FLUIDIGM®

PRD023 Rev 03

Cell-ID 20-Plex Pd Barcoding Kit

User Guide

For Research Use Only. Not for use in diagnostic procedures.

PRD023 Rev 03

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About This Guide

Safety Data Sheets

Read and understand the safety data sheet (SDS) before handling chemicals. To obtain the SDS for a chemical ordered from Fluidigm Corporation, either alone or as part of this system, go to fluidigm.com/sds and search for the SDS using either the product name or the part number.

Some chemicals referred to in this user guide may not have been provided with your system. Obtain the SDSs for chemicals provided by other manufacturers from those manufacturers.

Introduction

The Cell-ID™ 20-Plex Pd Barcoding Kit enables unique barcoding of up to 20 samples (Figure 1) so they can be combined and subsequently stained and acquired as 1 multiplexed sample, followed by software debarcoding and individual sample analysis. Multiplexing samples improves data quality because the 20 samples are stained, processed, and acquired in a single tube eliminating sample-specific staining and data collection variation.

This user guide includes protocols for barcoding samples and for debarcoding multiplexed files. For more information on how palladium barcoding improves data quality and increases workflow efficiencies, see The Benefits of Palladium Barcoding on Data Quality and Workflow Application Note (FLDM-00012).

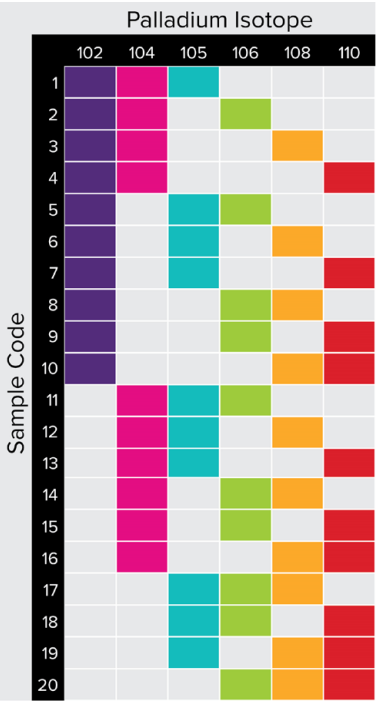
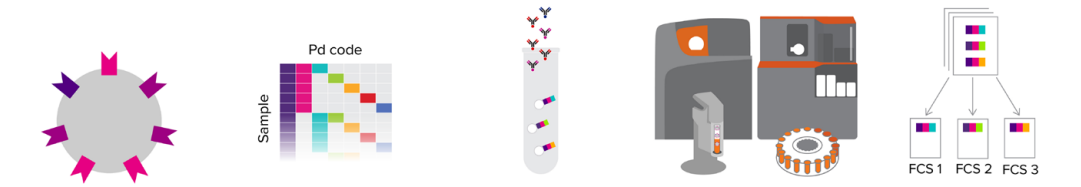


Figure 1. Schematic representation of the barcoding patterns of 20 samples barcoded with 6 different palladium isotopes. Each barcode consists of a unique combination of the 3 palladium isotopes.

Barcoding Workflow

The barcoding protocol employs fixation and gentle permeabilization to ensure uniform cell labeling with the palladium (Pd) barcode while preserving cellular epitopes. After samples are barcoded and combined, proceed with the appropriate downstream protocol for your assay.



1 Fix and permeabilize samples.

2 Barcode up to 20 samples by adding 3 unique isotopes (3-digit Pd code).

3 Stain combined samples in a single tube with metal-conjugated antibodies or validated panel.

4 Acquire data from a single tube of combined samples on a CyTOF suspension mass cytometry system.

5 Decode with Debarcoder software to create individual FCS files for each original sample.

Fluidigm Kit Contents

The following reagents are included in Cell-ID 20-Plex Pd Barcoding Kit (Cat. No. 201060), which provides the necessary reagents to barcode 3 sets of 20 test samples.

Contents	Volume	Storage Temperature
3 sets of 20 barcodes in PCR tubes	10 µL each	–20 °C
Maxpar® 10X Barcode Perm Buffer	50 mL	4 °C
Maxpar Cell Staining Buffer	500 mL	4 °C
Maxpar Fix I Buffer (5X)	15 mL	4 °C
Maxpar PBS	500 mL	4 °C

Required Materials Not Supplied in the Kit

Product Name	Source	Part Number
Consumables		
2 mL microfuge tubes	Major laboratory supplier (MLS)	—
Polypropylene conical tubes with screw cap, 15 mL or 50 mL capacity	MLS	—
Polypropylene round-bottom tubes, 5 mL capacity, 12 x 75 mm	Falcon™	352063
Polystyrene round-bottom tubes with 35 µm cell-strainer cap, 5 mL capacity, 12 x 75 mm	Falcon	352235
1 mL Norm-Ject® latex-free syringes and compatible 0.1 µm syringe filters	VWR (or MLS)	53548-001

Product Name	Source	Part Number
Carousel tubes for CyTOF® XT only:		
Polypropylene skirted conical tube with screw cap, 15 mL capacity, 20 x 76 mm	Sarstedt, Inc.	60.732.001
Viability Reagents—use one of the following:		
Cell-ID Cisplatin	Fluidigm	201064 (100 µL)
Cell-ID Cisplatin-194Pt		201194 (100 µL)
Cell-ID Cisplatin-195Pt		201195 (100 µL)
Cell-ID Cisplatin-196Pt		201196 (100 µL)
Cell-ID Cisplatin-198Pt		201198 (100 µL)
Acquisition Reagents for CyTOF 2 and Helios™:		
EQ™ Four Element Calibration Beads	Fluidigm	201078 (100 mL)
Maxpar Water (for CyTOF 2 and Helios with the HT Injector)		201069 (500 mL)
Maxpar Cell Acquisition Solution (for Helios with the WB Injector)		201240 (200 mL) 201241 (200 mL x 6)
Acquisition Reagents for CyTOF XT™:		
EQ Six Element Calibration Beads	Fluidigm	201245 (100 mL)
Maxpar Cell Acquisition Solution Plus for CyTOF XT		201244 (1 L)
Other Reagents		
Cell-ID Intercalator-Ir	Fluidigm	201192A (125 µM)
Maxpar Fix and Perm Buffer		201067 (100 mL)
Human TruStain FcX™ (Fc-Receptor Blocking Solution)	BioLegend®	422301 (50 tests)/ 422302 (200 tests
Pierce™ 16% Formaldehyde (w/v), Methanol-free	Thermo Fisher Scientific™	28906 (10 x 1 mL)/ 28908 (10 x 10 mL)
Equipment		
Three centrifuges, one for PCR plates/tubes, one for 5 mL tubes, and one for 1.5 mL microfuge tubes	MLS	—
Vacuum aspirator	MLS	—
Vortexer	MLS	—
Stand-alone CyTOF Software for Debarcoding		
For CyTOF 2 and Helios: CyTOF Software v6.3 (or later)	Fluidigm (download from fluidigm.com/software)	108520
For CyTOF XT: CyTOF Software v8.0 (or later)		130069

Barcoding Protocol

Before You Begin

Fix- and perm-sensitive surface epitopes: The barcoding protocol involves fixation and partial permeabilization of cells. Consequently, the ability to recognize and bind to target proteins may be altered for certain antibody clones. It is known that fixation tends to decrease the signal (though in rare cases it can increase the signal), while permeabilization can either decrease or increase the signal. To determine the compatibility of desired clones of surface antibodies with barcoding, it is important to perform a small, preliminary experiment, with and without barcoding, using noncritical samples. For instance, compare surface marker staining profiles of a sample stained in three ways: live-cell-stained, fix-cell-stained, and barcoded and stained. If the amount of available barcodes limits the ability to perform of the preliminary experiment, follow through with the barcoding protocol using Fix I Buffer and Barcode Perm Buffer, but without adding the barcodes. In cases where signal is adversely affected and antibody clones against surface epitopes that are not fixative-sensitive are unavailable, surface staining with fix- and perm-sensitive antibodies on the individual samples may be done prior to the fix and partial permeabilization step for barcoding.

NOTE For inquiries about fixation-sensitive epitopes and compatible antibody clones, contact your local Fluidigm Field Applications Specialist for assistance.

Antibody compatibility: Antibodies labeled with 106Cd and 110Cd metal isotopes are not compatible for use with the Cell-ID 20-Plex Pd Barcoding Kit due to direct mass overlap with the 106Pd and 110Pd metal isotopes in the kit.

Buffers: All Fluidigm products containing saponin are tested for compatibility with this product. The use of alternative saponin-based reagents with this product may result in high background and/or nonspecific staining on Fluidigm CyTOF suspension mass cytometry systems. We recommend that you perform a pilot test with any saponin-based reagents from other suppliers to determine their compatibility with this product.

Quantum dots (Qdots): Qdots labeled with antibodies may be used to boost signal for a particular antigen of interest. Most commercial Qdots contain natural abundance cadmium, which has eight naturally occurring isotopes of masses 106–116 that fall within the detectable range of a mass cytometer. These cadmium isotopes overlap with the mass range for the palladium barcodes and therefore cannot be used together for barcoding.

Cell viability staining: In Step 1 of the protocol, cells can be stained with one of the Cell-ID Cisplatin reagents to identify viable cells (see Required Materials not Supplied in Kit). We recommend that Cell-ID Cisplatin, instead of Cell-ID Intercalator-103Rh be used as a viability stain for barcoding experiments on Fluidigm CyTOF suspension mass cytometry systems. Search for the applicable Technical Data Sheet available at fluidigm.com for usage instructions (see Appendix: Related Documents).

NOTE the following:

- Samples resuspended in serum-containing media, for example complete RPMI with FBS, should be washed in serum-free media or PBS before beginning viability staining with Cell-ID Cisplatin.
- Cell-ID Cisplatin reagent channels may not be compatible with platinum (Pt)-labeled antibodies due to direct mass overlap. Pt-labeled antibodies are compatible with non-overlapping, monoisotopic Cell-ID Cisplatin reagents.

Number of cells per barcode: Barcode signal intensity is inversely proportional to cell concentration. The barcodes in each tube have been pre-optimized to stain 1–3 million cells in 1 mL. In order to ensure consistent barcode staining intensity, count cells prior to barcoding and adjust so that 1–3 million cells will be stained for each sample.

NOTE the following:

- The presence of unwanted cells such as red blood cells (RBCs), or of platelets, debris, etc., will impact the barcode signal intensity of the sample. To achieve the best-quality barcode staining it is important to minimize and/or account for unwanted cells and debris when barcoding samples. See Sample Validation recommendations under Tips and Tricks for staining less common sample preparations.
- For barcoding small sample sizes that are less than 1 million cells, scale down the amount of barcode and barcode staining volume. Perform a pilot experiment to validate this protocol change on samples and panels using noncritical samples. Make aliquots of the remaining barcode and store at –20 °C for one-time future use.

Thawing barcodes: Immediately before beginning the protocol, obtain the necessary barcodes and return the remaining kit components to the –20 °C freezer. Allow 10 minutes for the barcodes to warm up, then briefly centrifuge to ensure that all liquid reaches the bottom of the tube.

Formaldehyde solution: It is critical to prepare fresh formaldehyde (FA) solution to effectively fix cells stained with the Maxpar antibodies. Be sure to open the single-use formaldehyde ampule and prepare the FA solution immediately before use in the fixation process [see Fix Cells (Fresh Fix)].

Centrifugation speeds: For cell centrifugation steps, centrifugation should be performed at 300 x *g* for 5 min before cell fixation, and at 800 x *g* for 5 min after cell fixation. The increased centrifugation speed after cell fixation results in greater cell recovery. When centrifuging sample volumes greater than 5 mL in a single tube, increase centrifugation time to 10 min for greater cell recovery.

Reagents and Solutions to Prepare in Advance

Fix I Buffer: Prepare 1 mL for each sample to barcode by mixing 1 part Maxpar Fix I (5X) buffer with 4 parts Maxpar PBS. Prepare fresh for same day use.

Barcode Perm Buffer: Prepare 4 mL for each sample to barcode by mixing 1 part Maxpar 10X Barcode Perm Buffer with 9 parts Maxpar PBS. Prepare fresh for same day use.

Antibodies: Centrifuge the stock antibody at 12,000 x *g* for 5 min to sediment antibody aggregates.

Antibody cocktail: Prepare antibody cocktails according to the staining protocol appropriate for your antibody panel. The antibody cocktail should be prepared fresh for same day use and temporarily stored at 2–8 °C before use.

Intercalation Solution: Prepare 1 mL of intercalation solution for each sample to barcode by adding 1 µL of 125 µM Cell-ID Intercalator-Ir into 1 mL of Maxpar Fix and Perm Buffer to a final concentration of 125 nM (a 1,000X dilution of the 125 µM stock solution) and vortex to mix. Include 10% volume overage for multiple samples processed together. Prepare fresh for same day use.

1.6% FA solution: Prepare a fresh 1.6% formaldehyde (FA) solution from the 16% formaldehyde stock ampule. Use a 1 mL Norm-Ject latex-free syringe and compatible 0.1 µm syringe filter to filter the stock formaldehyde, and then dilute 1 part of the filtered stock formaldehyde with 9 parts Maxpar PBS.

Tips and Tricks

Multiwell handling: The barcoding protocol is amenable to multiwell pipetting and aspiration. Instead of individual 5 mL tubes, users may barcode cells in a deep V bottom 96-well plate (Corning, Product No. 3961) or other multiwell polypropylene plate or tube with a minimum 2 mL volume capacity prior to transferring to the combined tube. Take care when aspirating to ensure that the pellet is not disrupted or lost and that consistent residual volumes of supernatant remain in the well or tube. The careful use of a multiwell aspiration apparatus is recommended (Zunder, E.R. et al. 2015).

Reagent handling: Retrieve, mix, and centrifuge reagents as directed. See the individual reagent Product Information Sheet or Technical Data Sheet for usage instructions.

Reagent titration: We recommend titrating antibodies and other reagents for optimal performance and data quality in individual experiments. See the individual reagent Product Information Sheet or Technical Data Sheet for usage instructions.

Antibody usage: Maxpar metal-conjugated antibodies have been optimized to stain up to 3 million live cells with 1 µL of antibody in 100 µL of staining volume. Users should perform a titration of cells with their optimized antibody panel to assess whether a higher concentration of cells (up to 30 million cells/mL) may be used to achieve optimal staining performance. If a higher concentration of cells may be used, less antibody is required and the final staining volume of the barcoded cells should be adjusted accordingly.

Sample validation: Cell-ID 20-Plex Pd Barcoding Kit was validated for use with peripheral blood mononuclear cells (PBMC). It is recommended that users with less common sample preparations/cell types validate their samples for use with the barcoding reagent. For instance, samples with a high degree of unwanted cells (for example, RBCs and platelets) or debris may demonstrate diminished barcoding efficiency with the described protocol. It is recommended that users perform a pilot experiment to ensure effective barcoding.

Barcoding Protocol Steps

Prepare Samples

1 Perform cell viability staining on cells prior to fixation.

2 Resuspend cells in Maxpar Cell Staining Buffer and aliquot 1–3 million cells into individual 5 mL polypropylene tubes for each sample to be barcoded.

NOTE Up to 20 samples can be prepared using this protocol.

3 Centrifuge each sample at 300 x *g* for 5 min, carefully aspirate supernatant, and gently vortex.

Fix and Permeabilize

IMPORTANT It is essential to thoroughly disrupt the pellet by gentle vortexing before adding the fixative.

1 Resuspend each 1–3 million cell sample completely in 1 mL of 1X Fix I Buffer and incubate for 10 min at room temperature.

2 Centrifuge each sample at 800 x *g* for 5 min, carefully aspirate supernatant, and gently vortex.

NOTE The increased centrifuge speed after cell fixation results in greater cell recovery.

3 Repeat wash by adding 1 mL of 1X Barcode Perm Buffer per 1–3 million cell sample, centrifuge samples at 800 x *g* for 5 min, carefully aspirate supernatant, and gently vortex.

Barcode

1 Resuspend each 1–3 million cell sample to be barcoded completely in 800 µL of 1X Barcode Perm Buffer.

2 Resuspend each barcode completely in 100 µL of 1X Barcode Perm Buffer and transfer them to the appropriate samples. Immediately pipet to completely mix the sample.

IMPORTANT It is essential to thoroughly disrupt the pellet by gentle vortexing before adding the barcodes. Thorough mixing is required for uniform barcode staining.

3 Incubate samples at room temperature for 30 min. After 15 min, gently vortex each tube to mix.

4 Centrifuge each sample at 800 x *g* for 5 min, carefully aspirate supernatant, and gently vortex to resuspend.

5 Repeat wash by adding 2 mL of Maxpar Cell Staining Buffer per 1–3 million cell sample, centrifuge samples at 800 x *g* for 5 min, carefully aspirate supernatant, and gently vortex.

6 Resuspend each sample completely in 100 µL of Maxpar Cell Staining Buffer.

7 Combine all barcoded samples into 1 tube. For larger scale experiments, the maximum wash volume will dictate the optimal tube size depending on the number of cells stained:

Number of Cells	Optimal Tube Size (mL)
1–3 million	2
3–15 million	5
15–45 million	15
45–60 million	50

8 Rinse each individual barcoded sample tube an additional 2 times with 100 µL of Maxpar Cell Staining Buffer and transfer to the combined tube from Step 7 to maximize cell recovery.

9 Reserve a small volume (approximately 10 µL) from the combined tube to count cells in the multiplexed sample, to ensure optimal antibody staining.

NOTE Combining barcoded samples may lead to cell loss and therefore a lower cell count than the initial cell count before barcoding.

10 Centrifuge cells at 800 x *g* for 5 min, carefully aspirate supernatant, and gently vortex.

Stain Cells with Antibody

Proceed with the staining protocol appropriate for your antibody panel. Scale, stain, and wash volumes to accommodate the number of cells in the multiplexed sample.

NOTE the following:

- See Antibody usage under Tips and Tricks to learn how to effectively scale your antibody panel on barcoded samples.
- Perform centrifugation at 800 x *g* during antibody staining steps.

Fix Cells (Fresh Fix)

IMPORTANT It is essential to thoroughly disrupt the pellet by vortexing before adding the fresh fixative.

1 Add 1 mL of the 1.6% FA solution per 1–3 million cells (for example, for 10 million cells, add 4 mL of 1.6% FA solution) and gently vortex to mix well.

2 (Optional) Reserve a small volume (approximately 10 µL) to count cells, to ensure optimal Cell-ID Intercalator-Ir staining.

3 Incubate cells at room temperature for 10 min. If you reserved volume, go to Step 4.

4 (Optional) During incubation, count cells in the reserved volume. Make sure to note the cell concentration.

5 After incubation is complete, centrifuge cells at 800 x *g* for 5 min.

6 Carefully aspirate and remove supernatant. Gently vortex to resuspend cells in residual volume.

Stain Cells with Cell-ID Intercalator-Ir

1 Add 1 mL of the intercalation solution per 1–3 million cells (for example, for 10–12 million cells, add 4 mL of the intercalation solution) and gently vortex. Ensure cells are well resuspended.

2 Incubate the samples at room temperature for 1 hr or leave at 2–8 °C overnight.

STOPPING POINT Samples can be stored in the intercalation solution at 2–8 °C for up to 48 hr before sample acquisition.

Prepare Cells for Acquisition

IMPORTANT Run cells on the same day they are washed from intercalation solution.

Samples stained with the Cell-ID 20-Plex Pd Barcoding Kit can be acquired on CyTOF 2, Helios (HT or WB Injector), or CyTOF XT systems. Follow the applicable section below to prepare cells for acquisition. For more information, refer to your instrument user guide or select the Help tab in CyTOF Software v7.0 (or later) (see Appendix: Related Documents).

For Helios (HT or WB Injector) and CyTOF 2

IMPORTANT For Helios users, check with your Helios operator to confirm whether the HT or WB Injector is in use.

1 After incubation in the intercalation solution, thoroughly resuspend the cells by gently vortexing. Count a 10 µL aliquot of the cells, then centrifuge tubes containing cells in intercalation solution at 800 x *g* for 5 min. Carefully aspirate supernatant and resuspend cells in residual volume by gently vortexing.

NOTE Wash steps after staining may lead to cell loss and therefore a lower cell count than the initial cell count before staining.

2 Wash cells by adding 2 mL of Maxpar Cell Staining Buffer per 1–3 million cell sample and gently vortex to mix. Centrifuge at 800 x *g* for 5 min, carefully aspirate supernatant, and resuspend cells in residual volume by gently vortexing.

3 Repeat wash by adding 2 mL of Maxpar Cell Staining Buffer per 1–3 million cell sample.

4 Aliquot samples into new 5 mL polypropylene tubes in Maxpar Cell Staining Buffer according to instrument recommendations:

Instrument	Recommended Concentration
Helios (WB or HT Injector)	0.5–1 x 10^6 cells/mL (up to 4 x 10^6 cells/4 mL)
CyTOF 2	0.25–0.5 x 10^6 cells/mL (up to 2 x 10^6 cells/4 mL)

5 Centrifuge at 800 x *g* for 5 min and carefully aspirate supernatant.

STOPPING POINT Pelleted aliquots can be stored in Maxpar Cell Staining Buffer at 2–8 °C on the day of acquisition until ready to acquire samples.

6 Gently vortex to resuspend cells in residual volume. Wash cells by adding 2 mL of the appropriate acquisition reagent per 1–3 million cell sample, as shown below. Gently vortex to mix, centrifuge at 800 x *g* for 5 min, and carefully aspirate supernatant.

Instrument	Acquisition Reagent
Helios (WB Injector)	Maxpar Cell Acquisition Solution
CyTOF 2 or Helios (HT Injector)	Maxpar Water

7 Repeat Step 6 for a total of 2 washes.

NOTE The second wash is essential to remove residual Maxpar Cell Staining Buffer from samples before acquisition.

8 Prepare a sufficient volume of 0.1X EQ Four Element Calibration Beads (EQ4 beads) to resuspend all aliquots to the recommended cell concentration for acquisition by diluting 1 part beads to 9 parts of the appropriate acquisition reagent, as shown below.

Instrument	Acquisition Reagent	Cell Concentration
Helios (WB Injector)	Maxpar Cell Acquisition Solution	0.5–1 x 10^6 cells/mL
Helios (HT Injector)	Maxpar Water	0.5–1 x 10^6 cells/mL
CyTOF 2	Maxpar Water	0.25–0.5 x 10^6 cells/mL

9 Gently vortex to resuspend cells in residual volume.

10 Immediately before sample acquisition, resuspend cells in the diluted 0.1X EQ Four Element Calibration Beads and filter sample through appropriately sized cell strainers (for example, 35–45 µm mesh).

For CyTOF XT

1 After incubation in the intercalation solution, thoroughly resuspend the cells in the intercalation solution by gently vortexing. Count a 10 µL aliquot of the cells, then centrifuge tubes at 800 x *g* for 5 min.

NOTE Wash steps after staining may lead to cell loss and therefore a lower cell count than the initial cell count before staining.

2 Carefully aspirate supernatant and resuspend cells in residual volume by gently vortexing.

3 Wash cells by adding 2 mL of Maxpar Cell Staining Buffer per 1–3 million cell sample and gently vortex. Centrifuge tubes at 800 x *g* for 5 min.

4 Carefully aspirate supernatant and resuspend cells in residual volume by gently vortexing.

5 Repeat Steps 3–4 once for a total of 2 washes with Maxpar Cell Staining Buffer.

6 Wash cells by adding 2 mL of Maxpar Cell Acquisition Solution Plus for CyTOF XT per 1–3 million cell sample and gently vortex. Centrifuge tubes at 800 x *g* for 5 min.

7 Carefully aspirate supernatant and resuspend cells in residual volume by gently vortexing.

8 Repeat wash by adding another 2 mL of Maxpar Cell Acquisition Solution Plus for CyTOF XT per 1–3 million cell sample and gently vortex.

NOTE The second wash with Maxpar Cell Acquisition Solution Plus for CyTOF XT is essential to remove residual Maxpar Cell Staining Buffer from samples before acquisition.

9 Prepare samples for acquisition according to the sample format:

Sample Format	Sample Preparation Procedure
Suspension	**1** Centrifuge tubes at 800 x *g* for 5 min. **2** Carefully aspirate and discard supernatant. **3** Prepare a sufficient volume of 0.1X EQ Six Element Calibration to completely resuspend all samples to a recommended cell concentration of 0.5–1 x 10^6 cells/mL (including approximately 100 µL extra volume per sample), by diluting 1 part beads to 9 parts Maxpar Cell Acquisition Solution Plus for CyTOF XT. **4** Immediately prior to data acquisition, resuspend cells in the diluted 0.1X EQ Six Element Calibration Beads. **5** Filter cells through 35 µm cell strainer cap tubes into a new 15 mL skirted polypropylene carousel tube. **IMPORTANT** Cells must be filtered before CyTOF XT acquisition.
Pelleted	**1** Filter cells through 35 µm cell strainer cap tubes into new 5 mL polypropylene tubes. **IMPORTANT** Cells must be filtered before CyTOF XT acquisition. **2** Centrifuge tubes at 800 x *g* for 5 min. **3** Carefully aspirate (leaving approximately 100 µL in the tube) and discard supernatant. **4** Leave cells pelleted at 2–8 °C in the chilled Autosampler carousel until ready to run. Load EQ Six Element Calibration Beads in carousel Location 14 before startup. **NOTE** The instrument operator should keep the samples pelleted when loading them into the Autosampler carousel.

Acquire Data

1 Prior to starting acquisition:

- Select to collect the following channels in addition to the channels corresponding to the antibody panel:

Instrument	Category	Channels
CyTOF 2, Helios, or CyTOF XT	Barcodes	102Pd, 104Pd, 105Pd, 106Pd, 108Pd, and 110Pd
	Cell-ID Intercalator-Ir	191Ir and 193Ir
	Common environmental contaminants	120Sn, 127I, 131Xe, 133Cs, 138Ba, 190Os, and 208Pb
CyTOF 2, Helios, or CyTOF XT	Cell-ID (dead cells)	194Pt, 195Pt, 196Pt, and/or 198Pt
CyTOF 2 or Helios	EQ4 Beads*	140Ce, 151/153Eu, 165Ho, and 175Lu
CyTOF XT only	EQ6 Beads*	89Y, 115In, 140Ce, 159Tb, 175Lu, and 209Bi
	Maxpar® Direct™ Immune Profiling Assay™ (EQ4-MDIPA*)	140Ce, 151/153Eu, 165Ho, and 175Lu

* Select in Template Editor

- (CyTOF XT only) Do the following:
  - Check all sample acquisition settings in the Acquisition Protocol.
  - Add sufficient EQ6 beads for tuning and sample acquisition (if applicable) in Location 14 of the carousel.

2 Acquire barcoded sample on a CyTOF 2, Helios, or CyTOF XT instrument.

Debarcoding

The collected file contains combined data for all multiplexed samples and therefore must be debarcoded in order to perform downstream analysis of the individual samples. The Debarcoder application assigns each event in the multiplexed file to the barcode population matching its Pd isotope labeling pattern, performs filtering to eliminate uncertain events, and creates separate flow cytometry standard (FCS) files for each barcoded sample for downstream analysis.

The multiplexed file contains a mixture of desirable single-cell events and undesirable events such as debris, cross-sample aggregates, and cross-sample ion cloud fusions. Optimal debarcoding results in accurate assignment of the maximum number of desirable single-cell events with minimal inclusion of undesired or uncertain events in the debarcoded output files.

When a file is loaded into the Debarcoder, each event is assigned to a barcode population by matching the identities of its three brightest Pd isotopes to the corresponding barcode key. Any events with fewer than three Pd stains or with poor correlation to any other events in the file are removed from the data. The Debarcoder provides two calculated parameters that are used to eliminate undesired events from the debarcoded populations: Barcode Separation (BcS) and Mahalanobis Distance (MD). It is best to perform primary filtering using BcS, and use MD as a secondary filter only when debarcoding samples with a high incidence of cross-sample aggregates, such as cell-debris aggregates.

Barcode Separation

Barcode Separation (BcS) is a measure of the rescaled intensity difference between the isotopes with the third- and fourth-highest intensities. Rescaling is done by dividing each Pd intensity by the Mean + 2 SD value for the brightest Pd in the barcode. This corrects for differences in overall staining intensities that may result from cell-specific variation in Pd uptake or variations in cell number among the barcoded samples. The BcS value is low for debris events that have low intensity values for all Pd isotopes and for cross-sample cell aggregates that have high intensity values for four or more Pd isotopes. By applying a minimum BcS filter, these undesired events are filtered out of the barcoding results.

Mahalanobis Distance

Mahalanobis Distance (MD) quantifies the distance, in six-dimensional Pd intensity space, between an event and the distribution of its assigned barcode population. Cross-sample aggregates, including cell-debris aggregates, have higher intensities in at least one Pd isotope than the average single cell in the population and stray from the center of the single-cell cluster in Pd intensity space, resulting in higher MD values. Therefore, application of a lower MD value permits removal of events with inconsistent Pd intensities. These undesired events can be excluded by reducing the maximum MD filter value. However,

barcoded samples may contain valid cells that differ in Pd uptake from the majority of the events in the sample, and such events will be eliminated with a stringent MD filter. As a result, MD should be used cautiously as a secondary filter in experiments that do not contain significant cell-specific variations in Pd uptake in any of the barcoded samples.

Debarcoding Protocol

Download and Install the Debarcoder

The Debarcoder is a feature within CyTOF software v6.3 or later. The software can be installed on a stand-alone Windows® 7 or later 64-bit computer workstation.

IMPORTANT This software should **not** be installed on CyTOF 2 instrument workstations.

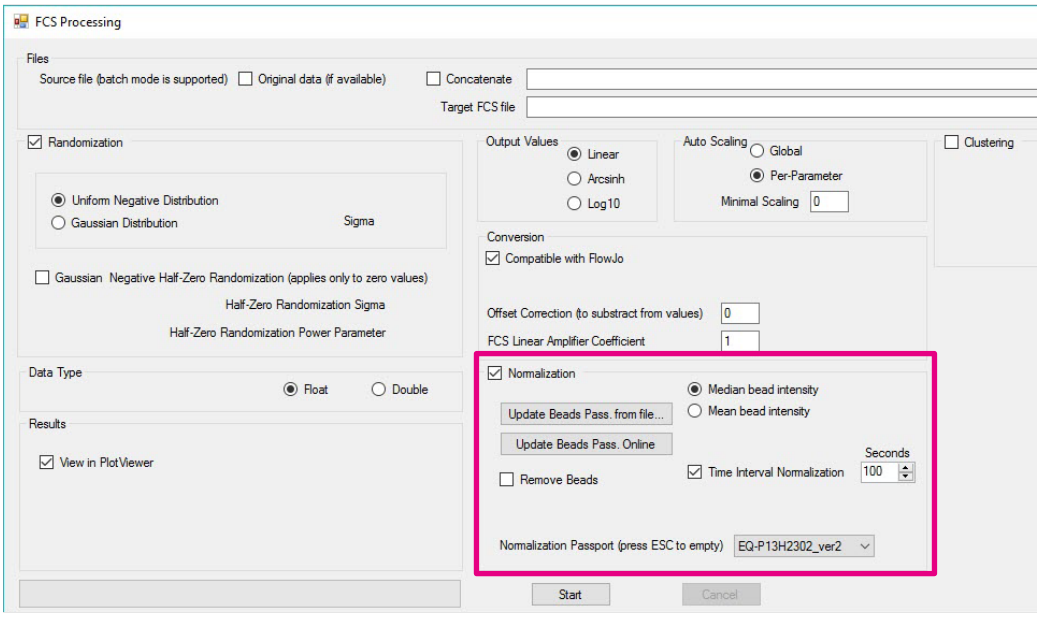
1. Go to fluidigm.com/software to download the latest installer for CyTOF Software v7.0 (for Helios) or CyTOF Software v8.0 (for CyTOF XT).
2. Go to fluidigm.com to search for and download the CyTOF Software v7.0 Release Notes (400338) or CyTOF Software v8.0 Release Notes (FLDM-00474), and follow the installation instructions.
3. Click the icon to launch the CyTOF Software.
4. Log in to Administrator mode. The CyTOF software interface opens.

Normalize the File

NOTE Samples acquired on CyTOF XT are automatically normalized. Transfer the normalized FCS files from their saved folder to a location for further analysis. Normalized files have the suffix **_Processed** after the Sample Name.

1. (CyTOF 2 and Helios) For CyTOF Software v7.0:
   a. Under the **Process** tab, click **FCS Processing**.

b Perform normalization on the barcoded file.



NOTE If the same barcoded sample is acquired over multiple barcoded files, they must be normalized first and then concatenated. Refer to the Normalization of Mass Cytometry Data Using EQ Four Element Beads Technical Note (400237) for more information.

2 If multiple sample tubes have been acquired, **Concatenate** to merge the normalized data files into one larger FCS file.

IMPORTANT It is necessary to concatenate before debarcoding.

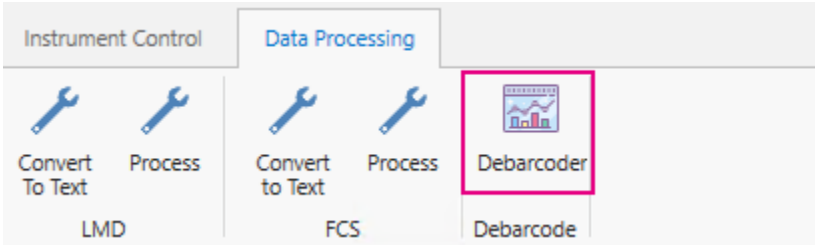
Debarcode the File

IMPORTANT Read the introductions to Debarcoding and Debarcoding Example sections for important guidance on use of the debarcoding filters.

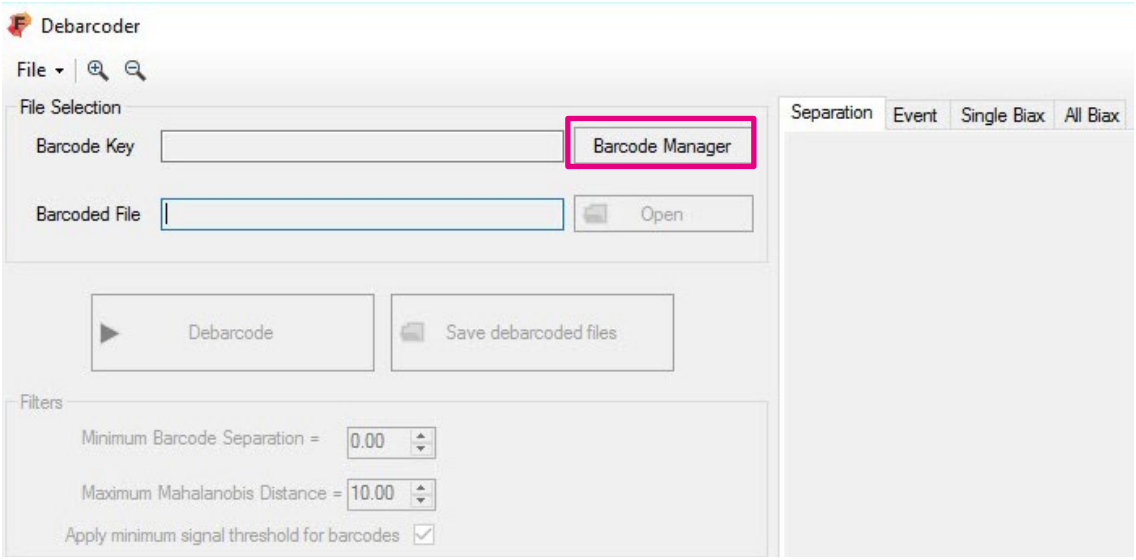
1 Open the Debarcoder:

- (CyTOF 2 and Helios) For CyTOF Software v7.0, in the **Process** tab, open the **Debarcoder**.

- (CyTOF XT) For CyTOF Software v8.0, in the **Data Processing** tab, open the **Debarcoder**.

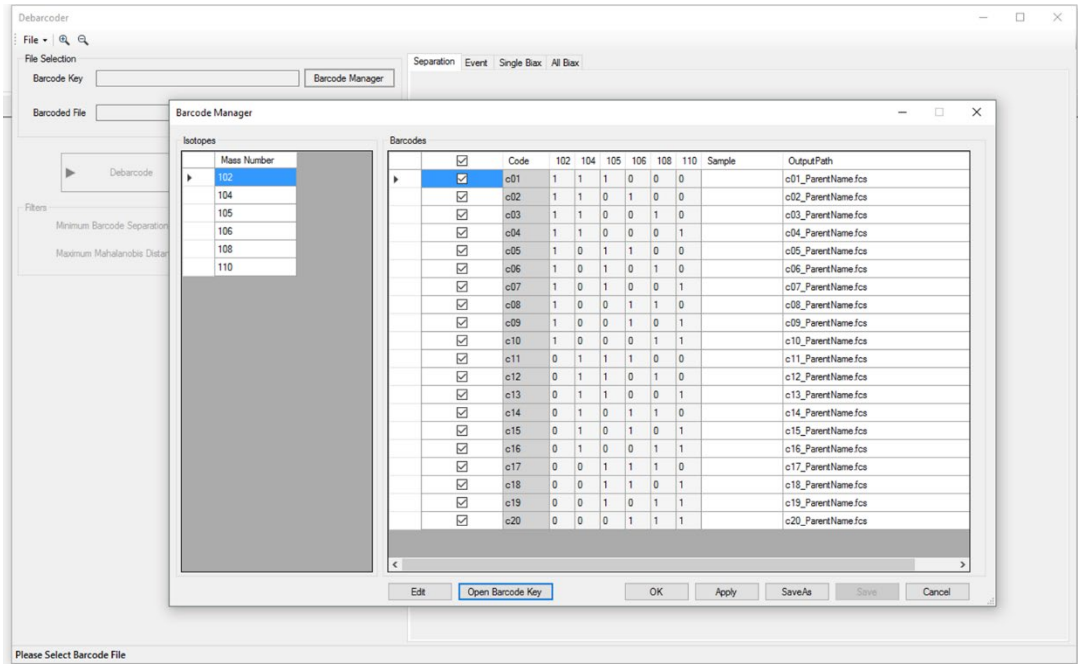


2 Click **Barcode Manager**.



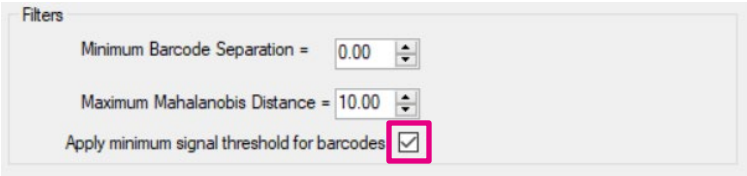
3 Go to fluidigm.com/singlearticles/palladium-based-barcoding to download the barcode key file.

4 Open the Barcode Key file (**Key_CellID_20-Plex_Pd.csv**) and modify the file. This file indicates the masses of the 6 Pd isotopes. The 3-digit Pd isotope combinations correspond to the code numbers found on the tubes in the kit. The Output Path represents the names of the files that will be generated when debarcoding is completed.



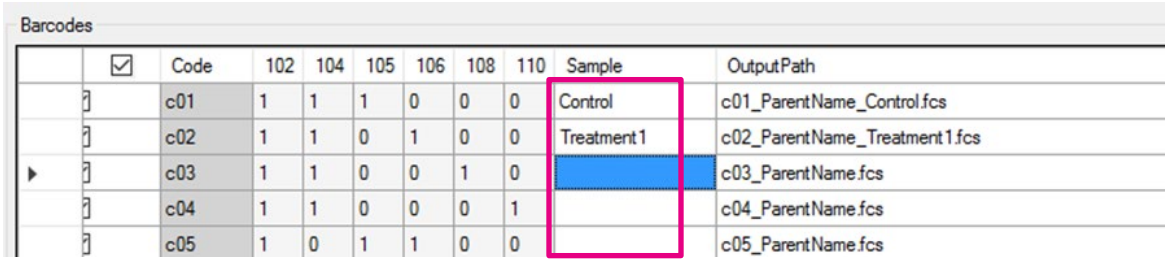
5 If necessary, in the Filters box uncheck the **Apply minimum signal threshold for barcodes** feature, which is checked by default.

NOTE This threshold is applied to remove events with signals lower than 35 dual counts per barcode channel. However, if the barcoding intensity or efficiency is low, unchecking this box enables the feature to increase recovery of low-intensity events by removing the lower signal threshold for barcodes.



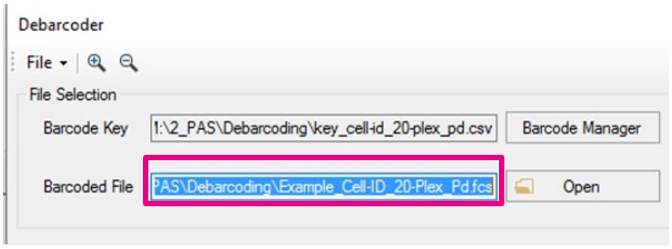
6 Modify the file:

a Enter unique sample names in the Sample column.



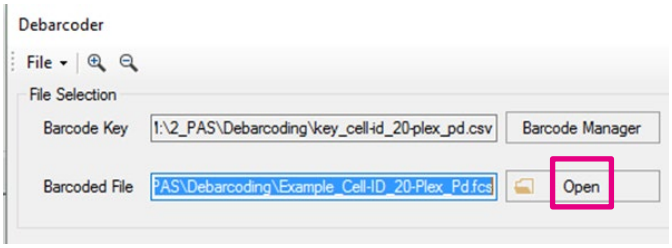
b Uncheck any barcodes that are not used in the experiment.

c (Optional) Click **SaveAs**. Save the resulting barcode key with a unique name.

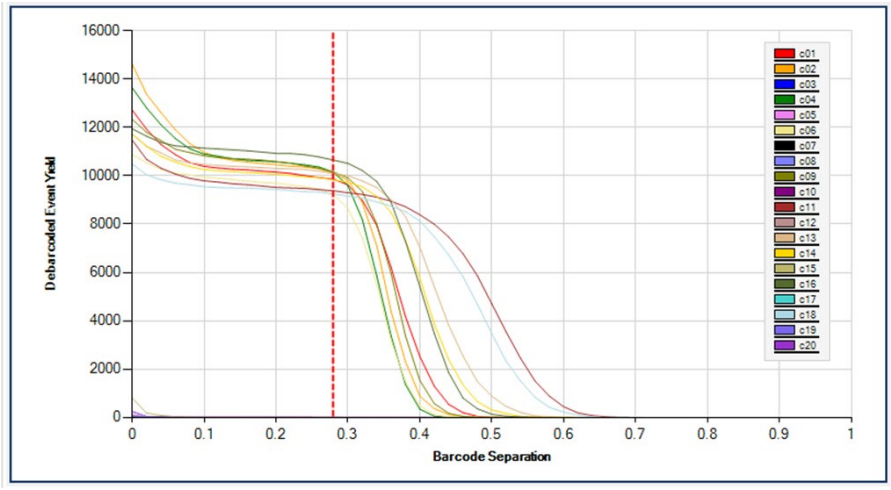


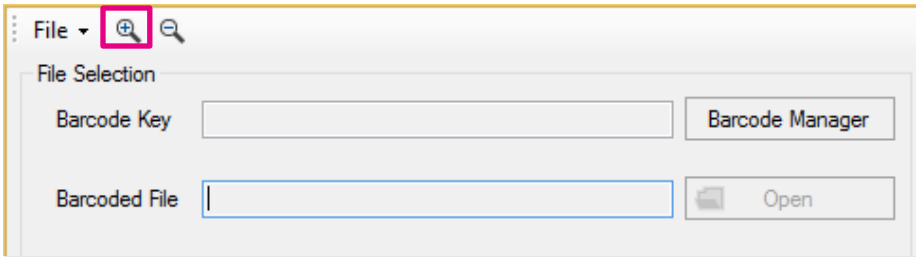
d Click **OK** to apply changes and close the Barcode Manager window.

7 Click **Open** to browse for the normalized barcoded FCS file, then click **Debarcode**.

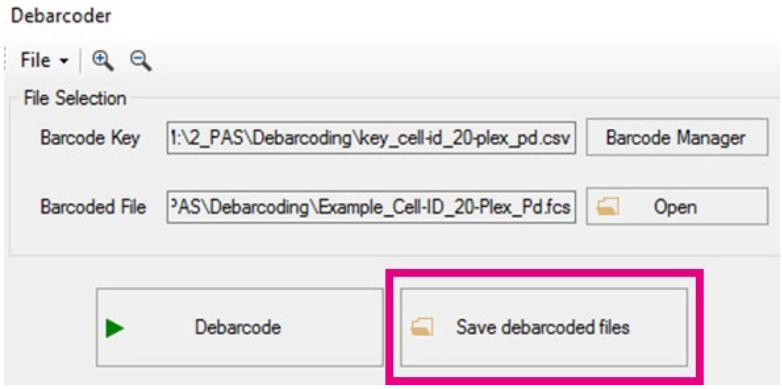


8 Filter barcode assignments by choosing optimal minimum Barcode Separation (BcS) and maximum Mahalanobis Distance (MD) values. Refer to the introduction to Debarcoding section above for more information.

Action	Procedure
Choose a minimum BcS	1 In the Separation tab, observe the barcode separation plots and choose a minimum BcS value just before the estimated event yield dramatically drops. The minimum BcS value is displayed as a vertical dotted red line on the plots. 

Action	Procedure
Choose a minimum BcS *(continued)*	**2** In the Event tab, magnify the Event plot by using the zoom icon at top-left in the Debarcoder until individual events are visible.  **3** In the Event plot, if there are events with poor intensity separation between the third and fourth isotopes, consider increasing the minimum BcS value. 
(Optional) Choose a maximum MD	**1** In the Single Biax or All Biax tab, select to **Color Plot by Mehalanobis Distance**. **2** Observe each code. **3** Choose a maximum MD value that eliminates outliers.

9 Click **Save debarcoded files** to generate and save FCS files for each debarcoded sample.



NOTE Save a screenshot of your debarcoding parameters for future reference or data validation.

10 After debarcoding, remove bead-cell doublet events prior to data analysis by gating on 140Ce-negative events and remove dead cells by gating on negative events in the channels for the viability reagents. For a complete manual cleanup strategy, see pages 6–8 of the Approach to Bivariate Analysis of Data Acquired Using the Maxpar Direct Immune Profiling Assay Technical Note (400248).

NOTE If you experience low barcoding staining or efficiency or other difficulties, contact your local Fluidigm Field Applications Specialist for assistance.

Debarcoding Example

Download the example Cell-ID 20-Plex Barcoding FCS file (**Example_Cell-ID_20-Plex_Pd.fcs**) from fluidigm.com/singlearticles/palladium-based-barcoding. For this example experiment, 10 samples containing the same number of cells were barcoded with unique barcodes and combined into one tube. Data for 160,000 events were acquired on the CyTOF 2 instrument.

Looking at the BcS vs. Debarcoded Event Yield plot in the Separation tab in Figure 2, a minimum BcS value of 0.24 can be set without negatively impacting the estimated yield in any of the samples.

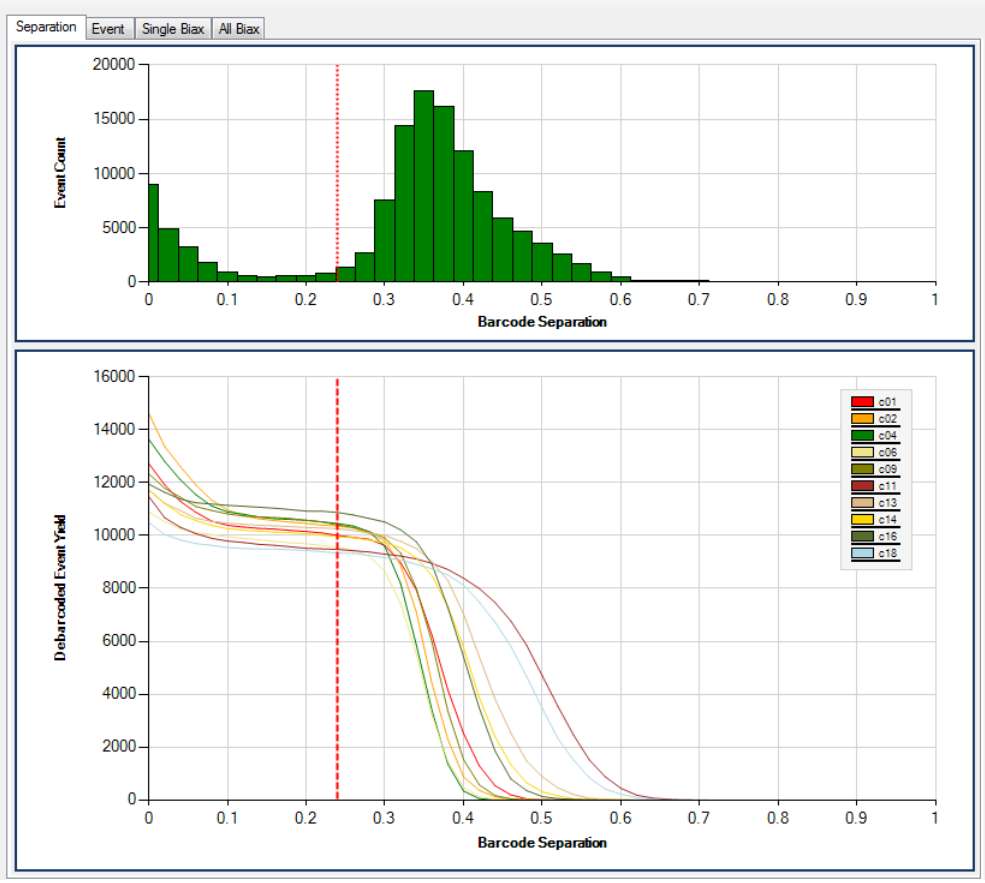


Figure 2. The BcS vs. Debarcoded Event Yield plot in the Plot Separation tab of the Debarcoder

The Event plot for barcode population 1 (Figure 3) shows clear separation between the three positive Pd isotopes and the three negative Pd isotopes, with very few events appearing in the gap. This is expected, since events with BcS values less than 0.24 are eliminated by the filter.

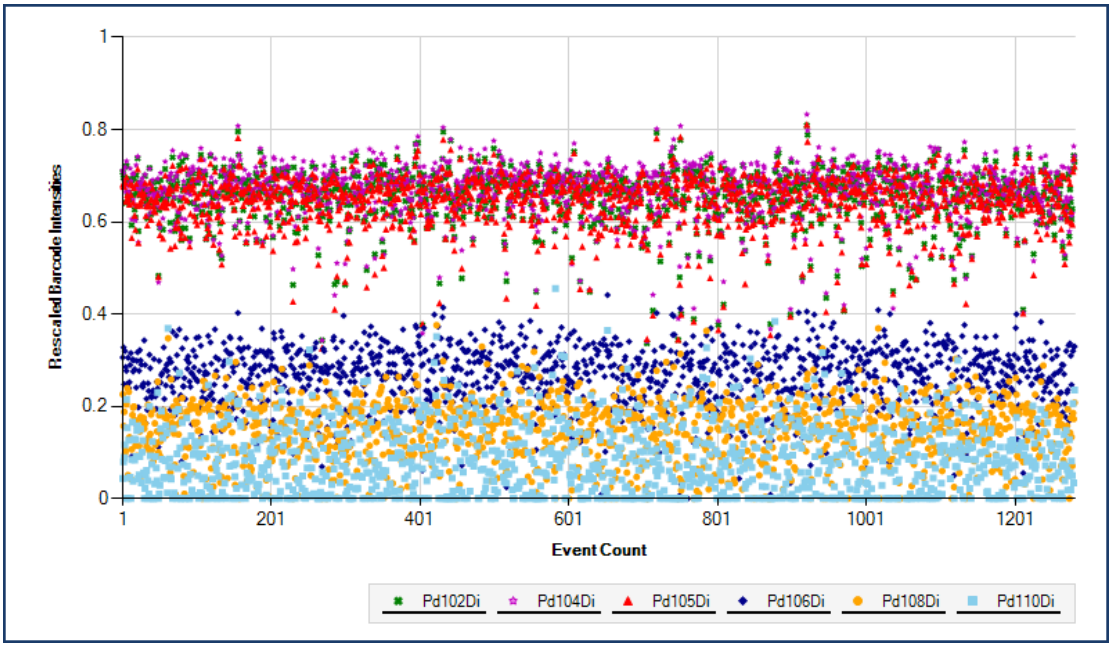


Figure 3. The Event plot in the Debarcoder

Examination of the All Biax plots for c01 reveals some outliers with high MD values, heat-mapped blue or green (top of Figure 4).

NOTE Most of these outliers have low intensities for the Pd isotopes in the code (102, 104, 105) and are therefore likely debris events.

These events were excluded by lowering the MD filter from 10 to 5 with minimal impact on estimated barcode yields (bottom of Figure 4).

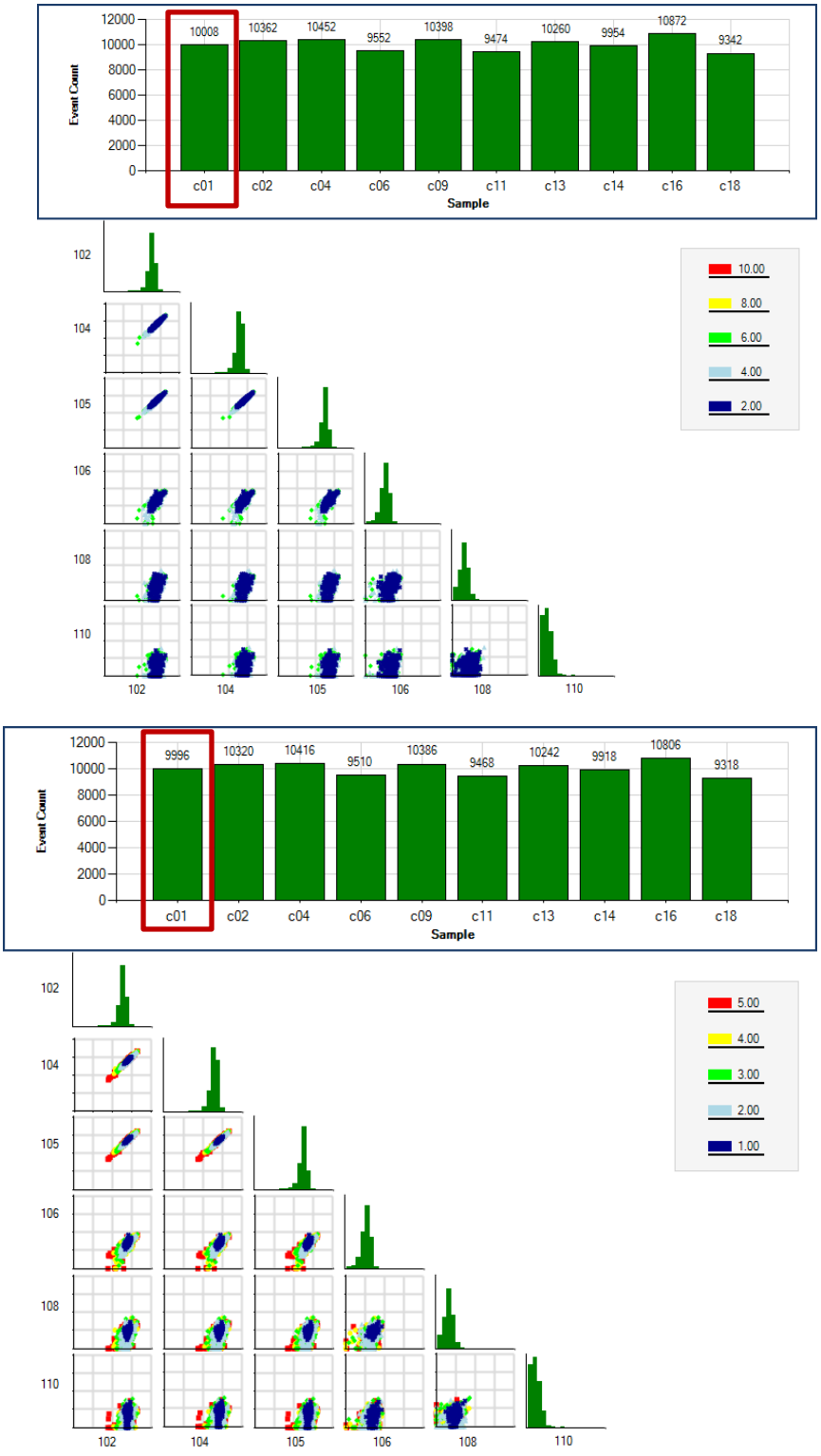


Figure 4. Upper bar graphs: The Event count plots for the samples in the Debarcoder. Lower biaxial plots: Sample c01 datapoints are heat-mapped according to MD values and the scale indicated to the right of each plot set. Debris events were removed by lowering the MD filter (lower).

The Event plots in Figures 5–7, magnified using the zoom icon (at top-left in the Debarcoder), show events with the maximum MD filter set to 10 (Figure 5), 5 (Figure 6), or 4 (Figure 7). Event 269 is likely debris because its positive Pd intensities are similar in value to the background in the other events. Event 286 is less clearly debris, so it should be included (because it can be analyzed more completely after debarcoding). As a result, we chose an MD filter value of 5.

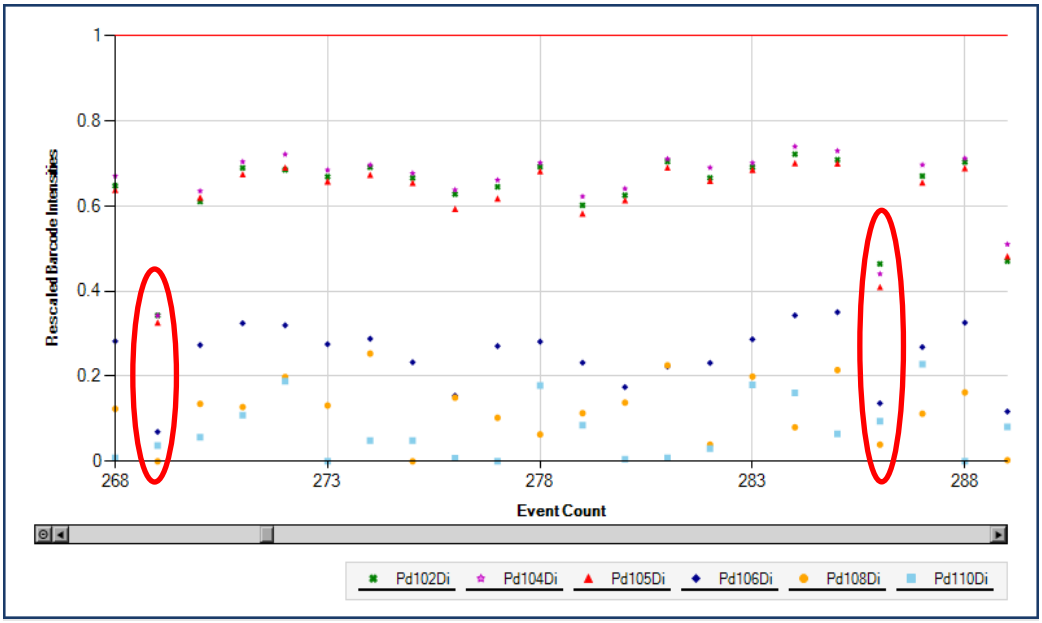


Figure 5. Magnified Event plots in the Debarcoder. The MD filter is set to 10.

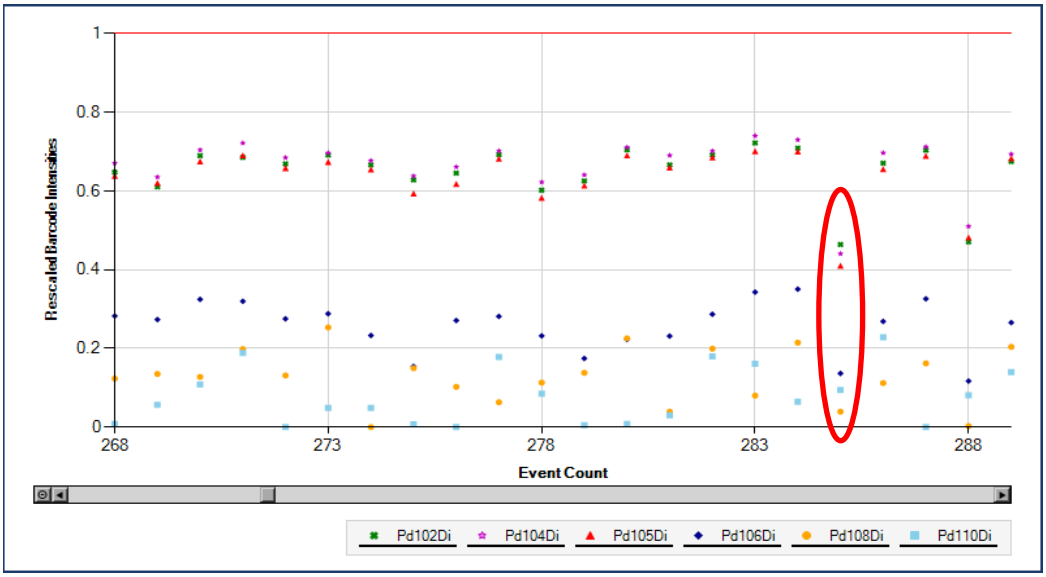


Figure 6. Magnified Event plots in the Debarcoder. The MD filter is set to 5. Event 269, seen in Figure 4 (MD=10), has been removed when the MD filter is set to 5.

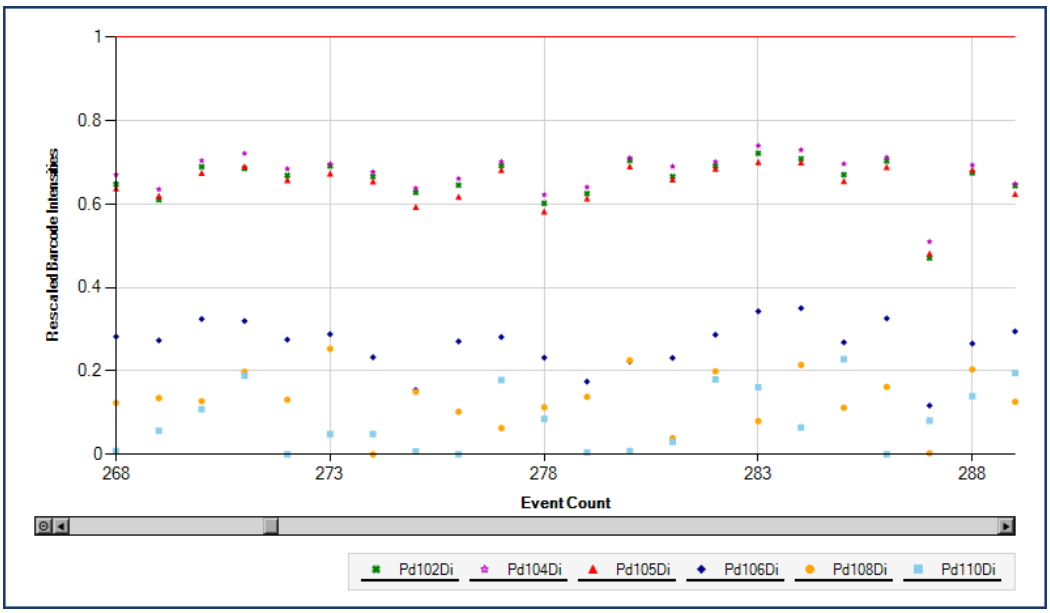


Figure 7. Magnified Event plots in the Debarcoding software. The MD filter is set to 4. Events 269 and 286, seen in Figure 4 (MD=10), have been removed when the MD filter is set to 4.

Appendix

References

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Related Documents

Go to fluidigm.com to download these related documents.

Title	Document Number
Cell-ID Cisplatin Product Information Sheet	PRD018
Cell-ID Cisplatin-194Pt Technical Data Sheet	TDS-00013
Cell-ID Cisplatin-195Pt Technical Data Sheet	TDS-00014
Cell-ID Cisplatin-196Pt Technical Data Sheet	TDS-00015
Cell-ID Cisplatin-198Pt Technical Data Sheet	TDS-00016
Cell-ID Intercalator-Ir Technical Data Sheet	TDS-00703
Cell-ID Intercalator-103Rh Technical Data Sheet	TDS-00702
CyTOF Software v7.0 Release Notes	400338
CyTOF Software v8.0 Help for CyTOF XT (installed with CyTOF Software v8.0)	FLDM-00045
CyTOF Software v8.0 Release Notes	FLDM-00474
CyTOF XT User Guide	FLDM-00254
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