August 2013

BD Lyoplate™ Human and Mouse Screen Analysis Instructions

For analysis using BD FACSDiva™ software and heatmap representation in Microsoft® Excel® 2007 and later

For use with the **BD Lyoplate™ Human Cell Surface Marker Screening Panel** (Cat. No. 560747) or the **BD Lyoplate™ Mouse Cell Surface Marker Screening Panel** (Cat. No. 562208). There are separate analysis templates for the human and mouse panels. Be sure to select the appropriate analysis template at **bdbiosciences.com/resources/stemcell**.

Purpose

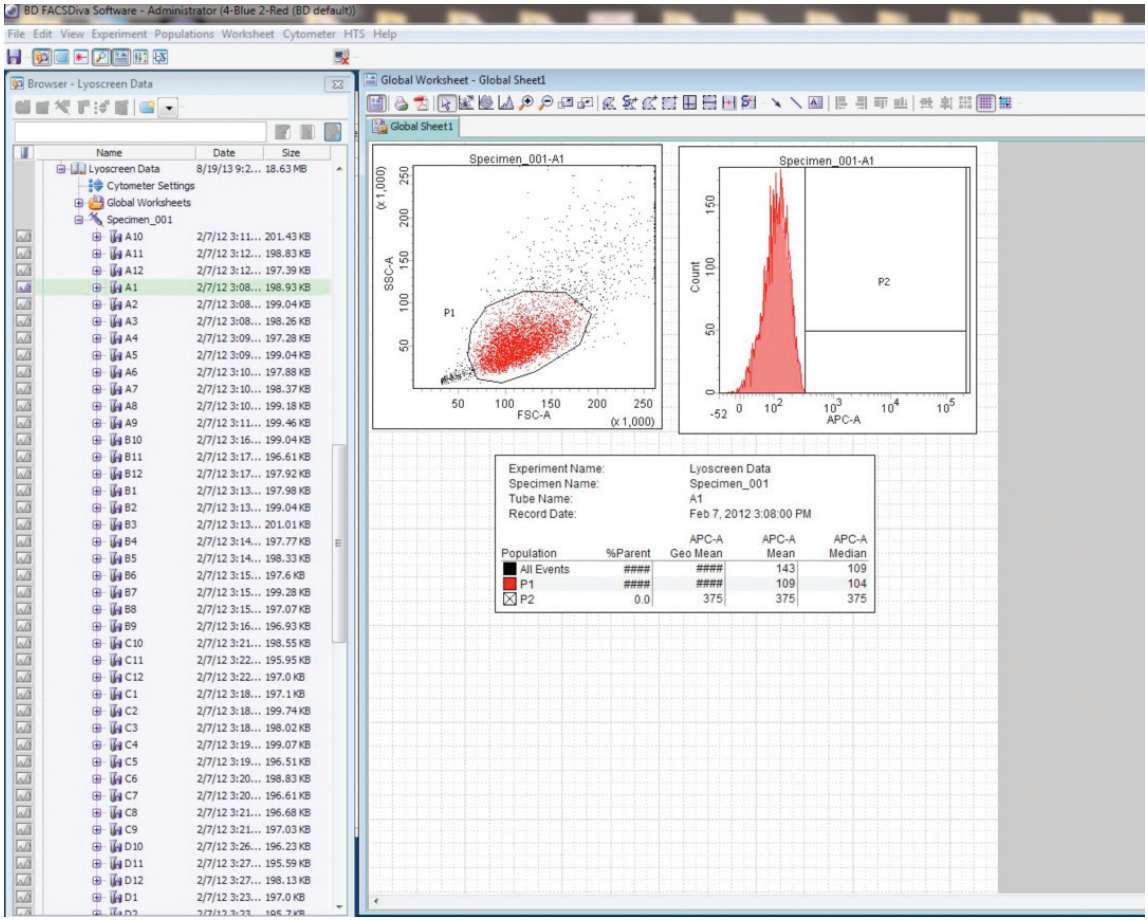
This Microsoft® Excel® 2007 (Excel) template is used to aid in the analysis of the data generated from running a BD Lyoplate™ screen after exporting specific statistics from BD FACSDiva™ software. The Excel template reorganizes the data into the corresponding 96-well BD Lyoplate format, performs normalization to isotype controls, generates heatmaps (specificity overlayed on colored tiles), and allows for easy comparison between multiple screens (on distinct cell populations or repetitions of the same cell population).

These instructions briefly describe a generic gating strategy and batch export of relevant statistics from BD FACSDiva software into an Excel template for initial analysis of FCS file data from the BD Lyoplate Cell Surface Marker Screening Panel. The Excel template is an aid that helps to identify the surface signature of a screened cell population. The identified hits should always be verified by going back to the raw data.

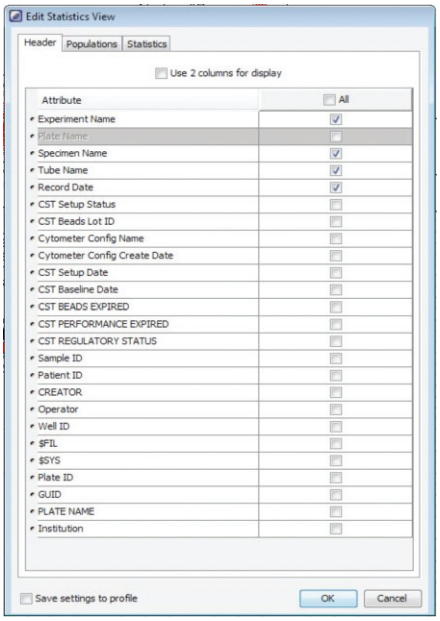
Analyzing data in BD FACSDiva software and exporting statistics to the Excel template

Open the experiment file or import the FCS files into BD FACSDiva software. It is not feasible to set isotype-specific gates within BD FACSDiva software and to export statistics individually for each sample. As an initial analysis, we recommend setting a gate on a control sample within the global worksheet that applies to all samples during batch export. Drawing one fluorescence gate for all included antibodies (nine isotypes) is not optimal, but it is a simplified analysis that is suitable for an initial screen. The isotype control data can be used to refine your analysis of a particular antibody identified after this initial analysis.

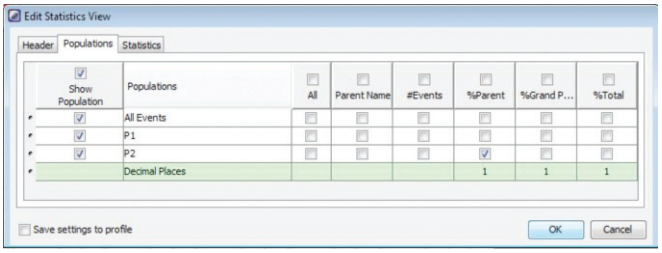
- Analyze your data by first setting a 2D gate (P1) around your cells of interest within a scatter plot, FSC vs SSC, of well A1 (or any control well with no primary antibody).
- View this gated population with an Alexa Fluor® 647 (or any secondary antibody used for the screen) histogram and draw a 1D gate (P2, subgate of P1) for everything to the right of your negative population (secondary alone from A1).
  - Place this gate carefully because it will be applied to data from all samples and might ultimately affect your critical range definition, and thus your filtered set of data parameters.



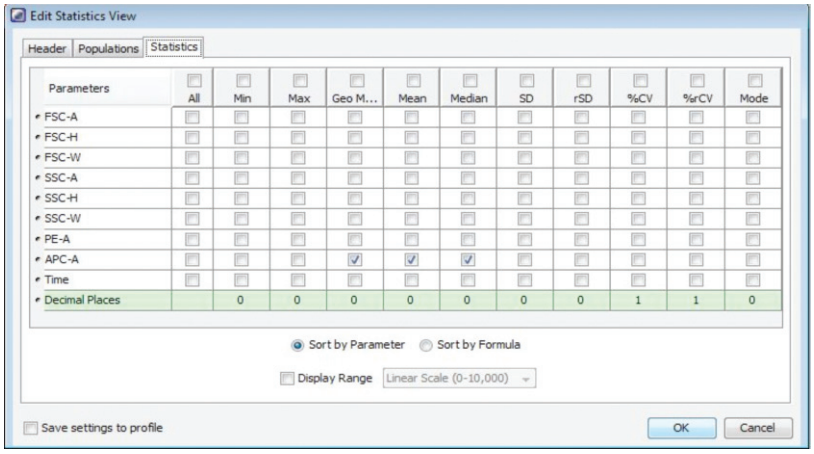
- Edit your Statistics View to display:
  - Header tab: only **Experiment Name, Specimen Name, Tube Name,** and **Record Date.**
    Note: the default settings may include other parameters. Scroll through the list and clear all other parameters.



  - Populations tab: select only **%Parent** for the positive fluorescence gated population used to collect your BD Lyoplate data (P2), and leave the **Show Population** checkboxes selected (all events, P1, and P2).



  - Statistics tab: select only **Geo Mean, Mean**, and **Median** for the fluorescence parameter used for your BD Lyoplate screen (Alexa Fluor® 647).



- Batch analyze the data.
  - Set up the batch analysis (found by right clicking the experiment in the Browser window) by selecting the checkboxes next to **Statistics** and **Freeze Biexponential Scales.** Select the **Auto** option, enter **0** for **View Time**, specify a name and location for the output file, and then click the **Start** button. The batch export process for all three plates can take 5 to 10 minutes.

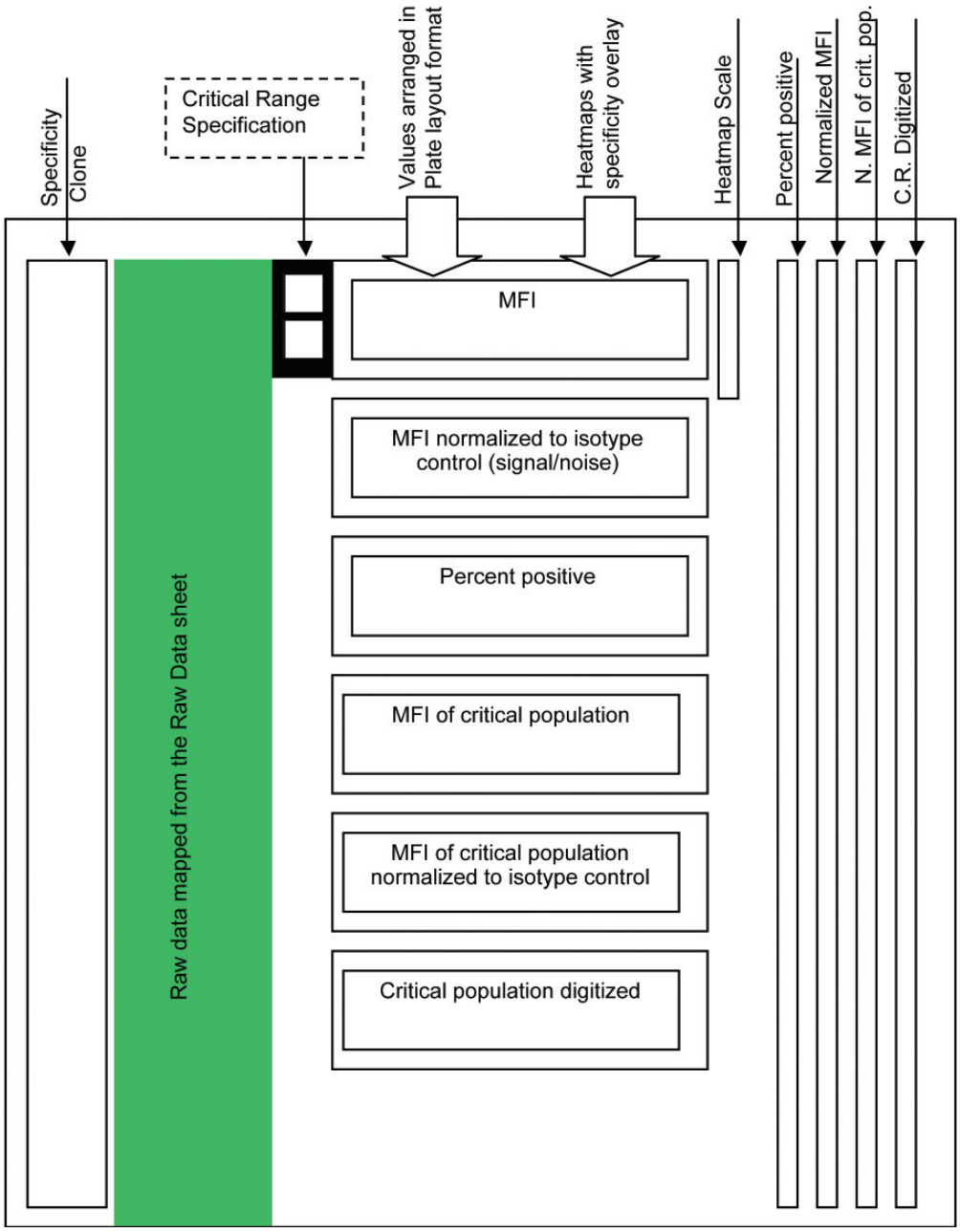
You can open your new CSV file in Excel. Keep in mind that the data will be organized in the sequence that the batch analysis was run. If the experiment was not run in the normal sequence (for example, Plate 1, Plate 2, Plate 3 in horizontal orientation), then r*eorganize the data rows into sequential order* before copying and pasting into the Excel 2007 template (do not delete, add, or reorganize any columns). If the experiment was run in sequential order, but the alignment is off (for example, the import of data files into a new BD FACSDiva file leads to alphabetical rather than sequential order), then reorganize the data to the correct sequence by using the custom sort function. The Excel 2007 custom sort can be used to accomplish this by sorting based on the **Record Date** column from **oldest to newest**.

Additional step for BD FACSDiva software version 7 and later: You will need to delete column E (Date Analyzed) in your sequentially organized CSV file before proceeding. Highlight the entire column by clicking the E above the column. Once highlighted, right-click and select **Delete**.

Highlight all of the data within the sheet, and then copy and paste into the Diva Raw Data sheet of the Excel template (click the A1 cell first, then paste to maintain alignment). No additional manipulation should be done on this sheet within the Excel template. The relevant data is mapped and redisplayed on the BD FACSDiva Analysis sheet.

> Note: perform one last check of proper alignment by scrolling down the BD FACSDiva Analysis sheet to verify that the specificity template mapping, column A, aligns with the FCS file tube name, column B.

Excel "Analysis Sheet" Template Map



Description of Excel template components

The green color highlights the cells that are filled from your pasted data in the BD FACSDiva Analysis sheet. The data includes the percent positive, A-mean, A-mean of the positive population, median, and Geo-mean for all included specificities. Before continuing analysis, an alignment check of your data should be performed. The tube name from your pasted data is mapped into column B. Ensure that the tube name from your imported data aligns with the template layout, column A, for all 200+ rows.

Cells L3 and L7 allow you to define a critical range based on the percent positive. This lets you filter the data so that only relevant results are displayed. The default critical population is between 5% and 100% positive. This range can be adjusted to filter your data depending on the experiment and specific analysis.

The six boxed regions to the right display manipulations of this raw data. Each boxed region displays the data twice and is organized in the BD Lyoplate layout (values are aligned in a 12 x 8-cell grid corresponding to the 12 x 8 96-well plate format). The cells in the left half of the box display values, and the cells on the right provide a heatmap corresponding to the same values with corresponding specificity overlays.

- The first (top) boxed region provides the MFI, directly mapped from the exported raw data region (just simply rearranged into the 96-well plate format).
- The second boxed region provides the mean fluorescence intensity normalized by the corresponding isotype control for that particular antibody. This is simply the MFI of the antibody divided by the MFI of the isotype control (signal/noise). This is especially important when comparing screens performed on different cell populations in distinct experiments.
- The third boxed region provides the percent positive, again directly mapped from the exported raw data region.
- The fourth boxed region provides the MFI of the critical population. This is simply the mean of the positive population multiplied by 1 if the user-defined critical range (cells L3 and L7) is met and 0 if it is not (ie, with filter settings of 5% to 100%, for all specificities with less than 5% of the population positive, the MFI of the critical population is nullified [= 0]).
- The fifth boxed region provides the normalized MFI of the critical population.
- The sixth boxed region provides a digital readout for the specified critical region. This plot generates a 1 for every specificity that has a percent positive value that falls in the critical region defined previously by the user (cells L3 and L7), and a 0 if it doesn't fall within this critical region. This heatmap region can be multiplied by any other plate values to clean out extraneous data. For example, the MFI of the positive population, which is the raw statistic from your batch export, can be somewhat misleading. Even for completely negative specificities, your well placed gate will likely include a very small population (<1%), and a value will be generated for the MFI of the positive population even if it represents only a few cells. In most cases this is not important data (likely dead autofluorescent cells or debris), but it will distort your heatmaps. You can remove this unwanted data by setting your critical region from 5% to 100%, then simply multiplying the critical population digitized cell values by the MFI of the positive population cell values (which is exactly what the MFI of the critical population represents).

The statistics that can be compared between various BD Lyoplate screens are the percent positive, normalized MFI, and the normalized MFI of the critical population. These statistics, in addition to the digitized critical range, are displayed on the far right in sequentially ordered single columns (as opposed to the BD Lyoplate format). These columns can be copied and pasted into a new Excel file so that they can be aligned adjacent to data generated from other BD Lyoplate screens. New heatmaps will need to be generated (using conditional formatting rules) for BD Lyoplate screen comparison analysis that includes all pasted values for a particular statistic (using one heatmap scale for values across all BD Lyoplate screens compared). Aligning data from multiple BD Lyoplate screens allows for quickly identifying surface expression differences among cell populations (mimicking the output from an RNA microarray). Refer to Excel Help for details about applying conditional formatting to generate colored heatmaps.

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