BD Lyoplate™ Human Screen Analysis Instructions

For analysis using FCS Express or FlowJo™ and heatmap representation in Excel 2007

For use with the **BD Lyoplate™ Human Cell Surface Marker Screening Panel** (Cat. No. 560747). Please check that your catalog numbers for the FCS Express Excel templates and BD Lyoplate product match.

Purpose

FCS Express and Microsoft® Excel® 2007 templates are used to analyze data generated from running a BD Lyoplate™ screen on human cells. The Excel template reorganizes the data into the BD Lyoplate format, performs normalization to isotype controls, identifies potential bimodal populations, generates heatmaps (specificity overlayed on colored tiles), and allows for easy comparison between multiple screens (ie, on distinct cell populations or repetitions on the same cell population). The FCS file data can be analyzed using any software that enables export of statistics (FCS Express, BD FACSDiva™ software, FlowJo, etc). However, the FCS Express template provided facilitates easy batch export and pasting of statistics into the Excel template.

The FCS Express template provides a tool for quickly analyzing FCS file data from the BD Lyoplate Human Cell Surface Marker Screening Panel. It automatically generates histogram overlays for all 242 antibodies with the corresponding isotype control. The FCS Express file (landscape format) can be saved directly as a Microsoft PowerPoint® presentation (.ppt file) or PDF file. Additionally, the batch export function has been programmed to output all of the proper statistics with correct alignment for easy pasting into the Excel template. The statistics data format can easily be moved in to the designated area, Raw Data sheet, within the Excel template for automatic generation of a heatmap representation of your data.

Analyzing data from a BD Lyoplate screen in FCS Express

- Make sure that when exporting your data from BD FACSDiva software (or other data acquisition software communicating with your instrument) that you export the FCS files rather than the experiment (with your experiment still open, select **File > Export > FCS files**). Choose to export only these three parameters: SSC-A, FSC-A, and A647 (or your secondary of choice) by clearing the checkboxes next to all other parameters collected.

- Open the FCS Express template (this may take a few minutes depending on your processor speed).

- Open the FCS file list window by selecting **FCS File > File List**.

- Import files by using the **Add** button and browsing for your FCS files. Make sure to import all 254 FCS files (This includes every well containing an antibody as well as three files for A1 from each plate, which serve as cells-alone controls). If the files are located in different folders, then you will need to perform multiple Add functions. If other wells from Plate 3 were collected during your experiment, do not import them during this preliminary analysis.

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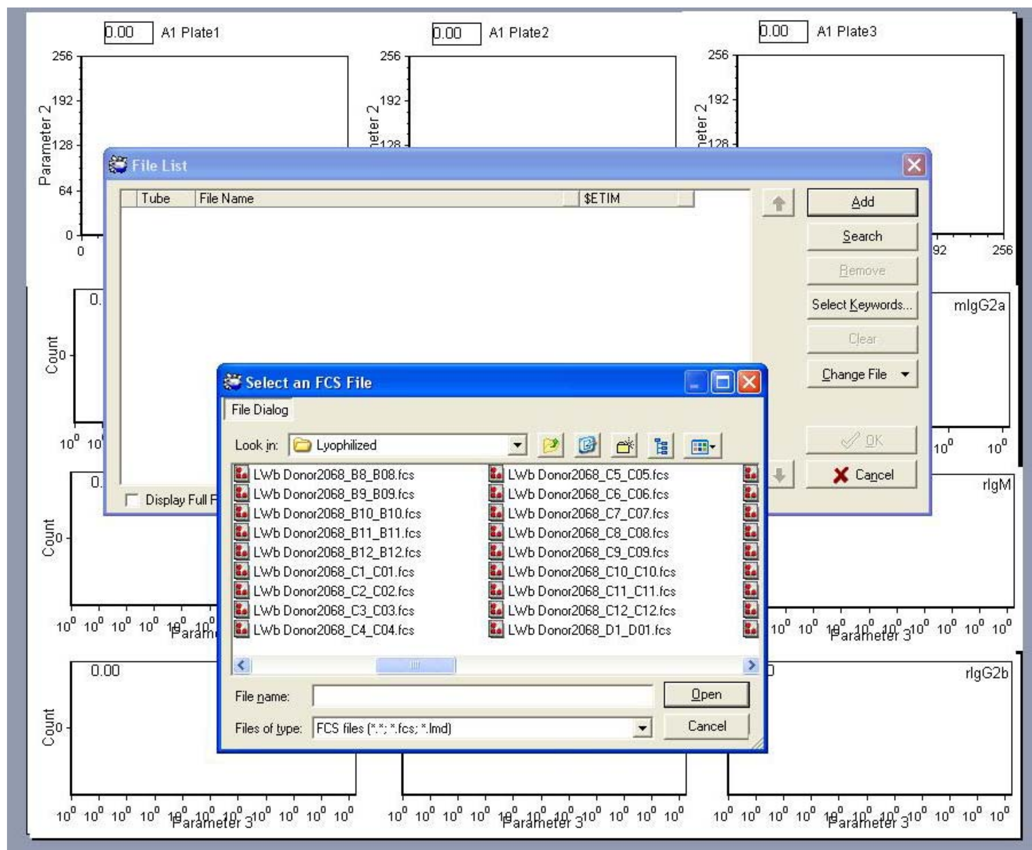

- Once these 254 FCS files have been imported, organize them in ascending order (Plate1 A1 →Plate1 H12 followed by Plate2 A1 → Plate2 H12 followed by Plate3 A1 → Plate3 H4). The first column, Tube, is the preset template layout defined in FCS Express (it doesn't move as you organize). The second column, File Name, is the FCS file name, and the third column is some variable keyword extracted from your imported FCS files. For the easiest way to organize your files *if you ran your experiment in sequential order*, select $ETIM, time at end of data collection, as the third column keyword (using the **Select Keyword** button). This should already be your default third column setting. You can organize according to sequence of data collection by clicking the small gray button in the column heading. Make sure the files are in ascending (not descending) order. Manual organization of your files within the **File List** window can be done using the larger blue arrow buttons.

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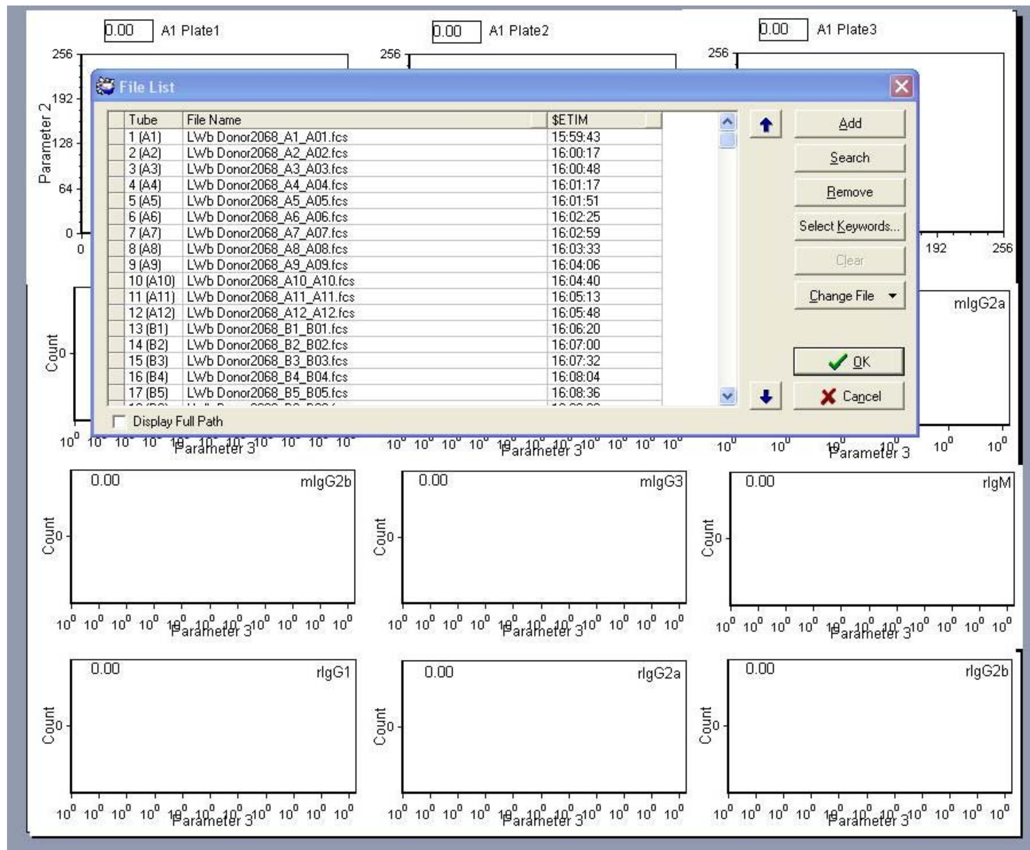

Note that the template layout (column 1) is incorrect from Plate 3 D5 onward. This is a limitation within FCS Express. Plate 3 D5 in the template column should correspond to the experiment file (FCS file) from Plate 3 E1 imported from your experiment. Plate 3 D6 in the template column should correspond to the experiment file from Plate 3 E2 imported from your experiment, and so on, finishing with Plate 3 F2 corresponding to the FCS file from Plate 3 H4 in your experiment.

- Click **OK** within the **File List** window and all plots within the 23-page template should fill. The first page allows you to set the cells gate (on FSC and SSC) and then to set all nine isotype control gates (on histograms of fluorescence). The next 22 pages provide histogram overlays for every antibody in the screening panel and its corresponding isotype control. The small box in the top left corner of the histograms displays the percent positive.

- Set your cells gate on any of the three scatter plots at the top of page 1. Then set all nine isotype control gates on the histograms below. The following example shows the gates set on granulocytes from an experiment using white blood cells.
- Save your FCS Express analysis with a new name (.fey). The analysis can also be saved as a PowerPoint and/or PDF file for presenting and sharing.

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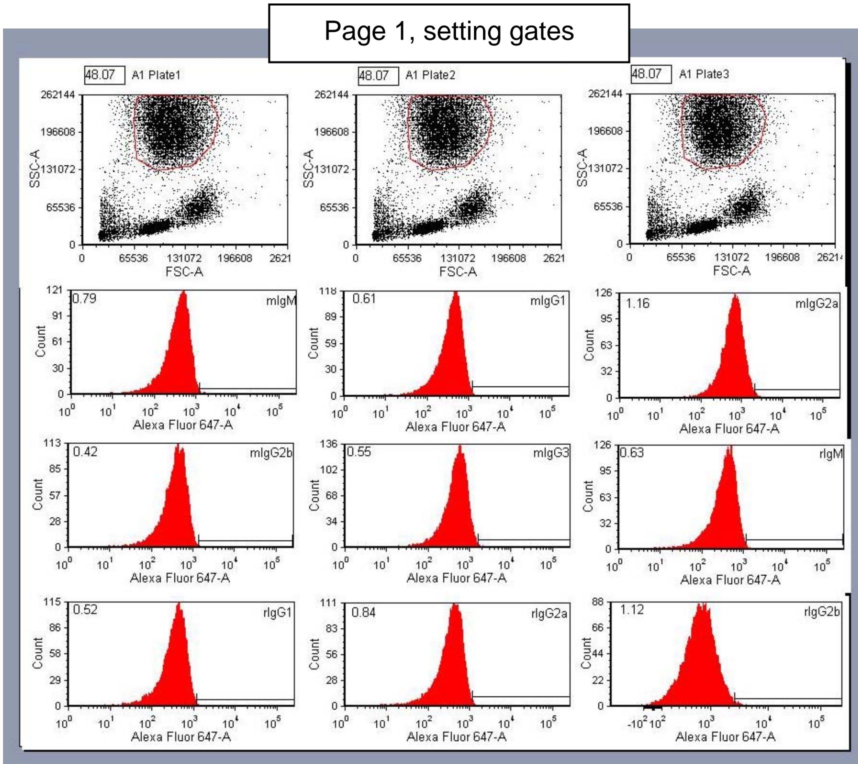

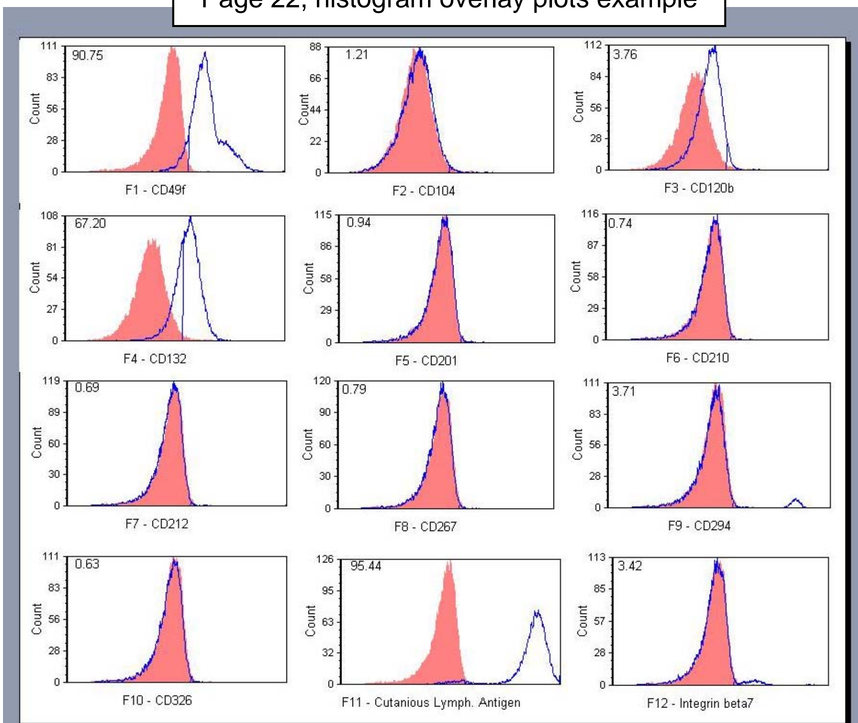

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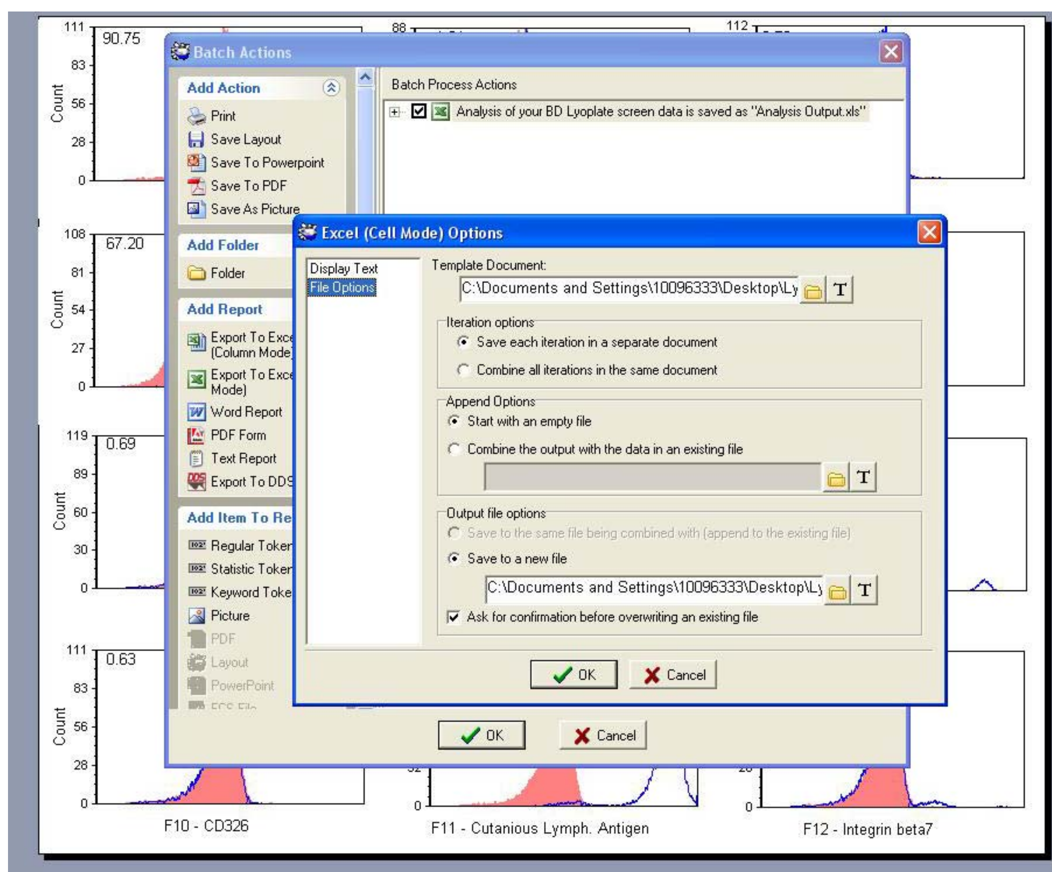
Exporting a BD Lyoplate screen analysis in FCS Express to Excel

First, FCS Express needs to know where to find the Excel template. Under **Tools > Batch Processing**, open the **Batch Processing Actions** window. Find the Excel Batch Action named **Analysis of your BD Lyoplate screen data** and double-click it to open the **Excel Options** window. In the template document bar, find the 2007 Lyoplate HeatMap Template for Data Analyzed Using FCS Express file on your computer. Select the **Save to a new file** option, and then designate a new name and location for the output file. Click **OK.** An error message warns you that you haven't used a token to specify this output file and that each new iteration will delete the previous one. This is fine because no new iterations will be used. Click **Continue**. Ensure that the **Excel Batch Process Action** checkbox is selected and click **OK.**



Once the data has been fully analyzed in FCS Express (all files imported and gates drawn), click the batch export button (lightning bolt button in the tool bar). Be sure to resave each exported analysis file (.xlsx) with a new name before performing your next analysis batch export, since the new exports will have the same name and replace your previous versions.

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Now you can open the batch export Excel file, and copy and paste the entire Iteration 1 sheet contents into the FCS Express Raw Data sheet of the template (ensure the exact alignment when pasting from Iteration 1 to FCS Express Raw Data). No additional manipulation should be done on this sheet within the Excel template. The relevant data is mapped and redisplayed on the FCS Express Analysis sheet.
Note: perform one last check of proper alignment by scrolling down the FCS Express Analysis sheet to verify that the specificity template mapping, column A, aligns with the FCS file tube name, column B.

Analyzing data from a BD Lyoplate screen in FlowJo and Exporting to Excel

Import the BD Lyoplate Screen FCS files into FlowJo. Instead of gating each marker on its specific isotype control, we recommend, as an initial analysis, setting a gate based upon a cells alone control histogram for your Alexa Fluor® 647 parameter (ie, A1 from any plate) and then dragging this gate into the All Samples group to apply it to all samples. Drawing one fluorescent gate for all 242 antibodies isn't ideal, but it is an easy way to quickly find hits within your data. The isotype control data can be used for further analysis after this initial analysis. Please refer to your FlowJo manual or the Help menu for guidance.

Use the **Add Statistic**s button to add Mean, Median, 95th %ile, and 5th %ile to the cells gate on all samples. Also add Mean and Freq. of parent to the fluorescent gate on all samples. Ensure that all samples are arranged in ascending order (Plate1 A1 →Plate1 H12 followed by Plate2 A1 → Plate2 H12 followed by Plate3 A1 → Plate3 H4). If the samples were run in sequential order, you can use the sort button to organize based on Collection Time.

Next, open the Table Editor. Drag and drop the six statistics from the file list into the **Table Editor** window. Rearrange the statistics from top to bottom (Tube, Freq. of parent, Mean, fluorescent gate Mean, Median, 5th %ile, 95th %ile), by dragging and dropping within the list. Click the **Create and view table** button to view a spreadsheet with all your values. Click the **Save as** button and save the file as an Excel document (.xls).

Now you can open the Excel file, then copy and paste the entire sheet contents into the FlowJo Raw Data sheet of the template (click the A1 cell first then paste to maintain alignment). No further manipulation should be done on this sheet within the Excel template. The relevant data is mapped and redisplayed on the FlowJo Analysis sheet.
Note: perform one last check of proper alignment by scrolling down the FlowJo Analysis sheet to verify that the specificity template mapping, column A, aligns with the FCS file tube name, column B.

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Excel "Analysis Sheet" Template Map

Specificity and Clone

Critical range specification

Values arranged in Plate layout format

Heatmaps with specificity overlay

Heatmap key

Percent

Normalized MFI

N. MFI of crit. pop.

C.R. digitized

Raw data mapped from the Raw Data sheet

MFI

MFI normalized to isotype control (signal/noise)

Percent positive

MFI of critical population

MFI of critical population normalized to isotype control

Critical population digitized

Bimodality factor

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Description of Excel Analysis Sheet template components

The green color highlights the cells that are filled from your Raw Data sheet, manually loaded via cut and paste from the FCS analysis export file. The data includes the percent positive, mean, mean of the positive population, median, 5th percentile, and 95th percentile for all 242 specificities.

The boxed regions to the right display manipulations of this raw exported 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 mean fluorescence intensity (MFI), directly mapped from the exported raw data region (rearranged into the 96-well plate format).
- The second boxed region provides the MFI normalized by the corresponding isotype control for that particular antibody. This is 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.
- 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 by the user (cells J3 and J7), and a 0 if it does not 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 and muddy your heatmaps. You can remove this unwanted data by setting your critical region from 5% to 100%, then 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 seventh and last boxed region provides the bimodality index. This is a measure of the symmetry of the Alexa647 histogram plot **(LOG(95%-50%)/LOG(50%-5%))**. A symmetric distribution indicates a single population and scores a bimodality index of 1. In some cases, two populations will show distinct peaks, but for those cases in which a subpopulation has only a small shift in MFI from the main population, it may be difficult to see qualitatively on the histogram plot (it may appear as a small shoulder or tail on the plot, which easily can be missed when reviewing 242 histogram overlays). In most cases when two populations are present with two distinct MFIs, the bimodality index will stray from the value 1 (less or greater than 1).

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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, which 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 quick identification of surface expression differences among cell populations (mimicking the output from an RNA microarray).

Alexa Fluor is a registered trademark of Molecular Probes, Inc.

Rev. 1