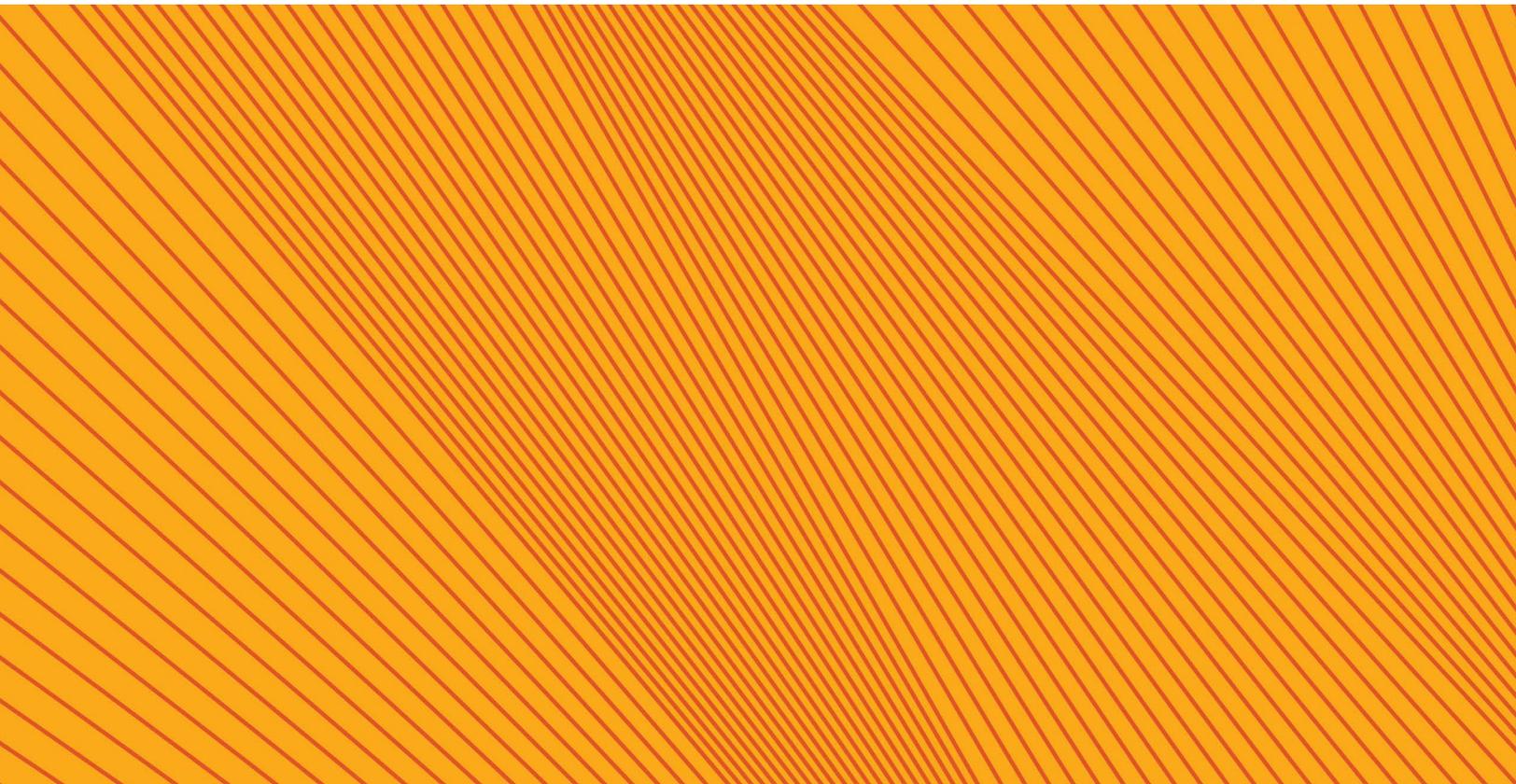


Cell-ID 20-Plex Pd Barcoding Kit

USER GUIDE



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About this Guide

Safety Data Sheets

Read and understand the SDSs before handling chemicals. To obtain SDSs for chemicals 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 a protocol for barcoding samples and instructions for debarcoding multiplexed files.

Kit Contents and Storage Conditions

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

		Palladium Isotope					
		102	104	105	106	108	110
Sample Code	1	•	•	•			
	2	•	•		•		
	3	•	•			•	
	4	•	•				•
	5	•		•	•		
	6	•		•		•	
	7	•		•			•
	8	•			•	•	
	9	•			•		•
	10	•				•	•
		102	104	105	106	108	110
	11		•	•	•		
	12		•	•		•	
	13		•	•		•	
	14		•		•	•	
	15		•		•		•
	16		•			•	•
	17			•	•	•	
	18			•	•		•
	19			•		•	•
	20				•	•	•

Barcoding Protocol

The barcoding protocol employs fixation and gentle permeabilization to ensure uniform cell labeling with the palladium 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	Company Name	Catalog Number
Centrifuge rotor that holds PCR plates	NA	NA
12 x 75 mm tubes, polypropylene, 5 mL	BD Falcon™	352002
Cell-ID Cisplatin or Intercalator-103Rh	Fluidigm	201064 201103A
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 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.

Important Notes before Starting:

- Perform centrifugation steps for 5 minutes at room temperature. For most cell types, perform pre-fixation centrifugations at 300 x *g* and post-fixation centrifugations at 800 x *g* (to improve cell recovery).
- 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.
- Immediately before beginning the protocol, remove 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 all liquid is in the bottom of the tubes.

Barcoding Protocol Steps

Fix and Permeabilize

- 1 Prepare 1–3 million cells resuspended in Maxpar Cell Staining Buffer in individual 5 mL tubes for each sample to be barcoded.
- 2 Stain dead cells with Cell-ID Cisplatin or Cell-ID Intercalator-103Rh according to protocol provided with those products.
- 3 Centrifuge cells, and discard supernatant.
- 4 Resuspend in 1 mL Fix I Buffer, and incubate for 10 minutes at room temperature.
- 5 Centrifuge cells, discard supernatant, and wash twice with 1 mL of Barcode Perm Buffer.

Barcode

- 6 Resuspend each sample to be barcoded completely in 800 μ L Barcode Perm Buffer.
- 7 Resuspend barcodes completely in 100 μ L Barcode Perm Buffer and transfer them to the appropriate samples. Mix the sample immediately and completely.
- 8 Incubate for 30 minutes at RT.
- 9 Centrifuge cells, discard supernatant, and wash twice with 2 mL of Maxpar Cell Staining Buffer.

Stain with Panel and Acquire Data

- 1 Resuspend in 100 μ L Maxpar Cell Staining Buffer.
- 2 Combine all barcoded samples into one tube.
- 3 Proceed with the staining protocol appropriate for your antibody panel. Scale staining and wash volumes to accommodate the number of cells in the multiplexed sample. Include EQ beads in the final resuspension.
- 4 Acquire barcoded sample on a CyTOF[®], CyTOF 2, or Helios instrument.
- 5 Collect the following channels in addition to the channels corresponding to the antibody panel:
 - Barcodes (102, 104, 105, 106, 108, and 110)
 - EQ beads (140, 151, 153, 165, and 175)
 - Cell-ID (dead cells) (195 for cisplatin; 103 for rhodium)

- Cell-ID Intercalator-Ir (191) and Ir (193)

6 Collect at least 300 events of the rarest population in the multiplexed sample.

NOTE If performing barcoding on the CyTOF instrument the mass range must be adjusted to 102-192. Please contact your Fluidigm Field Applications Specialist for more details.

Debarcoding Protocol

The collected file contains combined data for all multiplexed samples and therefore must be debarcoded in order to perform downstream analysis of the individual component 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 FCS files for each barcoded sample for downstream analysis.

Debarcoding Steps

Download and install the Debarcoder

NOTE The Debarcoder is a standalone application that must be installed on a Windows 7 64-bit computer.

- 1 Login to the [Fluidigm Proteomics Portal](#) by [clicking this link](#).
- 1 Download the Debarcoder application from the Data Processing tray.
- 2 Double-click the Debarcoder installer and follow installation instructions. A Debarcoder icon will appear on your desktop, and the executable will be installed in C:\Program File(x86)\Fluidigm\Debarcoder.
- 3 Launch the Debarcoder.

Debarcode the File

NOTE Please read [Parameters That Impact Debarcoding Performance](#) on page 10 and [Example Data](#) on page 12 for important guidance on use of the debarcoding filters.

- 1 Normalize the barcoded file using EQ Four Element Calibration Bead data.
- 2 Launch the Debarcoder.
- 3 Open the Barcode Manager.
- 4 Open barcode key file (Key_Cell-ID_20-Plex_Pd.csv). This file indicates the masses of the six Pd isotopes, the three-digit Pd isotope combinations corresponding to the code numbers found on the tubes in the kit, and the names of the files that will be output when debarcoding is completed.
 - a Enter unique sample names in the Sample column.
 - b Uncheck any barcodes that are not used in the experiment.
 - c Save the resulting barcode key with a unique name.
 - d Click **OK** to apply changes and close the Barcode Manager window.

- 5 Click **Open** to browse for the normalized barcoded FCS file, then click **Debarcode**.
- 6 Filter barcode assignments by choosing optimal minimum Barcode Separation (BcS) and maximum Mahalanobis Distance (MD) values. These are explained in detail in [Parameters That Impact Debarcoding Performance](#) below.
 - a **Barcode Separation:** In the Separation tab, observe the barcode separation plots and choose a minimum BcS value just before the event yield dramatically drops. This value is visualized as a dotted red line on the plots.
 - b In the Event tab, observe the Event plot. Magnify the plot until individual events are visible. If there are events with poor intensity separation between the third and fourth isotopes, consider increasing the minimum BcS value.
- 7 **Mahalanobis Distance** (use only if necessary; see [Parameters That Impact Debarcoding Performance](#) below and [Example Data](#) on page 12).
 - 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.
- 8 Click **Save debarcoded files** to generate and save FCS files for each debarcoded sample.

Parameters That Impact Debarcoding Performance

The multiplexed file contains a mixture of desirable single-cell events and undesirable events such as debris, cross-sample aggregates, and cross-sample ion fusions. Optimal debarcoding results in accurate assignment of the maximum number of desirable 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 less than 3 Pd stains or with poor correlation to any other events in the file are removed from the data. The Debarcoder provides two calculated parameters, Barcode Separation (BcS) and Mahalanobis Distance (MD), which are used to eliminate undesired events from the debarcoded populations. It is best to perform primary filtering using BcS, and only use MD as a secondary filter in special cases.

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. The use of rescaled values 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 values for all Pd isotopes and cross-sample cell aggregates, which have high intensity for four or more Pd isotopes. Such undesired events are filtered out of the barcoding results by applying a minimum BcS filter.

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. Therefore, such events stray from the center of the single cell cluster in Pd intensity space, resulting in higher MD values. 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.

The quality of sample preparation, barcode staining, and instrument run conditions impacts the optimal filter settings and the recovery of events in the debarcoded files. Samples that have excessive debris and/or cell aggregates or were run at exceedingly high concentration (and thus have high frequency of ion fusion events) have lower event recoveries when appropriate filtering is applied. Appropriate filtering ensures that the low recovery is associated with specifically eliminating debris and aggregates while retaining single cells. Although the Debarcoder has excellent filters that eliminate undesired events while retaining single cells, follow best practices of sample preparation, barcode staining, and instrument run conditions for optimal performance.

Example Data

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, ten 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.

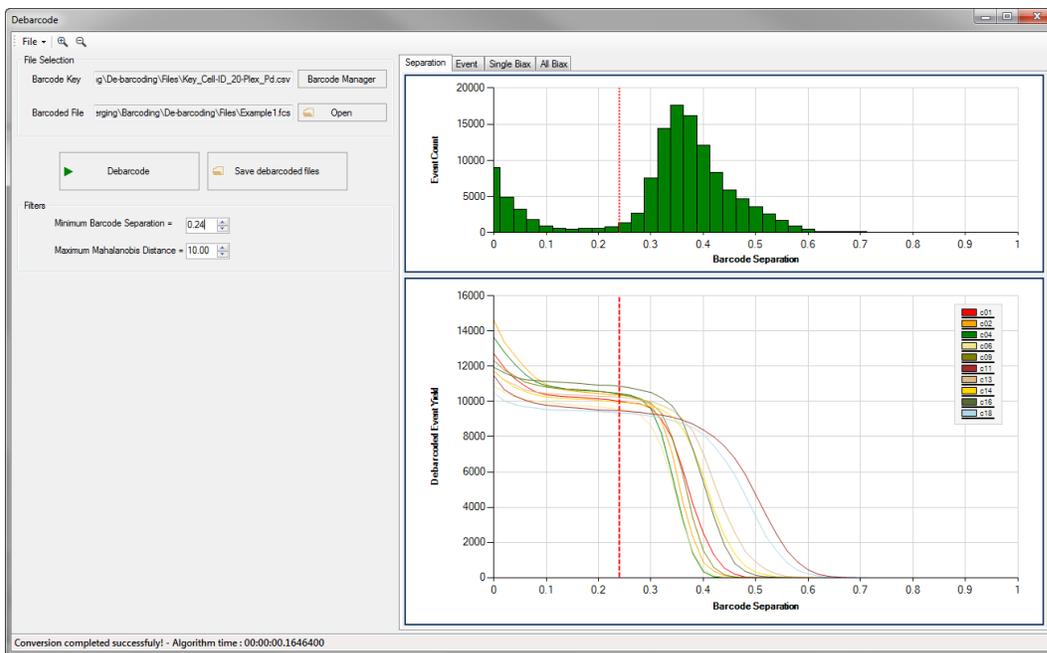


Figure 1. The BcS vs. Debarcoded Event Yield plot in the Plot Separation tab of the Debarcoding software.

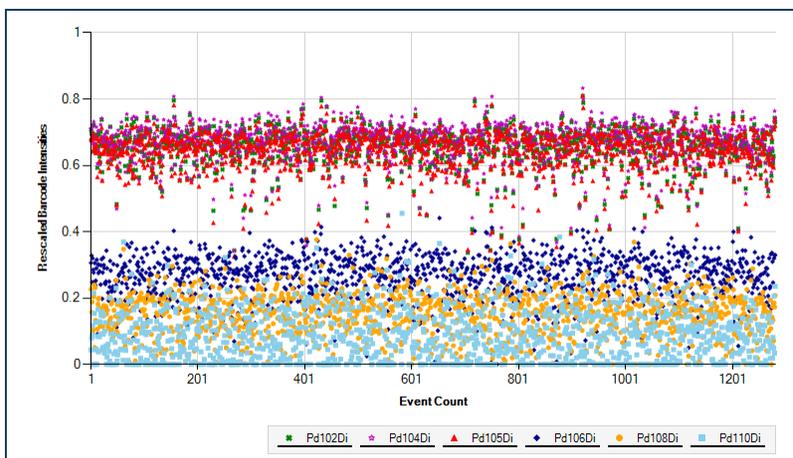


Figure 2. The Event plot in the Debarcoding software.

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 revealed some outliers with high MD values, heat-mapped blue or green (Figure 3, left). Note that 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, right).

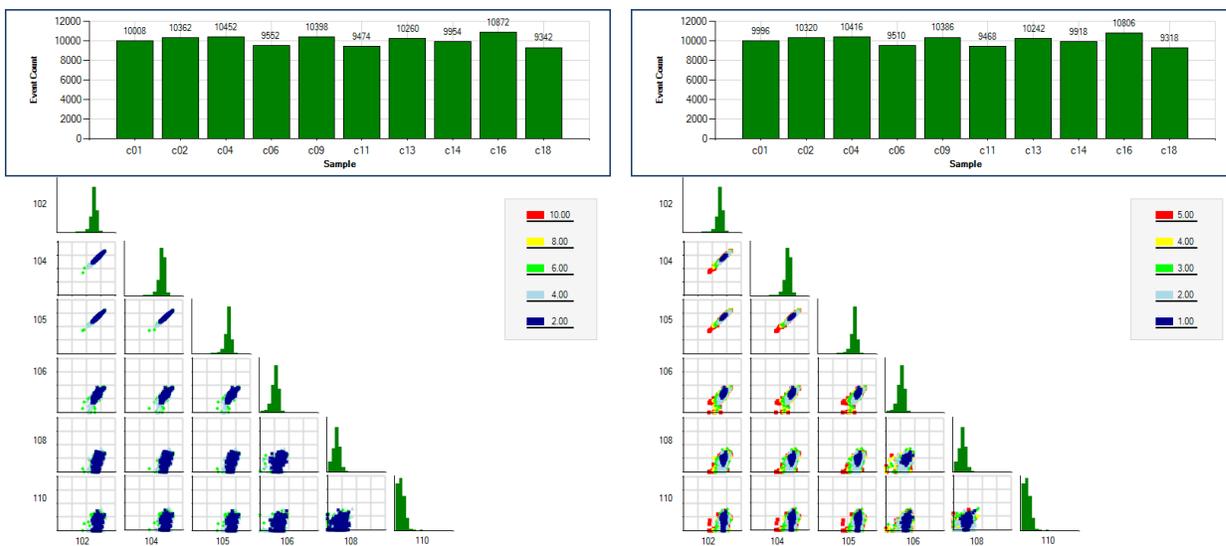


Figure 3. The Event plots for the samples in the Debarcoding software. Data points are heat-mapped according to MD values and the scale indicated to the right of each plot set. Debris events were removed by lowering the MD filter (lower right)

The Event plots in Figure 4, 5 and 6, magnified using the zoom icon (located 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 since its positive Pd intensities are similar in value to the background in the other events. Event 286 is less clearly debris, so should be included (as it can be analyzed more completely after debarcoding). As a result, we chose an MD filter value of 5.

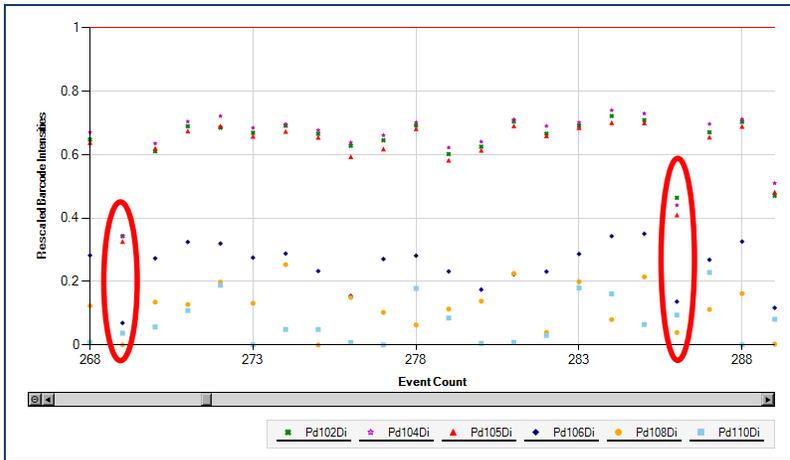


Figure 4. Magnified Event plots in the Debarcoding software. The MD filter is set to 10.

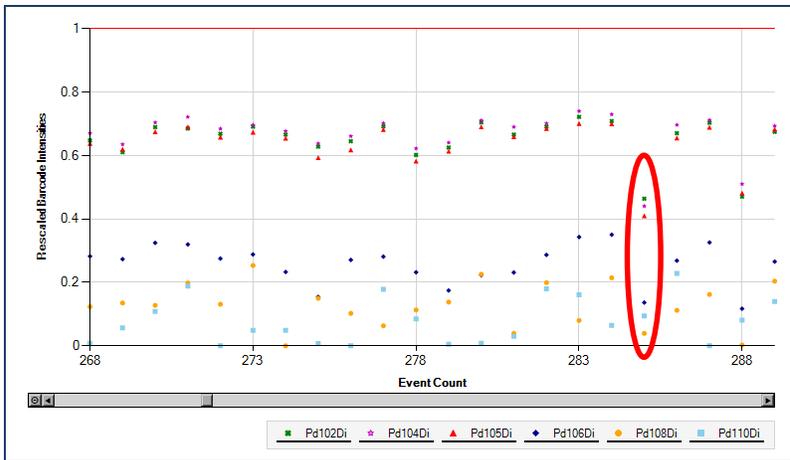


Figure 5. Magnified Event plots in the Debarcoding software. The MD filter is set to 5.

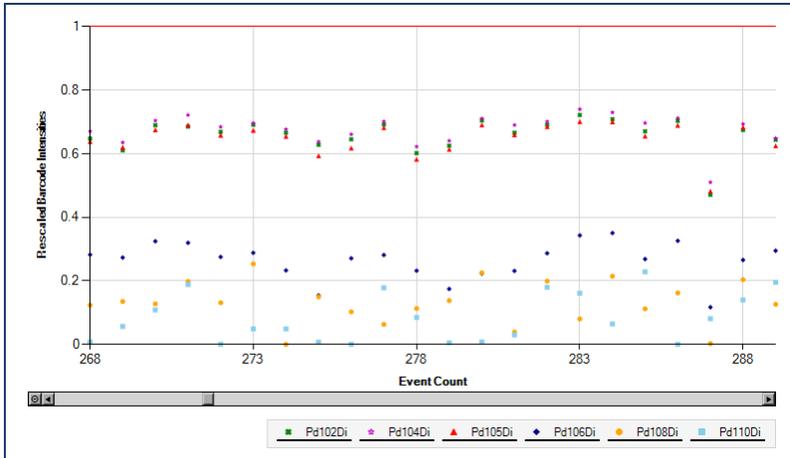


Figure 6. Magnified Event plots in the Debarcoding software. The MD filter is set to 4.



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