one year old, as we have noticed decreased staining intensity after one year, even with proper storage and preparation. Additionally, be careful when modifying fixation reagents, as 103Rh is not compatible with all fixation methods, such as methanol fixation.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Joanna Baginska, joanna_baginska@dfci.harvard.edu.

Materials availability
There were no new reagents generated.

Data and code availability
FCS files from healthy donors will be available upon request.

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AUTHOR CONTRIBUTIONS
Conceptualization, J.B.; methodology, C.A., M.H., M.B., E.H., J.D., and J.B.; investigation, C.A., M.H., and J.B.; resources, C.A., M.H., J.B., F.S.H., and M.S.; writing – original draft, C.A., M.H., and J.B.; visualization, M.H. and J.D.; funding acquisition, F.S.H. and M.S.; supervision, J.B.

DECLARATION OF INTERESTS
Dr. Hodi reports grants and/or personal fees from Bristol-Myers Squibb, Merck, EMD Serono, Novartis, Surface, Compass Therapeutics, Apricity, Sanofi, Pionyr, 7 Hills Pharma, Torque, Bicara, Pieris Pharmaceutical, Checkpoint Therapeutics, Genentech/Roche, Bioentre, Gossamer, Ivance, Trillium, Catalym, Immunocore, Amgen, and Zumutor outside the submitted work. In addition, Dr. Hodi has a patent (#20100111973) with royalties paid and patents (#7250291, #9402905, #10279021, #10106611, #20170248603, #20160046716, #20140004112, #20170022275, #20170008962, and #20170343552) pending.

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