

Cell-ID 20-Plex Pd Barcoding Kit

USER GUIDE

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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 20 samples so they can be combined and subsequently stained and acquired as one multiplexed sample, followed by software debarcoding and individual sample analysis. Multiplexing samples improves data quality because the 20 samples are stained, processed, and acquired as one sample, eliminating sample-specific staining and data collection variation.

This user guide includes protocols for barcoding samples and for debarcoding multiplexed files.

Kit Contents and Storage Conditions

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

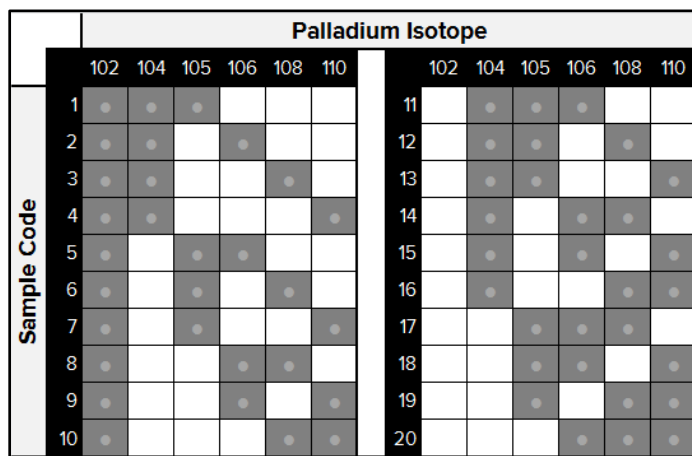


Figure 1 Schematic representation of the barcoding patterns of 20 samples barcoded with six different Palladium isotopes. Each barcode consists of a unique combination of the three palladium isotopes.

Barcoding Protocol

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.

Required Reagents, Equipment, and Materials not Supplied in Kit

Product Name	Company Name	Part Number
Centrifuge rotor that holds PCR plates	Various	Various
BD Falcon™ 12 x 75 mm tubes, polypropylene, 5 mL	BD	352002
Cell-ID Cisplatin	Fluidigm	201064
Cell-ID Intercalator-Ir		201192A/201192B
EQ™ Four Element Calibration Beads	Fluidigm	201078

Solutions to Prepare in Advance:

- **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. Store at 4 °C for up to one week.
- **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. Store at 4 °C for up to one week.
- **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 mix by vortexing.

NOTE Cell-ID Intercalator-Ir concentration can be titrated for individual cell types and experiments for optimal Cell-ID Intercalator-Ir staining. It is recommended that the Cell-ID Intercalator-Ir concentration in the staining solution not exceed 1 µM.

Important Notes before Starting:

- **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 performance 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 As a point of reference, large antibody production companies have established lists of antibodies and clones that have been tested and demonstrated to be sensitive to fixation and/or permeabilization. Refer to their websites for more information.

- **Centrifugation speeds:** For cell centrifugation steps, centrifugation should be performed for 5 minutes at 300 x *g* before cell fixation, and for 5 minutes at 800 x *g* after cell fixation. The increased centrifugation speed after cell fixation results in greater cell recovery.
- **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 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.

NOTE 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.
- **Mass range on first-generation CyTOF® instrument:** The mass range on the first-generation CyTOF instrument must be adjusted to 102–192 to accommodate the barcoding channels. Due to the limited mass range on the first-generation CyTOF

instrument, Cell-ID Intercalator-¹⁰³Rh is the only viability stain available for use with barcoding. In addition, ¹⁹¹Ir is the only channel available for use with Cell-ID Intercalator-Ir, which has both ¹⁹¹Ir and ¹⁹³Ir. Contact your Fluidigm technical support specialist and field application scientists for assistance when performing barcoding experiments on a first-generation CyTOF instrument.

- **Viability staining:** It is recommended that Cell-ID Cisplatin, instead of Cell-ID Intercalator-¹⁰³Rh be used as a viability stain for barcoding experiments on CyTOF 2 and Helios™ instruments. Refer to [\(Optional\) Cell-ID Cisplatin Viability Staining](#).

NOTE Samples resuspended in serum-containing media, e.g. complete RPMI with FBS, should be washed in serum-free media before beginning viability staining with Cell-ID Cisplatin.

- **Quantum dots (Qdots):** Qdots labeled with antibodies are often 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.
- **Troubleshooting:** If you experience low barcoding staining or efficiency or other difficulties, contact your local field application scientist for assistance.

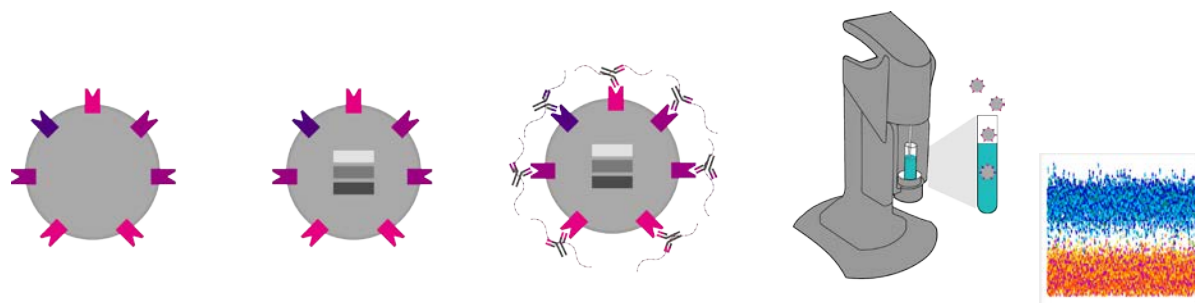
Tips and Tricks

- **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. 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.
- **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. Staining less than 3 million cells individually will use the same amount of antibody as staining 3 million cells. For example, staining three individual samples with 1 million cells would require 1 µL of antibody, whereas with barcoding, 3 barcoded samples with 1 million cells per sample would require 1 µL of antibody. Therefore, barcoding offers the potential for reagent savings. After 20-plex barcoding, cell staining is done in a single tube that may contain up to 60 million cells in a 2 mL volume, maintaining the maximum cell staining concentration of 30 million cells per mL (or 3 million cells per 100 µL). Based on this, it has been found that barcoded samples may require less antibody per sample than individually stained samples to achieve the same level of staining intensity.

Users should perform a titration of cells with their 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:** 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 Workflow



1 Fix

Samples are fixed.

2 Barcode

Samples are barcoded by adding three unique metal isotopes.

3 Stain

Samples in a single tube are stained with metal-conjugated antibodies/panels.

4 Acquisition

CyTOF[®]/CyTOF 2/Helios[™] Acquisition

5 Debarcode

Barcoding Protocol Steps

Prepare Samples

- 1 Prepare 1–3 million cells resuspended in Maxpar Cell Staining Buffer into individual 5 mL tubes for each sample to be barcoded.
- 2 Centrifuge each sample at 300 x *g*, carefully aspirate supernatant, and gently vortex.

(Optional) Cell-ID Cisplatin Viability Staining

NOTE If not performing viability stain, proceed to Fix and Permeabilize.

- 1 Resuspend cells to 20 x 10⁶/mL in PBS.
- 2 Prepare a working solution of 10 μM Cell-ID Cisplatin by diluting in PBS (500X dilution from stock).
- 3 Add an equal volume of 10 μM Cell-ID Cisplatin to cell suspension from Step 1 (for example, 500 uL 10 uM Cell-ID solution added to 500 uL cell suspension gives a final concentration of 5 μM cisplatin).
- 4 Mix well and incubate at room temperature for 5 minutes.
- 5 Quench cisplatin staining by washing with Maxpar Cell Staining Buffer using 5–10X the volume of the stained cells. Centrifuge at 300 x *g*, aspirate supernatant, and gently vortex.
- 6 If Cell-ID was performed with all the cells in one tube, add enough CSB to be able to split cells into 1–3 x 10⁶ cells per tube. Centrifuge at 300 x *g* for 5 min, aspirate supernatant, and gently vortex.

NOTE If cisplatin staining is poor, wash cells in PBS prior to cisplatin staining.

Fix and Permeabilize

- 1 Resuspend samples completely in 1 mL of 1X Fix I Buffer and incubate for 10 minutes at room temperature.
- 2 Centrifuge each sample at 800 x *g* for 5 minutes, carefully aspirate supernatant, gently vortex, and wash twice with 1 mL of 1X Barcode Perm Buffer.

Barcode

- 1 Resuspend each sample to be barcoded completely in 800 μL of 1X Barcode Perm Buffer.

- 2 Resuspend each barcode completely in 100 μ L of 1X Barcode Perm Buffer and transfer them to the appropriate samples. Pipette to mix the sample immediately and completely.
- 3 Incubate samples for 30 minutes at room temperature. Gently tap tube to mix after 15 minutes.
- 4 Centrifuge each sample at 800 x *g* for 5 minutes, carefully aspirate supernatant, gently vortex, and wash twice with 2 mL of Maxpar Cell Staining Buffer.
- 5 Resuspend each sample completely in 100 μ L of Maxpar Cell Staining Buffer.
- 6 Combine all barcoded samples into one 5 mL tube.
- 7 Rinse sample tubes an additional 2 x with 100 μ L each and combine with tube from step 6 to maximize cell recovery. Centrifuge cells at 800 x *g* for 5 minutes, carefully aspirate supernatant, and gently vortex. If volume is too large for tube, combine and centrifuge in two batches.

Antibody Staining

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 See [Antibody usage](#) under Tips and Tricks to learn how to effectively scale your antibody panel on barcoded samples.

NOTE Perform centrifugation at 800 x *g* during antibody staining steps.

Cell-ID Intercalator-Ir Staining

- 1 Add 2 mL of the intercalation solution to the cells and gently vortex. Ensure cells are resuspended well.
- 2 Incubate for 1 hour at room temperature or leave overnight at 4 °C.

NOTE Cells can be left at 4 °C in the intercalation solution for up to 48 hours.

- 3 Wash cells by adding 2 mL of Maxpar Cell Staining Buffer. Centrifuge at 800 x *g* for 5 minutes, carefully aspirate supernatant, and resuspend cells in residual volume by gently vortexing. Repeat for a total of two washes.
- 4 Wash cells with 2 mL of PBS. Centrifuge at 800 x *g* and carefully aspirate supernatant. Take an aliquot of cells in between water washes for a cell count.

NOTE Cell loss during wash steps will lead to a lower cell concentration than 10 million cells per mL.

- 5 Centrifuge at 800 x *g* for 5 minutes and carefully aspirate supernatant. Leave cells pelleted at 4 °C.
- 6 Prepare a sufficient volume of 0.1X EQ Four Element Calibration Beads in Maxpar Water to completely resuspend cells to a recommended cell concentration for acquisition:
 - CyTOF and CyTOF 2: 0.25–0.5 x 10⁶ cells/mL
 - Helios: 0.5–1 x 10⁶ cells/mL
- 7 Immediately prior to data acquisition, resuspend cells in 0.1X EQ Four Element Calibration Beads and filter sample through appropriately sized cell strainers (for example, 35 to 45 µm mesh).

Acquire Data

- 1 Acquire barcoded sample on a CyTOF, CyTOF 2 or Helios instrument.
- 2 Collect the following channels in addition to the channels corresponding to the antibody panel:
 - Barcodes (¹⁰²Pd, ¹⁰⁴Pd, ¹⁰⁵Pd, ¹⁰⁶Pd, ¹⁰⁸Pd, and ¹¹⁰Pd)
 - EQ beads (¹⁴⁰Ce, ¹⁵¹Eu, ¹⁵³Eu, ¹⁶⁵Ho, and ¹⁷⁵Lu)
 - Cell-ID (dead cells) (¹⁹⁵Pt for cisplatin)
 - Cell-ID Intercalator-Ir (¹⁹¹Ir and ¹⁹³Ir)
 - Common environmental contaminants (¹²⁰Sn, ¹²⁷I, ¹³¹Xe, ¹³³Cs, ¹³⁸Ba, ¹⁹⁰Os, and ²⁰⁸Pb)

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

IMPORTANT The Debarcoder is a feature within CyTOF software v6.7.1014 It must be installed on a stand-alone Windows® 7 64-bit computer workstation. This software should **not** be installed on first-generation CyTOF or CyTOF 2 instrument workstations.

- 1 Login to the [Fluidigm Proteomics Portal](#) by control-clicking this link.



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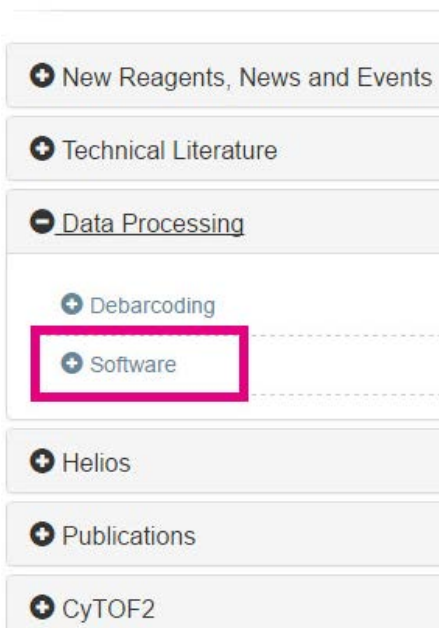
Login

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- 2 Select **Data Processing > Software**. Download the Software Package for Stand-alone Workstations.

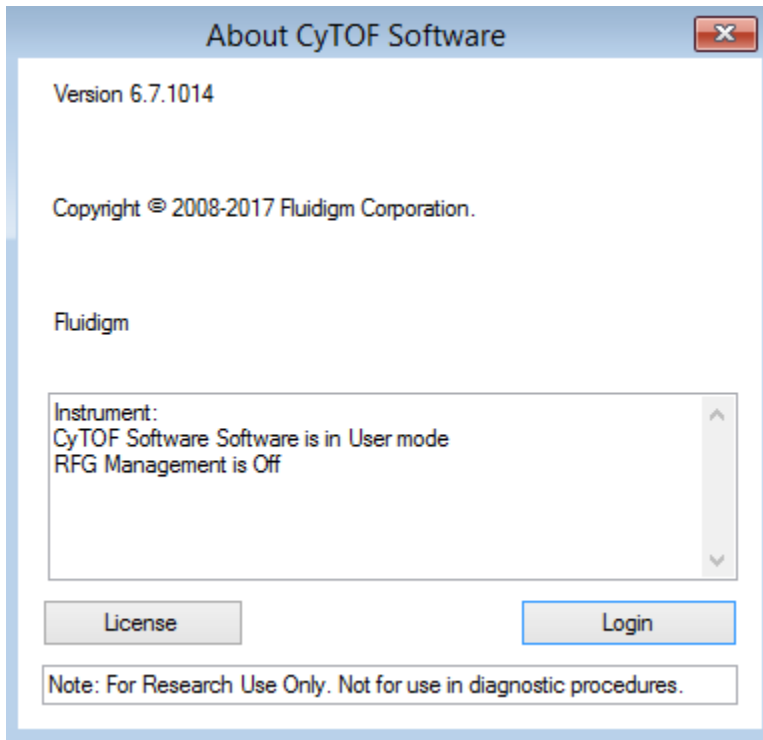
My DVS



- 3 Double-click the installer and follow installation instructions.

IMPORTANT Download the CyTOF Software 6.7 Release Notes (PN 400314) from the Portal and follow the installation instructions.

- 4 Click the icon to launch the CyTOF software.
- 5 Log in to Administrator mode.



The Helios software interface opens.

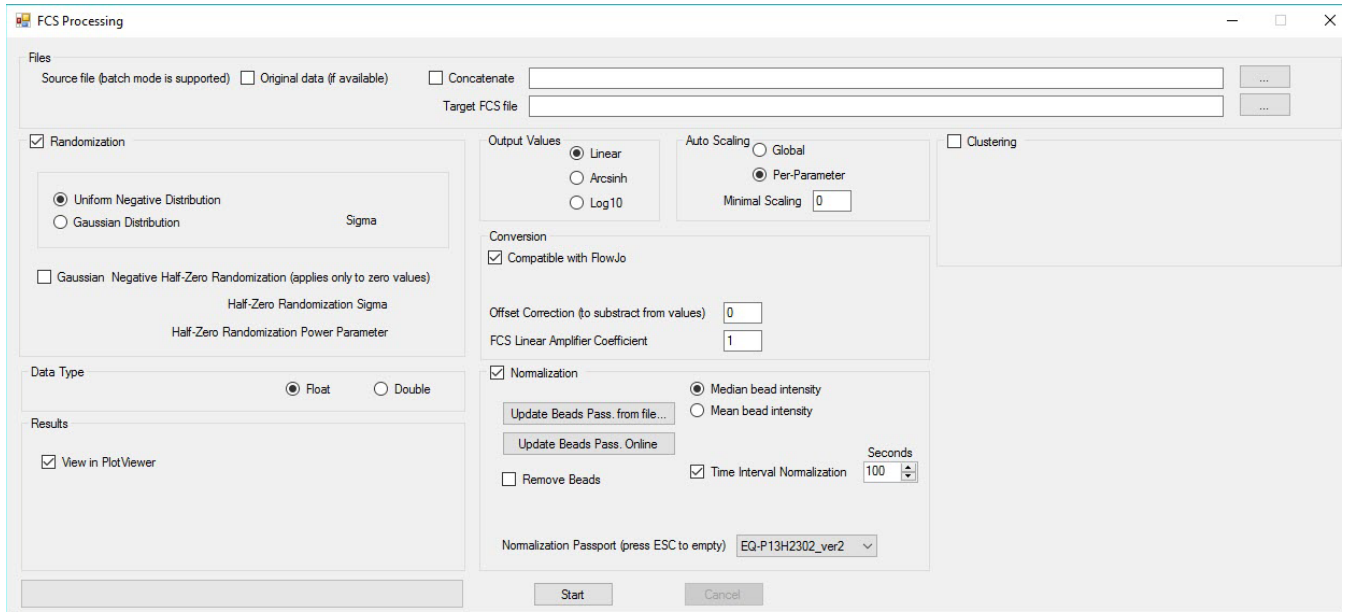
- Under the Process tab, click the **Debarcoder** icon to launch the program.



Debarcode the File

NOTE Read the introductions to [Debarcoding](#) and [Example Data](#) sections for important guidance on use of the debarcoding filters.

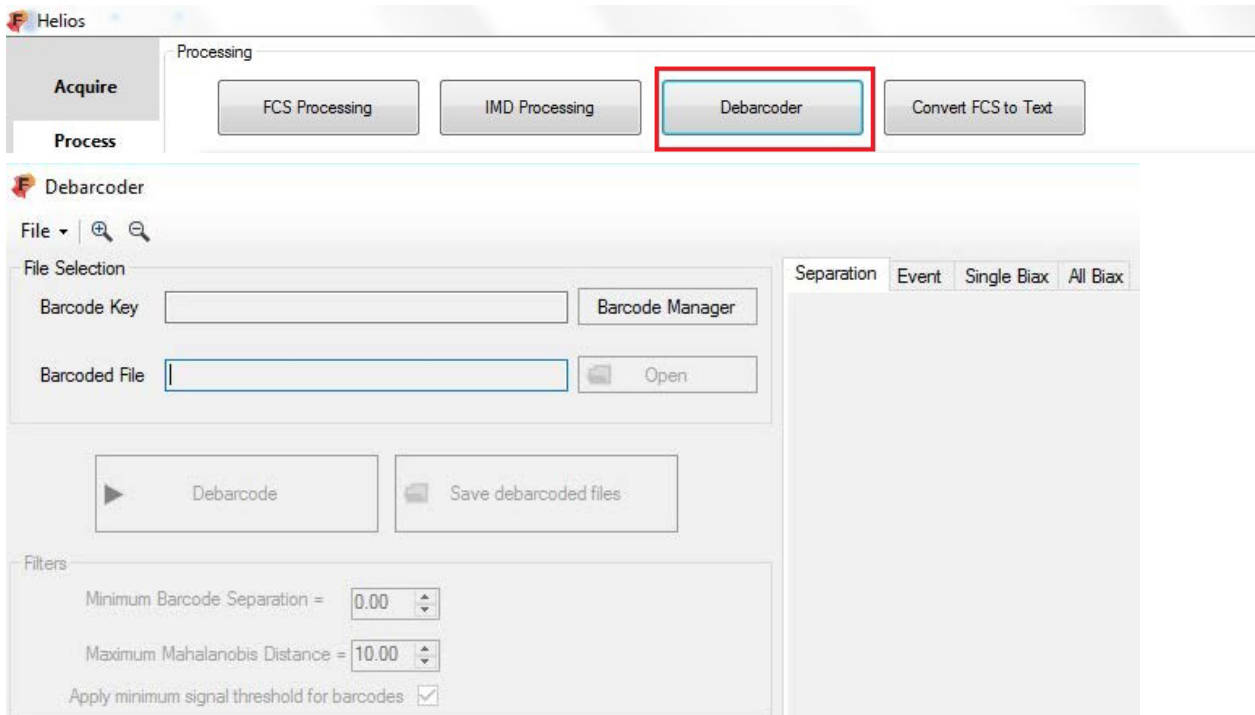
Perform normalization on the barcoded file.



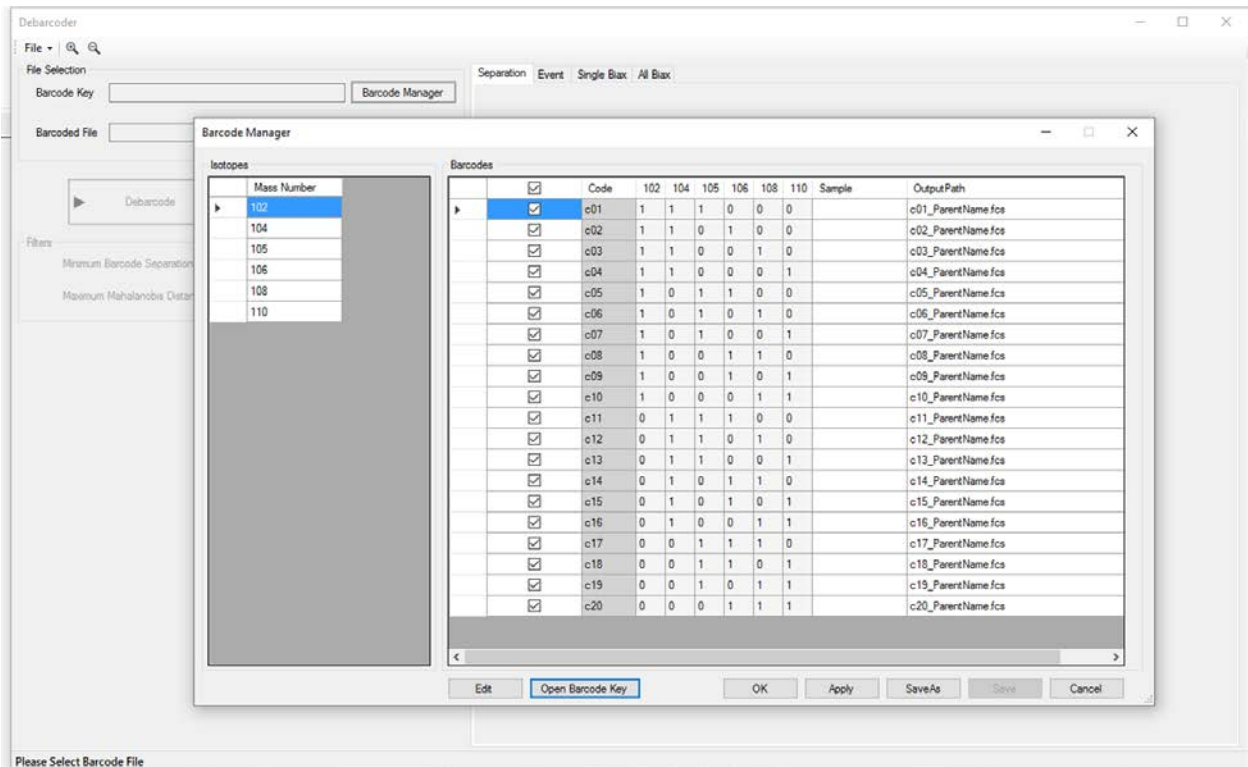
NOTE If the same barcoded sample is acquired over multiple barcoded files, they should be normalized first and then concatenated.

NOTE Refer to Normalization of Mass Cytometry Data Using EQ Four Element Beads (PN UG13-02_150501) for more information.

- 1 In the Process tab, open the **Debarcoder** and click **Barcode Manager**.



- 2 Open the barcode key file (Key_Cell-ID_20-Plex_Pd.csv) and modify the file. This file indicates the masses of the six Pd isotopes. The three-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.
- 3 In the Filters box there is a feature Apply minimum signal threshold for barcodes which is checked by default. 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 enables the feature to increase recovery of low intensity events by removing the lower signal threshold for barcodes.



Modify the file:

- a Enter unique sample names in the Sample column.

NOTE Uncheck the unused barcodes.

Barcodes										
	<input checked="" type="checkbox"/>	Code	102	104	105	106	108	110	Sample	OutputPath
	<input type="checkbox"/>	c01	1	1	1	0	0	0	Control	c01_ParentName_Control.fcs
	<input type="checkbox"/>	c02	1	1	0	1	0	0	Treatment1	c02_ParentName_Treatment1.fcs
	<input type="checkbox"/>	c03	1	1	0	0	1	0		c03_ParentName.fcs
	<input type="checkbox"/>	c04	1	1	0	0	0	1		c04_ParentName.fcs
	<input type="checkbox"/>	c05	1	0	1	1	0	0		c05_ParentName.fcs

- b Uncheck any barcodes that are not used in the experiment.
- c Click SaveAs. Save the resulting barcode key with a unique name.

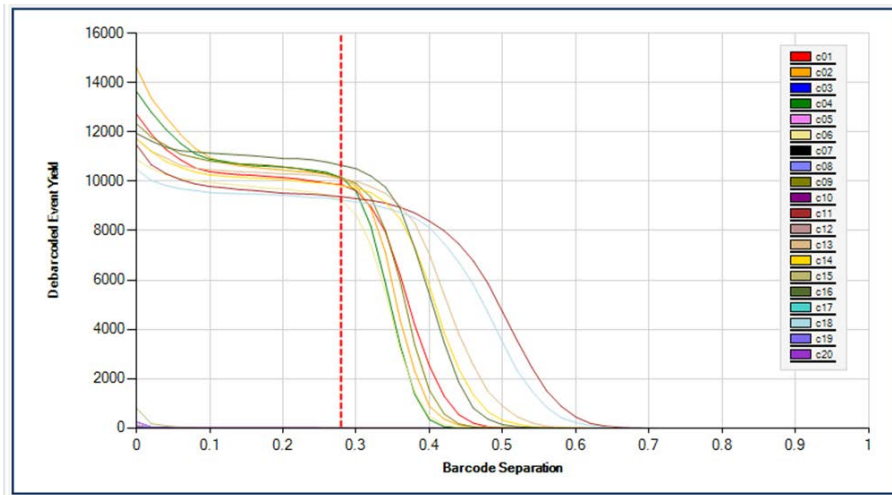
- d Click OK to apply changes and close the Barcode Manager window.

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

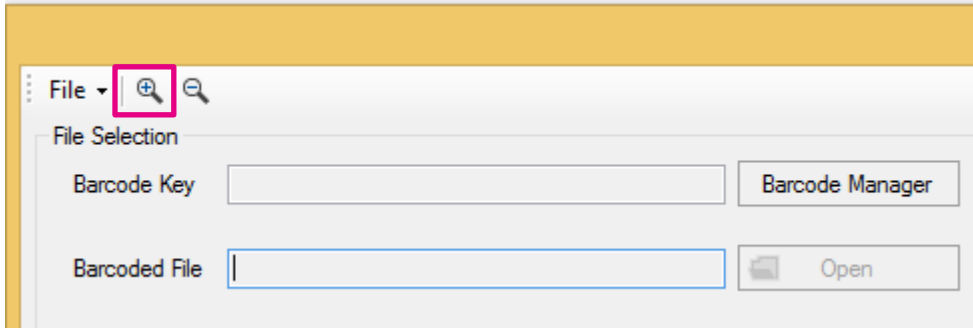
- 5 Filter barcode assignments by choosing optimal minimum Barcode Separation (BcS) and maximum Mahalanobis Distance (MD) values. Refer to the introduction to Barcoding section above.

Choose a minimum BcS:

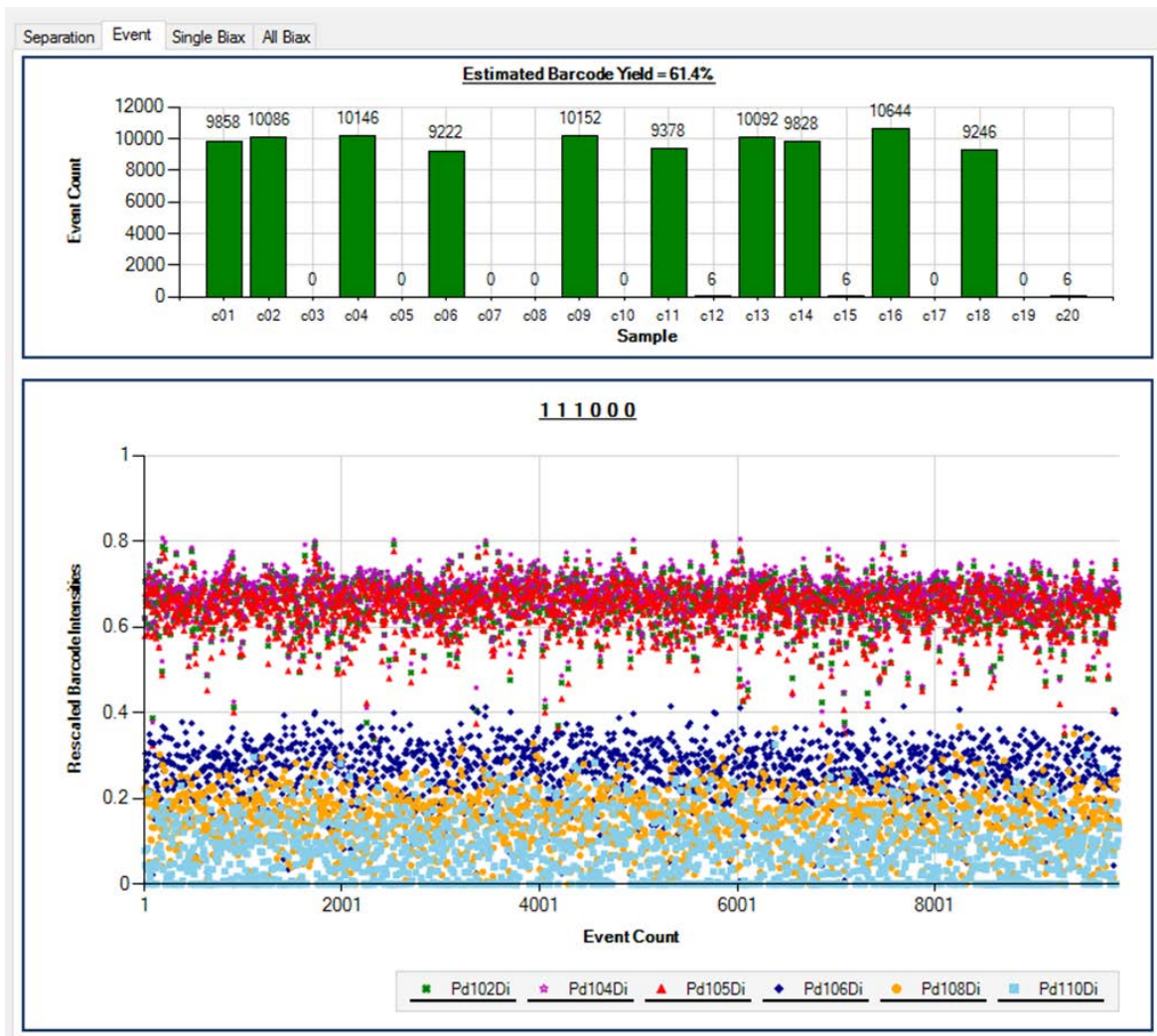
- a In the Separation tab, observe the barcode separation plots and choose a minimum BcS value just before the event yield dramatically drops. The minimum BcS value is displayed as a vertical dotted red line on the plots.



- b In the Event tab, magnify the event plot by using the zoom icon at top-left in the Debarcoder until individual events are visible.

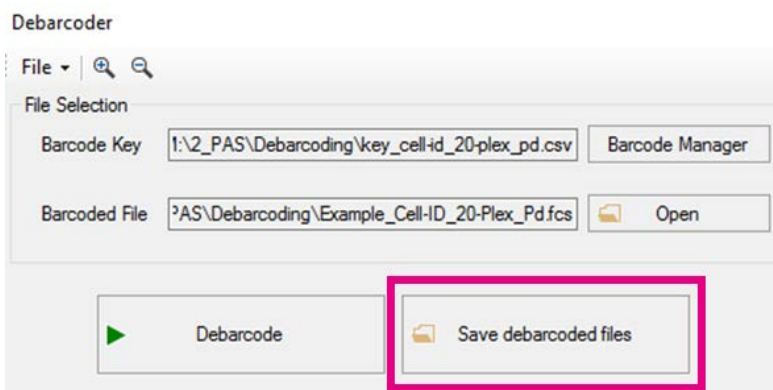


- c In the Event plot, if there are events with poor intensity separation between the third and fourth isotopes, consider increasing the minimum BcS value.



(Optional) Choose a maximum MD:

- a In the Single Biax or All Biax tab, color by MD.
 - b Observe each code.
 - c Choose a maximum MD value that eliminates outliers.
- 6 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.

- 7 After debarcoding, remove bead-cell doublet events prior to data analysis by gating on ¹⁴⁰Ce-negative events.

Debarcoding Example

Download the example data file (Example_Cell-ID_20-Plex_Pd.fcs) from the Data Processing tray in the Fluidigm Proteomics portal. For this 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 1, a minimum BcS value of 0.24 can be set without negatively impacting yield in any of the samples.

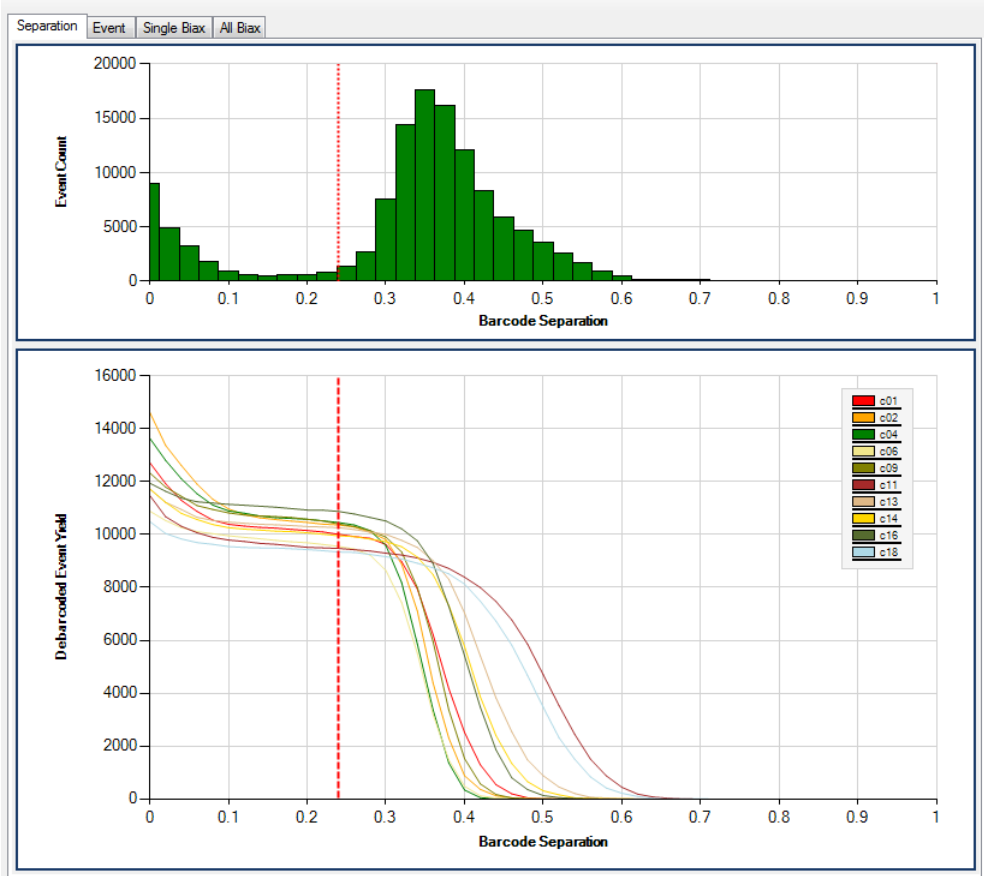


Figure 1. The BcS vs. Debarcoded Event Yield plot in the Plot Separation tab of the Debarcoder.

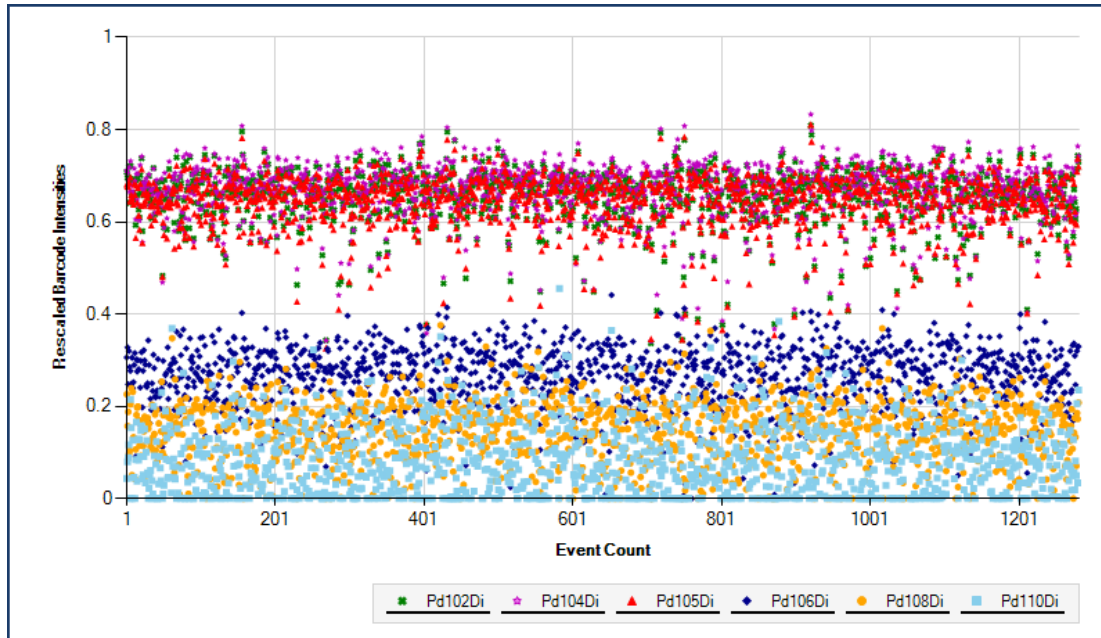


Figure 2. The Event plot in the Debarcoder.

The Event plot for barcode population 1 (Figure 2) 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.

Examination of the All Biax plots for c01 reveals some outliers with high MD values, heat-mapped blue or green (Figure 3, above).

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 barcode yields (Figure 3, below).

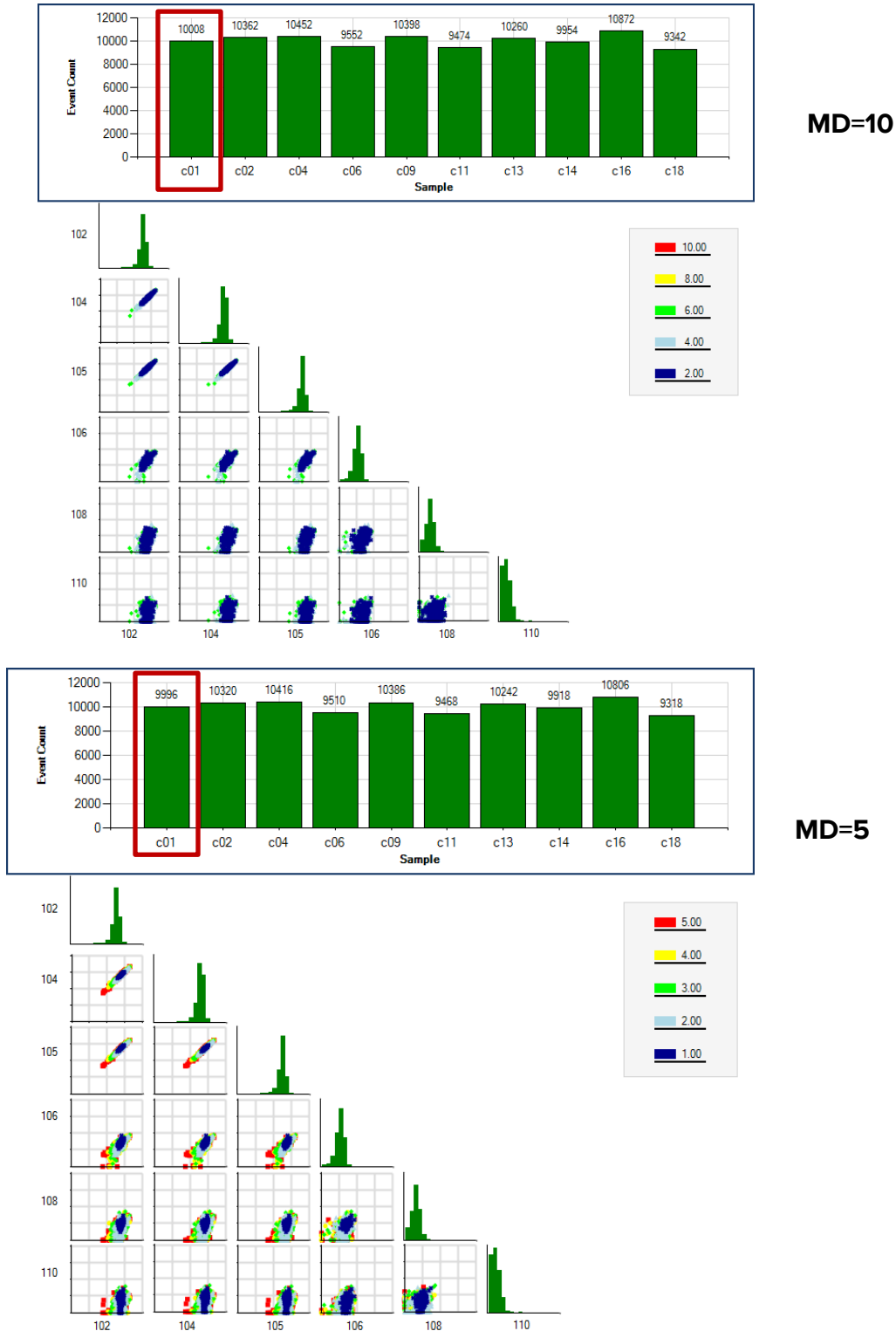


Figure 3. Upper bar graphs: The Event count plots for the samples in the Debarcoder. Lower biaxial plots: Sample c01 datapoints are heat-mapped according to MD values and the scale indicated to the right of each plot set. Debris events were removed by lowering the MD filter (lower).

The Event plots in Figures 4–6, magnified using the zoom icon (at top-left in the Debarcoder), show events with the maximum MD filter set to 10 (Figure 4), 5 (Figure 5), or 4 (Figure 6). 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.

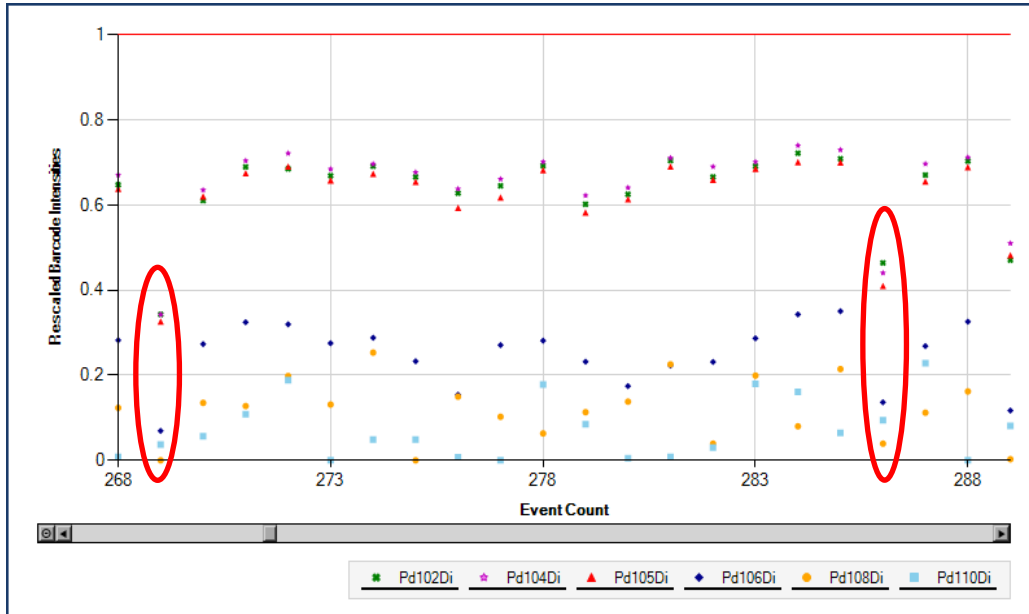


Figure 4. Magnified Event plots in the Debarcoder. The MD filter is set to 10.

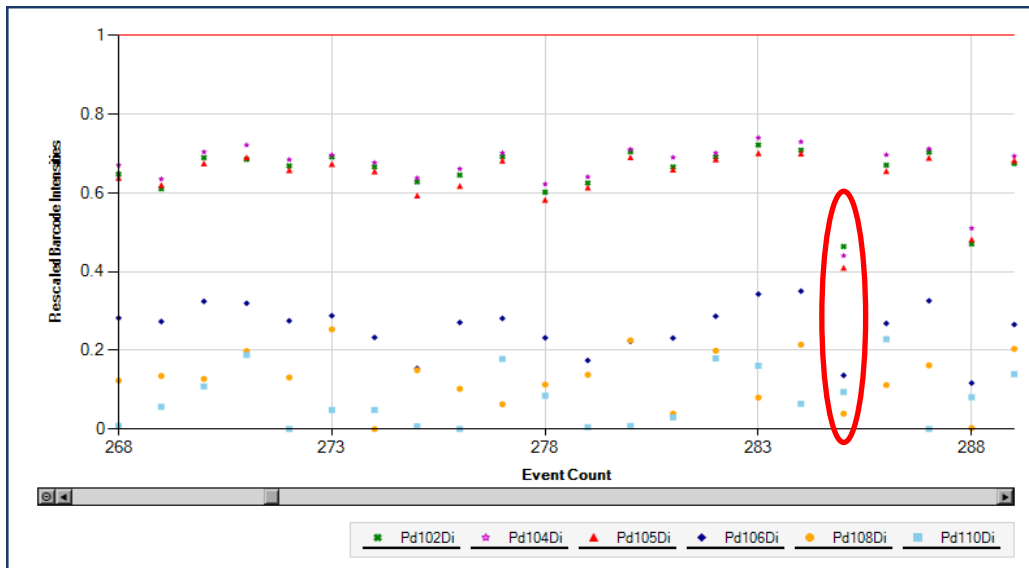


Figure 5. Magnified Event plots in the Debarcoder. The MD filter is set to 5. Event 269, seen in Figure 4 (MD=10), has been removed when the MD filter is set to 5.

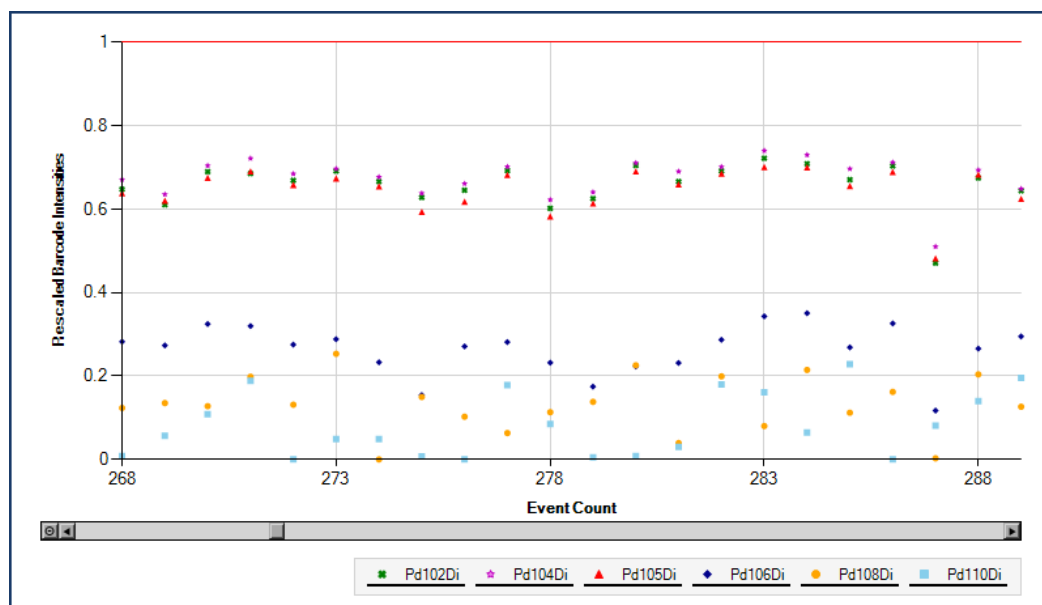


Figure 6. Magnified Event plots in the Debarcoding software. The MD filter is set to 4. Events 269 and 286, seen in Figure 4 (MD=10), have been removed when the MD filter is set to 4.

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