Miltenyi Biotec

Data acquisition and analysis of MACSPlex Cytokine Kits using the MACSQuant® Analyzer Express Modes in MACSQuantify™ Software version 2.11

Contents

1. **Description**
   - 1.1 **Background information**
   - 1.2 **Reagent and instrument requirements**
   - 1.3 **Estimation of time and buffer for data acquisition using the MACSQuant® Instrument**
2. **Data acquisition using the MACSQuant® Analyzer 10 or MACSQuant® X Express Modes**
3. **Analysis of data files with the MACSPlex Express Modes**
4. **Protocols for manual process**
   - 4.1 **Manual experiment definition with Express Modes**
   - 4.2 **Gating strategy for manual analysis without Express Mode**
5. **Troubleshooting**

1. Description

This product is for research use only.

1.1 Background information

MACSPlex Assays have been designed for determining concentrations of soluble cytokines in a single sample. The analysis is based on MACSPlex Capture Beads, which display defined fluorescence properties and can be identified using standard flow cytometry techniques.
In combination with the Express Modes of the MACSQuant® Instruments the MACSPlex Cytokine Kits are optimized for automated measurement. They simplify flow cytometric analysis via predefined instrument and experiment settings as well as acquisition and analysis templates. They apply an automated gating strategy to populations of interest that will be automatically adjusted for each data file to achieve optimal results.

The Express Modes **MACSPlex_Standard** and **MACSPlex_Sample** have been developed for use with the MACSPlex Cytokine Kits. The measurement and analysis using these Express Modes can be performed with the MACSQuant Analyzer 10 and the MACSQuant X. They are not suitable for use with the MACSQuant VYB.

MACSPlex_Standard is used for the measurement of the MACSPlex Cytokine Standard. It contains all relevant information for the determination of the standard values based on the serial dilution of the MACSPlex Cytokine Standard. If replicates of the serial dilution of the Standard are measured, mean values of the replicates will be calculated.

MACSPlex_Sample is used for the measurement of samples containing unknown cytokine amounts. To accurately calculate the cytokine concentration in an unknown sample both Express Modes, MACSPlex_Standard and MACSPlex_Sample, have to be used. The cytokine concentrations of unknown samples will be determined based on the last acquired MACSPlex_Standard values.

1.2 Reagent and instrument requirements

- MACSQuant Analyzer 10 (# 130-096-343) or MACSQuant X (# 130-105-100) with MACSQuantify™ Software version 2.11 or higher and at least Express Mode version **installExpressModes_211.10.19504**.
  - ▲ **Note:** The MACSQuant VYB cannot be used.
- Chill 96 Rack (# 130-094-459)
- MACSQuant Calibration Beads (# 130-093-607)
- MACSPlex Cytokine Kit, for example, MACSPlex Cytokine 12 Kit, human (# 130-099-169), MACSPlex Cytokine 10 Kit, mouse (# 130-101-740), MACSPlex Cytotoxic T/NK Cell Kit, human (# 130-119-473), or MACSPlex Cytokine Reagent Kits, human or mouse of choice.
  - ▲ **Note:** Do not mix human and mouse in one experiment.

1.3 Estimation of time and buffer for data acquisition using the MACSQuant® Instrument

Estimation for acquisition with default settings of Express Modes (150 µL sample uptake with High flow rate and Standard Mode with active mixing):

	MACSQuant Running Buffer	Time
1 sample	20 mL	2.5 minutes
96 samples	2000 mL	4 hours

2. Data acquisition using the MACSQuant® Analyzer 10 or MACSQuant® X Express Modes

▲ Use the autolabel function including the 2D code reader of the MACSQuant® Instrument to ensure the selection of the correct reagent lot and to upload all necessary MACSPlex information. These include population data, names of analyzed cytokines, median for B1, median for B2, the maximum number of standard positions within the serial dilution, dilution factors, and concentrations of each cytokine in the MACSPlex Cytokine Standard. When using MACSPlex Cytokine Kits all labeling steps are performed manually. The autolabel function is only used to identify reagents, to add required information to data files, and to load the predefined experiment for acquisition.

▲ For further information refer to the MACSQuant Instrument user manual and the MACSQuantify Software user guide.

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For a correct MACSPlex Filter Plate calibration please contact your administrator or refer to chapter "Calibration of the MACSPlex Filter Plate" of the MACSQuant® Instrument user manual.

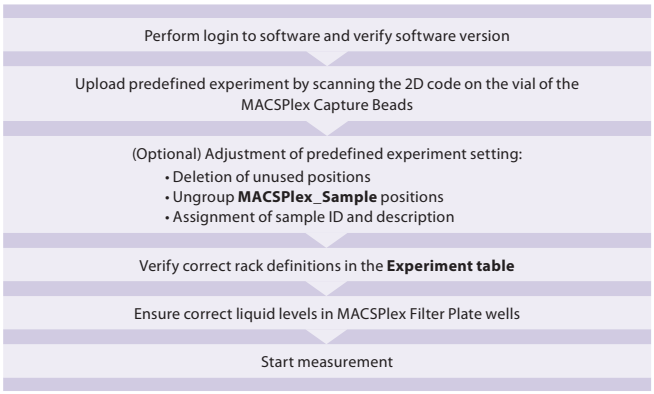


Figure 2.1: Overview of data acquisition

2.1 Login

Login as a custom mode user. Make sure the instrument is in acquisition mode, i.e., bottles are illuminated and the instrument status bar displays **Acquisition mode**.

For optimal results it is important to perform a PMT calibration of the instrument prior to use.

For a proper Express Mode acquisition and analysis performance please avoid special characters or annotations (e.g. "ö", "ë", "œ", "µ", etc.) for the data file name structure.

2.2 Verify software version

1. Select **Help** > **Info…** from the menu bar.
2. Verify that MACSQuantify™ Software version 2.11 or higher and at least Express Mode version 211.10.19504 (installExpressModes_211.10.19504) are installed on the MACSQuant Instrument.

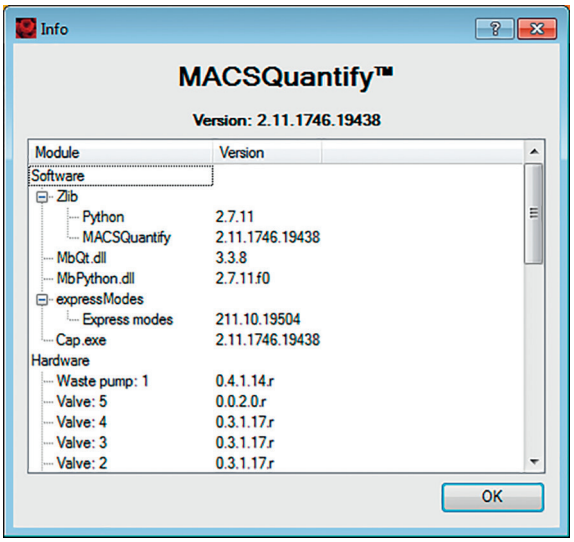


Figure 2.2: Information window for MACSQuantify Software and Express Mode version

2.3 Instrument settings

The instrument settings are automatically uploaded when using the MACSPlex Express Modes with MACSQuantify Software version 2.11 or higher after assigning the MACSPlex Capture Beads. The loading of the settings will be performed when the measurement starts.

2.4 Upload predefined experiment settings

In order to use the Express Mode, reagent information of the MACSPlex Kit needs to be identified. This is achieved by using the autolabel function and the 2D code reader of the MACSQuant Instrument. With the MACSQuantify Software version 2.11 a predefined experiment can be uploaded by using the 2D barcode of the MACSPlex Capture Beads vial, containing all relevant settings for the acquisition.
If the reagent label on the vial is damaged or the scanning of the barcodes is not successful, the predefined experiment can be selected manually as described in chapter 5.2 "Manual loading of predefined experiments".
When working with the MACSPlex Custom Cytokine Assays no predefined experiment settings are provided. For the creation of a MACSPlex Custome Cytokine experiment, please refer to chapter 4.1 "Manual experiment definition with Express Modes". An individual combination of up to seven cytokines is possible. Do not mix human and mouse in one experiment.

1. Click on the 2D code reader icon of the toolbar and wait for the code reader to start blinking. Present the vial containing the MACSPlex Capture Beads to the 2D code reader. Ensure the 2D code is facing the blinking code reader light in the optimal reading distance of 0.5–2.5 cm.

   ▲ **Note:** The predefined experiment is based on the MACSPlex Filter Plate. It contains in column 1 and 2 the MACSPlex_Standard positions (duplicate of serial dilution) with vertical rack processing order. Columns 3–12 are grouped and assigned to MACSPlex_Sample position. For the whole plate the autolabel for the appropriate MACSPlex Capture Beads and for NoLabel are selected and assigned. If the positions of the serial standard dilution on your plate are not positioned in column 1 and 2, please refer to chapter 4.1 "Manual experiment definition".

2.5 (Optional) Adjustment of predefined experiment settings

▲ If not complying with the predefined experiment in terms of, e. g., sample volume or order of serial dilution of standards, please refer to chapter 4.1.6 "Assignment of MACSPlex_Standard positions" or chapter 4.1.7 "Assignment of MACSPlex_Sample positions" .

2.5.1 Deletion of unused MACSPlex_Sample Positions

▲ Always delete unused positions in the predefined experiment settings.

▲ Grouping can be performed on several columns as long as the rack positions are adjacent.

▲ If the middle positions of a grouping are deleted the group will be splitted into two (indicated by two different numbers on the wells).

▲ If sample positions are not adjacent, the measurement can be performed without grouping positions. Then the grouping of sample positions needs to be performed during data analysis to enable batch analysis on all samples at once.

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1. To delete unused sample positions click on the relevant well or column. A closed green circle with an orange rim indicates sample position activation.
2. Click **Clear** to delete the activated positions.
3. (Optional) To ungroup the MACSPlex_Sample positions double-click on one well to activate all positions within the group (indicated by orange rim). Click **Ungroup** to ungroup the positions.

2.5.2 Assignment of Sample ID and Description

Additional user-defined information can be entered in tab **Experiment**, fields **Sample ID** and **Description.** Sample ID and Description can be allocated individually to each sample position. Please avoid special characters or annotations (e.g. "ö", "ë", "œ", "µ", etc.).

2.6 Check the rack definitions

Before starting the measurement, especially if the predefined experiment has been adjusted, it is recommended to check and verify the rack definitions using the **Experiment table**:

1. Select **View > Experiment table…** from the main menu.
2. Ensure that rack definitions for **Acquisition, Annotations, Autolabel,** and **Settings** are correct.
3. If the assignment of the MACSPlex_standard positions has been changed, check in the **Acquisition** tab that no double assignments of Sample IDs within one serial dilution of MACSPlex Cytokine Standards has been done.
4. Check the **Autolabel** and **Settings** tabs and make sure that for each selected position the correct MACSPlex Capture Beads, **NoLabel** function, as well as the correct Express Mode are selected.

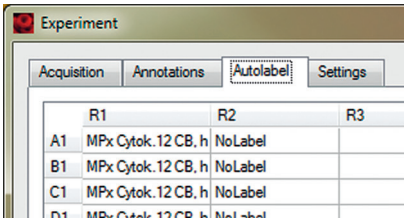


Figure 2.3: Control of rack definitions in the Autolabel tab of the Experiment table.

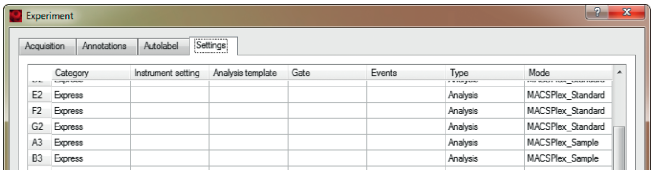


Figure 2.4: Control of rack definitions in the Settings tab of the Experiment table.

2.7 Control wells for liquid level

1. Place the MACSPlex Filter Plate on a Chill 96 Rack. Make sure that all residual drops under the plate are completely removed to prevent liquid carry over (refer also to the kit data sheet).
2. Ensure that all used wells of the MACSPlex Filter Plate contain the required amount of liquid, as described in the kit data sheet. In case of leaks remove any residual drops from under the plate completely, e.g., by placing the plate briefly onto a tissue. If required, fill up wells with the relevant amount of buffer.

2.8 Start the measurement

▲ Before starting the measurement, ensure buffer supply and an empty waste bottle of the MACSQuant® Instrument are sufficient (refer to section 1.3).

▲ If measuring a whole plate, use the orange protective cover to avoid optical whitening of APC staining.

1. Click **Start** measurement.
2. The acquisition window, defined by the selected Express Mode, will appear on the screen. After completion of the acquisition the Express Mode will automatically proceed to the analysis (for grouped positions, the analysis starts after measurement of the last position within the group). All relevant information (acquisition window, analysis window, type of Express Mode, instrument settings) are saved within the data file and do not have to be saved separately.

   ▲ **Note:** The time difference between the first MACSPlex_Standard measurement and the first MACSPlex_Sample measurement should not exceed two hours to ensure the assignment of the appropriate standard data to the respective sample analysis. Moreover, when performing more than one assay within two hours, do not reactivate former MACSPlex_Standard data files before or during a measurement to avoid the assignment of inappropriate former MACSPlex_Standard data files to the current sample analysis. Always monitor until the measurement has been started in case of warn windows appear.

3. Analysis of data files with the MACSPlex Express Modes

The analysis of the data files can be performed on the MACSQuant Instrument itself or on a PC with installed MACSQuantify™ Software version 2.11 or higer with at least Express Mode version **installExpressModes_211.10.19504**.

▲ **Note:** The Express Mode version must be the same on the MACSQuantify Software PC version as of the used MACSQuant Instrument.

▲ After finishing the measurement, do not perform the MACSPlex_Standard analysis on the MACSQuant Instrument if another MACSPlex Cytokine Assay is going to be measured within the next two hours.

3.1 Analysis of data files using the Express Modes

1. When performing the data analysis on a PC, make sure that the MACSQuantify Software is set to the correct instrument configuration: Select **Edit > Configuration...** from the main menu to select the MACSQuant Instrument that was used for data acquisition.
2. Right-click within the **Samples** tab and select **Add…** or **Open...** from the context menu to upload data files to the MACSQuantify Software.

   ▲ **Note:** If MACSPlex_Sample positions were acquired ungrouped, group the appropriate data files to enable batch analysis. Mark the data files in the sample list, right-click on the marked files, and select **Group**.
   Assure that only samples from the same experiment are grouped. Do not mix them with MACSPlex_Standard files. The order of the grouped samples in the grouped file (marked by "_") is based on the order of the files in the sample list (from top to bottom).

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3. Right-click on the file name and select **View with Analysis.<*name of the Express Mode*>** (i. e., MACSPlex_Standard or MACSPlex_Sample) for accessing the Express Mode analysis template. The appropriate analysis page will be displayed after analysis has been finished.

▲ **Note:** The analysis of the grouped data file can take a certain amount of time due to the high number of events in the grouped file.

▲ **Note:** Perform analysis always by using **View with….** If using the Analysis Mode tool to scroll through samples in list, the calculation of the cytokine concentration results is not applied to the correct sample.

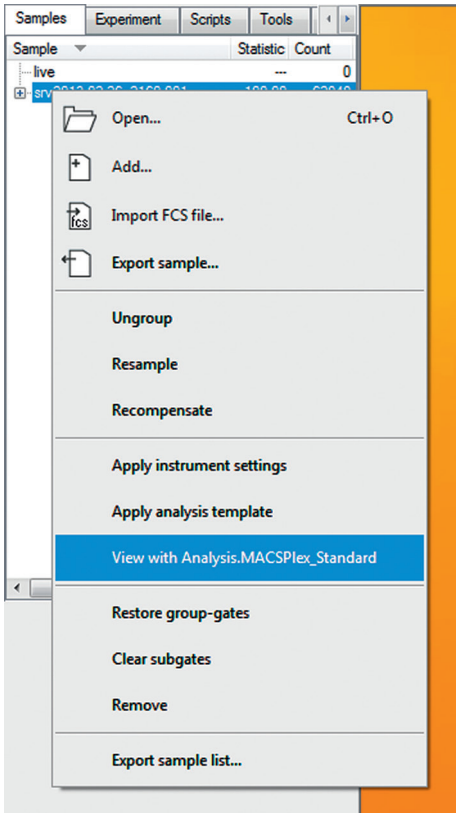


Figure 3.1: Selecting the Express Mode for analysis of the data files.

For MACSPlex_Standard data files the data will be displayed in a single analysis window without further user interaction.

For the grouped MACSPlex_Sample data during analysis it can be choosen between standard values or change standard values. For futher details, please refer to chapter 3.3 "Analysis of MACSPlex_Sample data files with changed standard values". At the end of the analysis an information window is displayed about the automatically exported results and its directory before the final analysis pages are displayed. By clicking **OK** in the info window, the display of the analysis pages will be finalized.

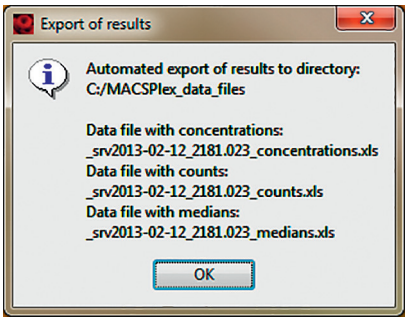


Figure 3.2: Information window about exported results.

▲ **Note:** The possibility to change standard values and the automatic export of results is only performed on the PC not on the MACSQuant® Instrument.

4. The plot windows of the MACSPlex_Standard and MACSPlex_Sample analysis display all relevant information, including trend of the APC median of each standard concentration in the MACSPlex Standard samples. Also final concentrations of each cytokine are shown.

The forward scatter area (FSC-A) versus side scatter area (SSC-A) density plot allows the identification of the singlet beads and the B1-A (FITC) versus B2-A (PE) density plot for the identification of the different color-coded bead populations.
The major plot of the MACSPlex_Standard analysis window shows the increase of the APC median values for increasing standard concentrations for all cytokines of the MACSPlex Cytokine Kit (figure 3.3). The MACSPlex_Sample analysis window shows a diagram with the logarithmic overview of the determined cytokine concentration of all cytokines in the unknown sample (figure 3.4).

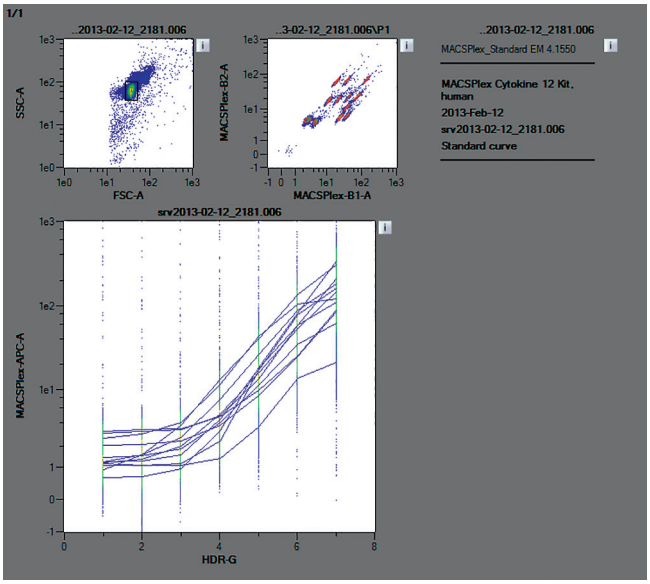


Figure 3.3: MACSPlex_Standard analysis window, displaying all relevant information. HDR-G abbreviates the group parameter in the MACSQuantify™ Software. In case of MACSPlex Assays, each position of HDR-G represents a measured standard cytokine concentration.

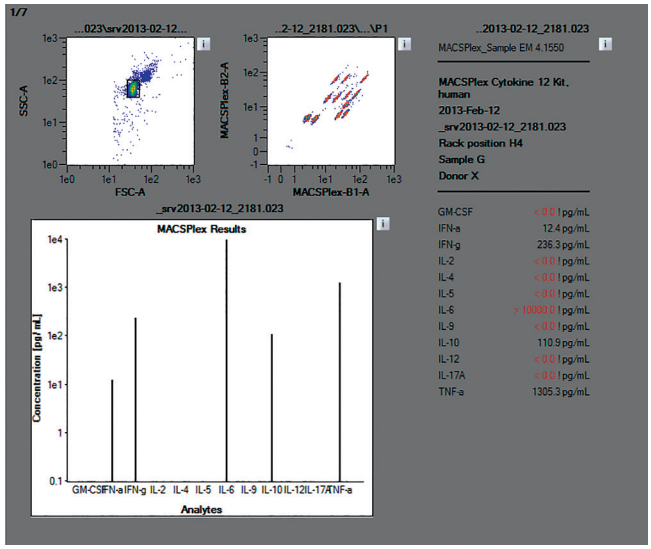


Figure 3.4: MACSPlex_Sample analysis window from the MACSPlex Cytokine 12 Kit. The statistics table displays the Express Mode name and version number, the MACSPlex Kit name, the date of the measurement, the file name, the rack position of the sample, the sample ID, and the description of the data file, and lists the final concentration of each cytokine measured in the unknown sample.

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The automatic export contains a table with the sample identifying parameters well ID, sample ID, and description and the corresponding cytokine concentrations, R1 (APC) medians, or counts, respectively, for each analysis.

_srv2013-02-12_2181.023							
Cytokine conc. in pg/mL							
Well ID	Sample ID	Description	GM-CSF	IFN-a	IFN-g	IL-2	IL-4
H4	Sample G	Donor X	<0,0!	13,3	236,3	<0,0!	<0,0!
A5	Sample H	Donor V	<0,0!	20,2	24,6	<0,0!	<0,0!
B5	Sample I	Donor W	0,6	34,7	86,6	2,7	2,3
C5	Sample J	Donor S	<0,0!	20,2	1,3	<0,0!	<0,0!
D5	Sample K	Donor Z	3,8	60,5	62,8	0,6	22,1
E5	Sample L	Donor T	37	98,1	6378,7	9144,9	17,3
F5	Sample M	Donor U	3,9	82,6	53	0,4	20,9

Figure 3.5: Example from automatically exported concentrations of unknown samples.

If the unknown samples have been pre-diluted, the dilution factor will not automatically be included in the cytokine concentrations calculated by the software. These have to be calculated by the user.

If standard curve raw data are needed, including the APC medians for each concentration level of each MACSPlex population, they can be viewed in the csv-file created by the Express Mode MACSPlex_Standard. For more information on the export of csv files, please refer to the MACSQuantify™ Software user guide or contact Miltenyi Biotec Technical Support.

3.2 Additional information about analysis

During the acquisition of the unknown samples, the MACSPlex_Sample Express Mode utilizes the average APC medians from the MACSPlex_Standard measurements and saves this information as an extension of the data file. Based on this extension it calculates the cytokine concentration levels.

The regions for the singlet gate and for the color-coded bead populations are determined on the whole amount of events of the grouped data file. Afterwards the regions are linked to each sample position of the grouped file. Therefore the same regions are applied to all samples of the batch analysis.

The names of the regions are not automatically displayed in the analysis template. Click the **i** button next to the dot plot and select the **Region functions** tab. Select the functions **Name** or **Path** and confirm it by selecting **OK** or **Apply** to display the names of the regions.

The calculation of the cytokine concentrations in the unknown samples is based on a combination of monotone cubic Hermite interpolation and linear interpolation.

During the analysis of cytokine standard curves the range of monotonic increasing behavior is identified and sets the concentration range for the calculation (light grey area in figure 3.6). The upper and lower detection limits describe the APC median values that border the well-defined concentration-median assignment (dark grey area in figure 3.6). The calculation only takes place within the concentration range of monotonic increasing behavior. As shown as an example in figure 3.7, sample APC median values that are above the 2000 pg/mL APC median value are marked with "> 2000.0 ! pg/mL".

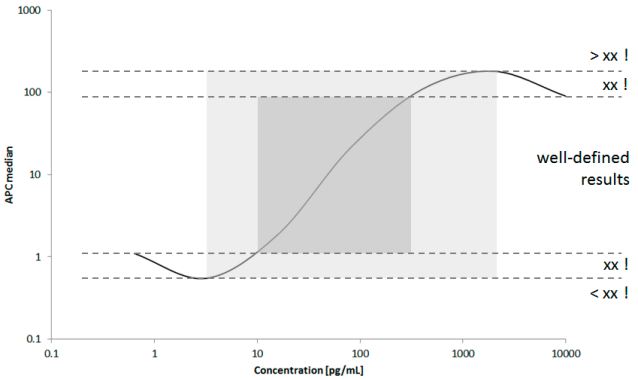


Figure 3.6: Demonstration of the different defined calculation areas within an exaggerated cytokine standard curve.

MACSPlex_Sample EM 4.1550

MACSPlex Cytokine 12 Kit, human
2013-Mar-12
srv2013-03-12_2160.045
Rack position C8
Sample B
Donor B

GM-CSF	4.2 pg/mL
IFN-a	> 2000.0 ! pg/mL
IFN-g	5.1 pg/mL
IL-2	< 0.0 ! pg/mL
IL-4	4.7 pg/mL
IL-5	0.6 pg/mL
IL-6	996.1 pg/mL
IL-9	3.8 ! pg/mL
IL-10	44.2 pg/mL
IL-12	50.1 pg/mL
IL-17A	22.2 pg/mL
TNF-a	1595.5 pg/mL

Figure 3.7: Example of a MACSPlex_Sample statistic with special marked results.

Special markings of cytokine concentration results:

"!": APC median values used for calculation of sample concentration are outside the defined median range and could correlate to more than one concentration of the standard curve. The value assigned is the one within the concentrations range of monotonic increasing behavior of the APC median (light grey area of figure 3.6).

"<…!": APC median values used for calculation of sample concentration are below the lowest APC median value of the standard curve. The corresponding standard cytokine concentration is displayed.

">…!": APC median values used for calculation of sample concentration are above the highest APC median value of the standard curve. The corresponding standard cytokine concentration is displayed.

"No result !": Calculation of the cytokine concentration for the relevant analysis was not possible as the standard APC median values do not show a proper increasing behavior. Other analysis within the experiment may also not have the expected behavior due to premixed cytokine standards.

All results that are calculated outside the well-defined range are also marked red.

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The complete analysis of standard and sample data files is a self-contained system. Later manual modifications of the gates do not have any effect on the calculated results. In case of manual intervention in the gating the final cytokine concentration of the unknown samples has to be determined manually.

3.3 Analysis of MACSPlex_Sample data files with changed standard values

The MACSPlex Cytokine Express Modes in the MACSQuantify™ Software version 2.11 has the option to perform the MACSPlex_Sample data file analysis based on changed standard values. This can be necessary if a MACSPlex_Standard data file analysis shows unexpected behavior. Therefore, please check the analysis of the MACSPlex_Standard data before processing the analysis of MACSPlex_Sample data.

The possibility to change standard values can only be performed on the PC not on the MACSQuant® Instrument.

▲ **Note:** Assure not to upload empty csv files. If more than one csv file containing the average cytokine standard values has been uploaded they all have to have the same analytes and standard cytokine concentration levels.

During MACSPlex_Sample data analysis a question window is displayed to choose between standard values or change standard values on the displayed question window.

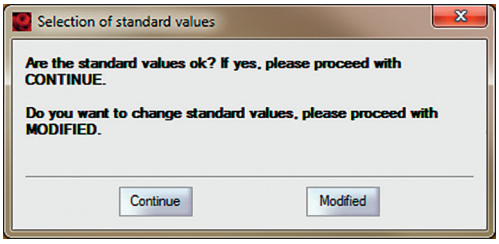


Figure 3.8: Question window if standard values are ok or not.

If the standard values of the experiment are ok, click **Continue** and the analysis proceeds with its general behavior based on the standard values of the experiment and the information window about the exported results is displayed (refer to figure 3.2).

If the standard values of the experiment are not ok, click **Modified** to proceed with changed standard values.
A question window is then displayed, asking how many sets of standard values are going to be uploaded. Select the number by clicking on the up and down arrow buttons and click **OK**. A set of standard values represents the analyzed values saved within a csv file of one MACSPlex_Standard data file.

▲ **Note:** This question window cannot be closed by clicking the **X** button.

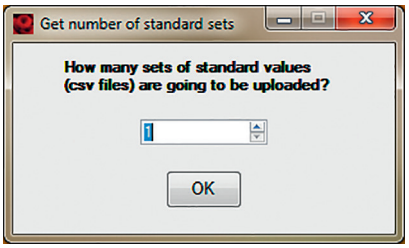


Figure 3.9: Question window how many standard sets are to be uploaded.

A separate window pops up showing the path of the csv files containing the standard values saved by the MACSPlex_Standard Express Mode (naming of csv files according to appropriate data file name). Select csv file of choice and confirm.
The user directory path has to be used to upload the csv file. When trying to upload a csv file from another directory a directory mismatch warn window appears (refer to figure 3.13).
When selecting more than one set of standard values the respective number of windows will be opened one after the other.

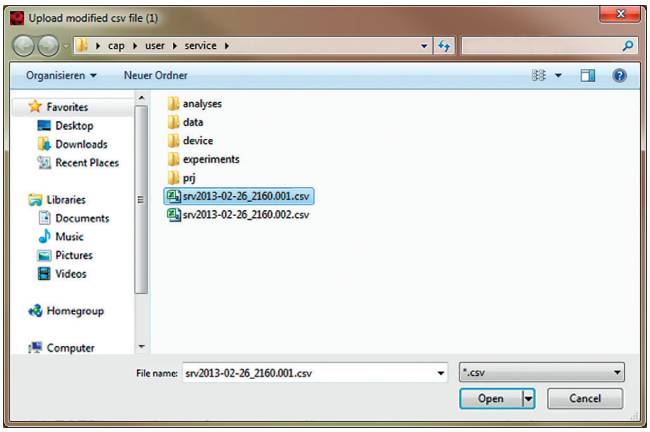


Figure 3.10: Opened explorer with user directory path to upload csv files.

Finally the information window about the automatically exported results is displayed (refer to figure 3.2).

The modified standard values of the MACSPlex_Sample analysis are stated in the statistic on the analysis pages (refer to figure 3.11 and 3.12)

▲ **Note:** In the exported results the modification of the standard values are not mentioned.

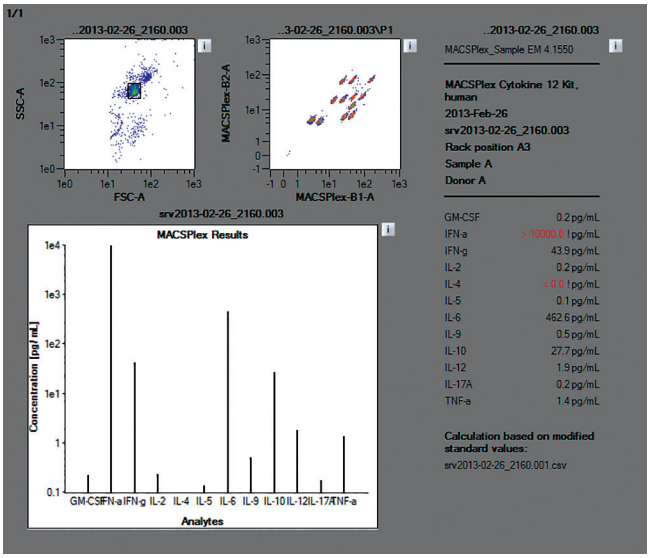


Figure 3.11: MACSPlex_Sample analysis from the MACSPlex Cytokine 12 Kit with changed standard values.

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MACSPlex_Sample EM 4.1550

MACSPlex Cytokine 12 Kit, human
2013-Feb-26
srv2013-02-26_2160.003
Rack position A3
Sample A
Donor A

GM-CSF	0.2 pg/mL
IFN-a	> 10000.0 ! pg/mL
IFN-g	43.9 pg/mL
IL-2	0.2 pg/mL
IL-4	< 0.0 ! pg/mL
IL-5	0.1 pg/mL
IL-6	462.6 pg/mL
IL-9	0.5 pg/mL
IL-10	27.7 pg/mL
IL-12	1.9 pg/mL
IL-17A	0.2 pg/mL
TNF-a	1.4 pg/mL

Calculation based on modified standard values:
srv2013-02-26_2160.001.csv

Figure 3.12: Statistic of MACSPlex_Sample analysis with changed standard values.

Directory mismatch

When trying to upload a csv file from another directory a directory mismatch warn window appears (refer to figure 3.13) and the upload of the chosen csv files is canceled (refer to figure 3.14).
In this case transfer csv files of choice to the user directory (path displayed in the "directory mismatch" warn window) and start the MACSPlex_Sample analysis again.

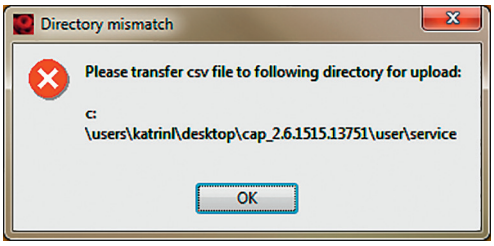


Figure 3.13: Directory mismatch warn window when trying to upload csv file from another directory than the user directory.

Cancellation of changed standard values

If the process to change the standard value is canceled (e.g. if trying to upload from a different directory than user directory or if trying to close the upload window using the **X** button) a warn window is displayed, stating the failure during the upload of the chosen csv files. In this case the MACSPlex_Sample analysis is processed based on the standard values of the experiment.
To use the MACSPlex_Sample analysis based on changed standard values, the analysis has to be started again.

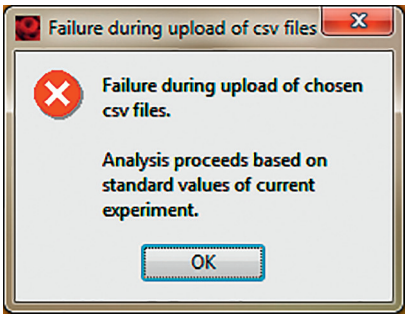


Figure 3.14: Warn window about the failure during upload of csv files.

4. Protocols for manual process

4.1 Manual experiment definition with Express Modes

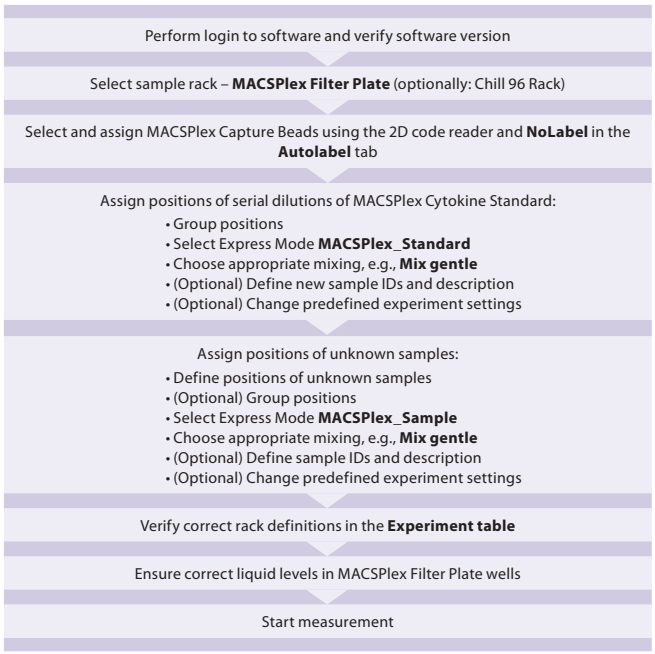


Figure 4.1: Overview of data acquisition

For a correct MACSPlex Filter Plate calibration please contact your administrator or refer to chapter "Calibration of the MACSPlex Filter Plate" of the MACSQuant® Instrument user manual.

4.1.1 Login

Login as a custom mode user. Make sure the instrument is in acquisition mode, i.e., bottles are illuminated and the instrument status bar displays **Acquisition mode**.

For optimal results it is important to perform a PMT calibration of the instrument prior to use.

For a proper Express Mode acquisition and analysis performance please avoid special characters or annotations (e.g. "ö", "ë", "œ", "µ", etc.) for the data file name structure.

4.1.2 Verify software version

1. Select **Help > Info…** from the menu bar.
2. Verify that the MACSQuantify™ Software version 2.11 or higher and at least Express Mode version 211.10.19504 (installExpressMode_211.10.19504) are installed on the MACSQuant Instrument (refer to figure 2.2).

4.1.3 Instrument settings

The instrument settings are automatically uploaded when using the MACSPlex Express Modes with MACSQuantify Software version 2.11 or higher after assigning the MACSPlex Capture Beads. The loading of the settings will be performed when the measurement starts.

4.1.4 Select the sample rack

1. In the sidebar **Experiment** tab, select the **MACSPlex Filter Plate** from the **Rack** drop-down list. When not using a MACSPlex Filter Plate select **Chill 96 rack**.

   ▲ **Note:** If the plate is not displayed in the window, activate the **Rack** icon of the toolbar.

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4.1.5 Select and assign MACSPlex Reagents

In order to use the Express Mode, reagent information and the predefined experiment of the MACSPlex Kit need to be identified by using the autolabel function and the 2D code reader of the MACSQuant® Instrument.

If the reagent labels on the vials are damaged or the scanning of the barcodes is not successful, the reagents can be manually selected (refer to section 5.1).

When using MACSPlex Custom Cytokine reagents do not exceed the maximum number of seven analytes. Do not mix human and mouse in one experiment.

1. Click on the **2D code reader** icon of the toolbar and wait for the code reader to start blinking. Present the vial containing the MACSPlex Capture Beads to the 2D code reader. Ensure the 2D code is facing the blinking code reader light in the optimal reading distance of 0.5–2.5 cm. Once scanned a MACSQuantify™ Software dialog box will appear with information about where to place the reagent on the reagent rack as long as no predefined experiment exists.

   When using the MACSPlex Custom Cytokine reagents repeat step 1 for each MACSPlex Capture Beads.

   ▲ **Note:** If a predefined experiment setting exists, the experiment will be uploaded and displayed instead of the dialog box. If you need the rack "MACSPlex Filter Plate" for your manually defined experiment, select all positions on the rack, clear them, and proceed with chapter 4.1.6. If you need another rack type, select the appropriate rack according to chapter 4.1.4 and proceed with step 2 to check the reagents (refer to figure 4.2).

2. The scanned reagent and a **NoLabel** check box will automatically be displayed in the **Autolabel** tab of the **Experiment** tab.

3. Check the respective MACSPlex Capture Beads and NoLabel boxes in the **Autolabel** tab located in the **Experiment** tab. The red exclamation mark indicates the activated autolabel function.

   ▲ **Note:** It is important to tick NoLabel option. It will disable pipetting steps, which are usually performed by the MACSQuant Instrument during an autolabeling procedure.

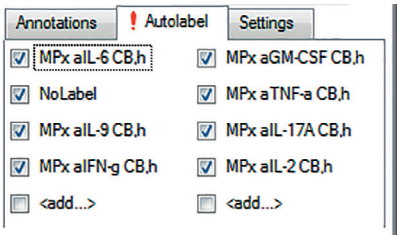


Figure 4.2: Tick MACSPlex Capture Beads, human and NoLabel boxes in the Autolabel tab (example given for MACSPlex Custom Cytokines with seven analytes).

4.1.6 Assignment of MACSPlex_Standard positions

▲ The first column(s) of your experiment on the plate have to contain the MACSPlex Cytokine Standards.

▲ Due to the Express Mode calculation processes the wells of the serial dilution have to be grouped so that one data file is created for the measurement of all wells.

▲ Due to the grouping the definition of standard positions and the selection of the Express Mode must be performed for each serial dilution individually.

▲ For standard dilution replicates, make sure that the same number of wells is selected and arranged in the same sequential order. If not complying with the kit protocol for final sample volume in plate or order of serial dilution of standard (refer to the kit data sheet), please refer to "(Optional) Change predefined experiment settings" below.

Define positions for serial dilutions of the cytokine standards

Repeat steps for each serial dilution individually.

1. Define the positions for the samples of one serial dilution of cytokine standards by selecting the respective wells in the **Rack** window. Do not exceed the maximum number of standard positions allowed for the used kit as indicated in the kit data sheet.

2. Group these positions by using the **Group** feature. Grouped positions will be assigned with the same group number.

Select the Express Mode for measurement of the MACSPlex Cytokine Standards

Repeat steps for each serial dilution individually.

1. Select the **Settings** tab.

2. Check the **Express** button.

3. Select **Analysis** from the **Type** drop-down list.

4. Choose **MACSPlex_Standard** from the **Mode** drop-down list (refer to figure 4.3).

5. A warn window appears in terms of sample mixing (refer to figure 4.4). Choose an appropriate mixing program from the **Mix sample** drop-down list (refer to figure 4.5).

   ▲ **Note:** **Mix gentle** is equivalent to **Mix sample** in the MACSQuantify Software version 2.6.

By selecting the Express Mode all experiment settings are automatically loaded. The loaded values are shown in the respective fields in the **Experiment** tab.

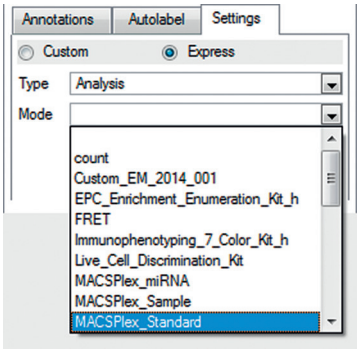


Figure 4.3: Selection of MACSPlex_Standard from the Settings tab.

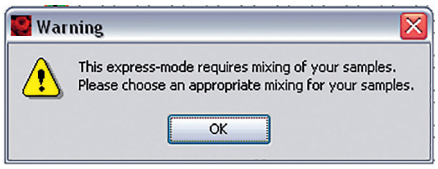


Figure 4.4: Warn window in terms of sample mixing.

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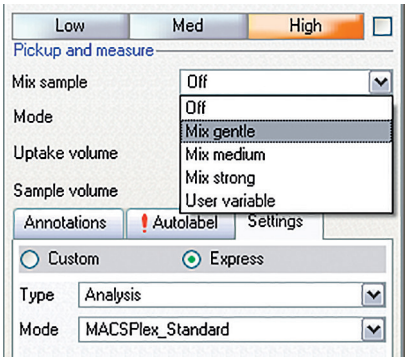


Figure 4.5: Selection of Mix gentle from the Mix sample drop-down list.

(Optional) Defining new sample IDs and a description for the serial dilution of the MACSPlex Cytokine Standard

The sample IDs are automatically applied to the well positions according to the predefined order as displayed in the **Sample ID** drop-down list. However, if the samples of the dilution series are not added to the plate in the predefined order, sample IDs have to be changed manually by choosing the respective value from the **Sample ID** drop-down list. For the correct assignment of the serial dilution to the Sample IDs refer to the kit data sheet. Make sure that no double assignments have been done.

Additional user-defined information can be entered in the field **Description**. Please note that only one description may be assigned per group of standards (all positions of the group have to be selected). Avoid special characters or annotations (e.g. "ö", "ë", "œ", "µ", etc.) in the **Description** field.

(Optional) Change predefined experiment settings

Logging in as a custom mode user allows you to change the default experiment settings defined by the Express Mode. This might be necessary if the protocol was not exactly followed, e.g., if the final sample volume per well in the MACSPlex Filter Plate has been changed from the recommendations given in the kit data sheet.

▲ **Note:** Changes to the Express Mode settings can affect the automated calculations during the analysis. For example, if the uptake volume has been reduced, there may not be enough events in the data file for an accurate analysis.

4.1.7 Assignment of MACSPlex_Sample positions

Define and group positions for unknown samples

1. To define positions click on the wells that contain your unknown samples. A closed green circle with an orange rim indicates sample position activation.
2. Group these positions by using the **Group** button. Group only rack positions that are adjacent and in columns.

   ▲ **Note:** Grouping can be performed on several columns as long as the rack positions are adjacent. If sample positions are not adjacent, measure without grouping and perform grouping of unknown samples during data analysis to enable batch analysis on all samples of your experiment at once.

Select the Express Mode for MACSPlex Samples

1. Make sure all relevant sample positions are selected, i.e., highlighted with an orange circle. A group can be selected by double-clicking on one of its positions.
2. Select the **Settings** tab.
3. Click on the **Express** button.
4. Select **Analysis** from the **Type** drop-down list.
5. Choose **MACSPlex_Sample** from the **Mode** drop-down list (refer to figure 4.3).
6. A warn window appears in terms of sample mixing (refer to figure 4.4). Choose an appropriate mixing program from the **Mix sample** drop-down list (refer to figure 4.5).

   ▲ **Note:** **Mix gentle** is equivalent to **Mix sample** in the MACSQuantify™ Software version 2.6.

By the selection of the Express Mode all experiment settings except the **Mix sample** parameter are loaded automatically. The loaded values are shown in the respective fields of the **Experiment** tab.

(Optional) Defining new sample IDs and a description for unknown samples

Use the **Sample ID** and **Description** fields to enter relevant sample information. For detailed information, please refer to chapter 4.1.6.

Sample ID and Description can be allocated individually to each sample position within the group.

Avoid special characters (e. g., "ö", "ë", "œ", or "µ" etc.) in the data file names, **Sample ID**, and **Description**.

(Optional) Change predefined experiment settings

Logging in as a custom mode user allows you to change the default experiment settings defined by the Express Mode. This might be necessary if the protocol was not exactly followed, e.g., if the final sample volume per well in the MACSPlex Filter Plate has been changed from the recommendations given in the kit data sheet.

▲ **Note:** Changes to the Express Mode settings can affect the automated calculations during the analysis. For example, if the uptake volume has been reduced, there may not be enough events in the data file for an accurate analysis.

For checking of the rack definitions, please refer to chapter 2.6.

For controling of wells for liquid level, please refer to chapter 2.7.

For information about how to start the measurement, please refer to chapter 2.8.

4.2 Gating strategy for manual analysis without Express Mode

Data files can also be analyzed manually using the MACSQuantify Software.

Grouped samples can be ungrouped after acquisition and analyzed individually if desired.

1. Right-click on the file name of the MACSPlex Cytokine Standard or Sample data file and select **Ungroup** to analyze manually. Once ungrouped, the samples cannot be analyzed by an Express Mode, which requires grouped samples. Perform ungrouping also on the unkown sample data files.
2. Create an analysis template that can be applied to cytokine standard and unknown sample data files.
3. Set up the following gating strategy:
   Detection of singlet beads: forward scatter (FSC) versus side scatter (SSC).

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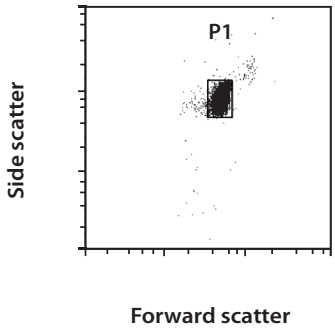


Figure 4.6: Detection of the singlet beads in a forward scatter versus side scatter plot.

4. Detection of MACSPlex Capture Bead populations: Select P1 and display MACSPlex-B1 (FITC) versus MACSPlex-B2 (PE)
   Draw an elliptic region around each single MACSPlex Cytokine Capture Bead population.

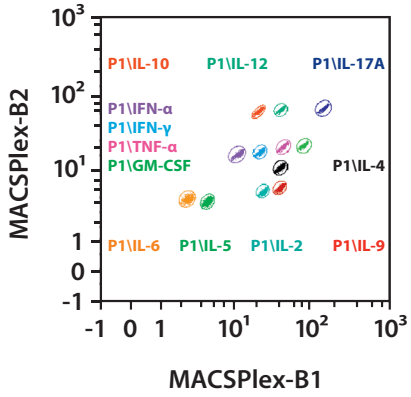


Figure 4.7: Detection of human MACSPlex Capture Bead populations in a MACSPlex-B1 (FITC) versus MACSPlex-B2 (PE) dot plot.

5. Right-click on each region and choose **Region Properties…** to assign cytokine names. Different colors for each cytokine can also be choosen. Please refer to the kit data sheet for the correct assignments of cytokines to the MACSPlex Cytokine Capture Bead populations.

6. For APC median determination display the APC median of each MACSPlex Capture Bead population in a separate statistic by formatting the **Region Functions** and the **Feature Functions** in the **statistic properties**. This can be done by clicking on the **i** button next to the statistic.

File	srv2012-12-14_2181.003.mqd	2012-Dec-14 08:21	
Sld	0pgmL	Descr.	Reihe

Path	APC-A Median
P1\IL-6	0,80
P1\IL-5	0,80
P1\IFN-a	1,15
P1\IL-2	0,77
P1\IFN-g	1,17
P1\IL-10	2,23
P1\IL-9	0,74
P1\IL-4	0,91
P1\TNF-a	1,15
P1\IL-12	2,24
P1\GM-CSF	1,06
P1\IL-17A	1,98

Figure 4.8: Statistic format displaying the APC median of each MACSPlex Capture Bead population (example given for MACSPlex Cytokine 12 Kit, human).

7. Further calculation:

   Calculate the cytokine standards: If using MACSPlex Cytokine Standard serial dilution replicates, average the APC median for each concentration level for each cytokine and determine the cytokine standard curves (cytokine concentration versus APC median).

   Calculate the cytokine concentration in the unknown samples using the cytokine standard curves.

5. Troubleshooting

The following section offers solutions for problems that might be encountered when using the MACSPlex Express Modes for the MACSPlex Cytokine Assays.

5.1 Scanning of barcodes was not successful

If the reagent labels on the vials are damaged or the scanning of the barcodes is not successful, the reagents can be manually selected using the **Reagents** window: Choose **Edit** > **Reagents…** from the menu bar to display the window.

1. Check reagent position R1. Select the category **MACSPlex** from the **Category** drop-down list.

2. Select the used MACSPlex Capture Beads from the **Reagent** drop-down list.

   ▲ **Note:** When clicking the **i** button in the Reagents window on the right hand side of the MACSPlex Capture Beads, an **Info** window will popup, displaying MACSPlex information shown in figure 5.2.

3. Check reagent position R2. Select the category **Universal** and select **NoLabel** from the **Reagent** drop-down list.

4. (Optional) When using MACSPlex Custom Cytokines, repeat step 1 and 2 for each MACSPlex Capture Bead by selecting R3–R8.

5. Click **Apply** to save the changes and close the window.

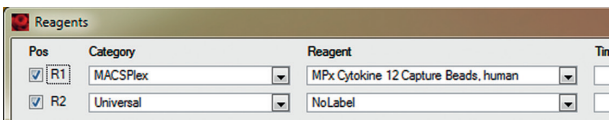


Figure 5.1: Manual selection of the MACSPlex Capture Beads and NoLabel settings.

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6. Check the selected reagents in the **Autolabel** tab located in the **Experiment** tab. The red exclamation mark indicates autolabel activation (refer to figure 4.2).

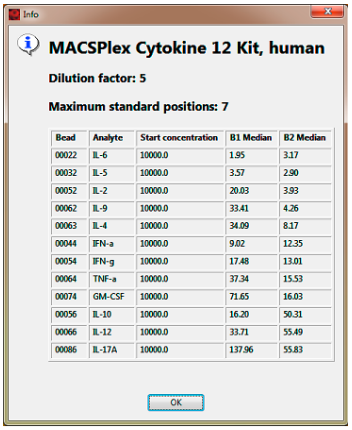


Figure 5.2: Example of MACSPlex information displayed for MACSPlex Cytokine 12 Capture Beads, human. When clicking the **i** button in the **Reagents** window, an **Info** window will popup, displaying MACSPlex information. This information will be provided when using the autolabel function.

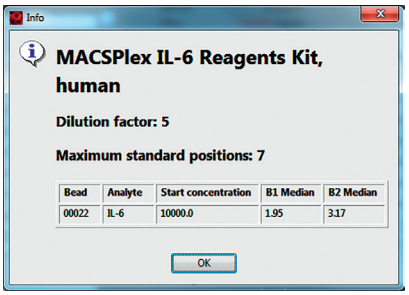


Figure 5.3: Example of MACSPlex information displayed for MACSPlex IL-6 Capture Beads, human. When clicking the **i** button in the **Reagents** window, an **Info** window will popup, displaying MACSPlex information. This information will be provided when using the autolabel function.

5.2 Manual loading of predefined experiments

If the predefined experiments were not able to be uploaded automatically, they can be loaded manually.

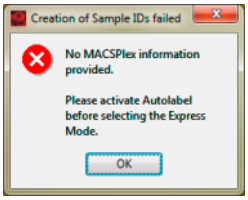
1. Click **Open** button of the toolbar or select **File > Open…** from the menu bar.
2. Choose **Experiment** and **Public**.
3. Select the subfolder **MQ10** for correct instrument configuration.
4. Select the used kit to upload the predefined experiment.

   ▲ **Note:** If the experiment does not match with the configuration an information window appears stating that annotations have been reset to default values.

5.3 Data acquisition warning windows

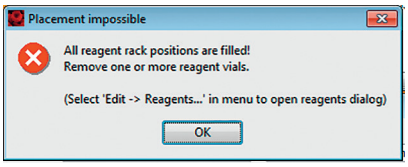
Some warning windows may appear after starting the measurement directly before picking the sample. Please wait until MACSPlex_ standard and MACSPlex_Sample is picked to make sure all settings are correct.

A warning window appears when selecting the MACSPlex_ Standard Express Mode for grouped samples, stating that the creation of Sample IDs has failed.



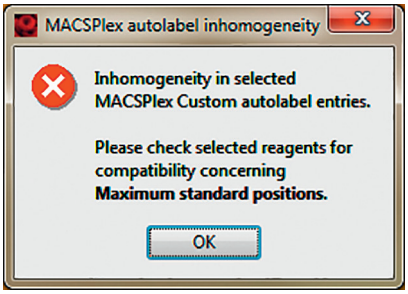
- MACSPlex reagents are not selected. Load the MACSPlex reagents by using the 2D code reader or select them manually by using the reagents window (including **NoLabel**).
- MACSPlex reagents are not assigned correctly. Check the reagents in the **Autolabel** tab.

A warning window appears when scanning the MACSPlex Custom Cytokine Capture Beads, stating that placement is impossible.



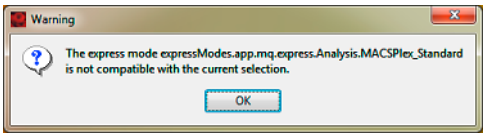
- Seven MACSPlex Custom Cytokine Capture Beads have been already scanned (including NoLabel), there are no open positions in the **Autolabel** tab. When working with the MACSPlex Express Modes and the MACSPlex Filter Plate the maximum number of seven analytes can be processed. Check the reagents in the **Autolabel** tab.

A warning window appears when selecting the MACSPlex_ Standard Express Mode for grouped samples, stating that there is an inhomogeneity in the selected MACSPlex Custom autolabel entries.



- MACSPlex reagents are not assigned correctly, e.g., MACSPlex Custom Cytokine reagents for human and mouse has been mixed. Check the reagents in the **Autolabel** tab.

A warning window appears when selecting the MACSPlex_ Standard Express Mode for grouped samples, stating that the Express Mode is not compatible with the current selection:

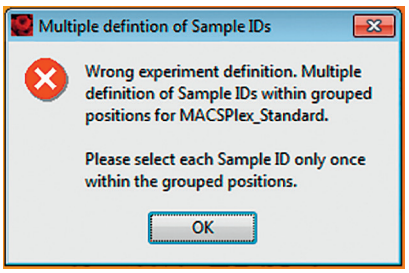


- No Chill Rack has been selected.
- Sample positions have not been grouped.

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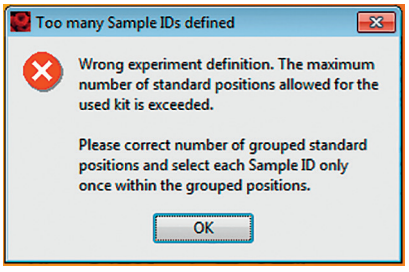
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A warning window appears when starting the MACSPlex_ Standard Express Mode, stating a multiple defintion of Sample IDs.



- Within the grouped positions for the current MACSPlex_ Standard measurement Sample IDs has be selected twice or more. Assure that each Sample ID is only selected once within the grouped positions.
- Delete already measured positions from the experiment before starting the measurement again.

A warning window appears when starting the MACSPlex_ Standard Express Mode, stating too many defined Sample IDs.



- Within the grouped positions for the current MACSPlex_ Standard measurement too many Sample IDs has been defined. Please refer to the kit data sheet for the maximum number of standard position for the used kit. Delete the redundant positions and assure that each Sample ID is only selected once within the grouped positions.
- Delete already measured positions from the experiment before starting the measurement again.

A warning window appears when starting the MACSPlex_ Sample Express Mode, stating that no MACSPlex information is provided.



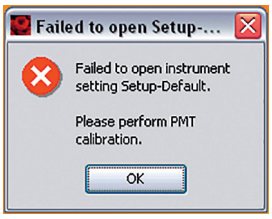
- MACSPlex reagents are not selected. Load the MACSPlex Reagents by using the 2D code reader or select them manually by using the **Reagents** window (including NoLabel).
- MACSPlex reagents are not assigned correctly. Check the reagents in the **Autolabel** tab.
- Delete already measured positions from the experiment before starting the measurement again.

A warning window appears when starting the MACSPlex_Sample Express Mode, stating that no standard data are available.



- Perform processing of MACSPlex_Standard positions with the MACSPlex Cytokine Standard prior to MACSPlex_Sample positions with unknown samples.
- The time difference between the MACSPlex_Standard and the first MACSPlex_Sample measurement has exceeded two hours. Repeat the analysis of the MACSPlex_Standard data files of the current experiment manually by right clicking on the file name within the **Samples** tab, selecting **View with Analysis.MACSPlex_Standard**.
- Delete already measured positions from the experiment before starting the measurement again.

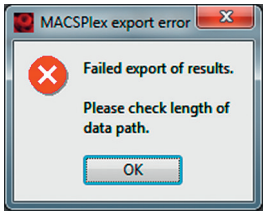
A warning window appears when starting the MACSPlex_ Standard or MACSPlex_Sample Express Mode, stating that the opening of the instrument setting Setup-Default has failed.



- Perform a PMT calibration of the instrument. For details, please refer to the MACSQuantify™ Software user guide.

5.4 Data analysis warning windows

An export warning window appears instead of the export information window:



- Export failed as the limit of data path length is reached. Relocate data files to a shorter data path.
- Export failed due to use of special characters (e.g., “ö”, ”ë”, “œ”, “µ”, etc.) in sample ID or description.

Failure of analysis:

For example, the warning window “Error during <name of Express Mode> analysis. Please contact MACSQuant® Support.” is displayed. Data files have to be analyzed manually. Please refer to section 4.2 “Gating strategy for manual analysis without Express Mode”.

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Reasons for the failure may be:

- Special characters (e.g., “ö”, ”ë”, “œ”, “µ”, etc.) have been used in data file name.
- No or very few events have been measured. Please, check the liquid level in the wells before starting the measurement.
- No MACSPlex reagents have been selected in the **Autolabel** tab (refer to figure 4.2). Check the MACSPlex reagents of the MACSPlex kit.
- Wrong reagents have been selected in the **Autolabel** tab.
- Within a set of MACSPlex_Standard measurements different numbers of standard positions have been used.
- MACSQuantify™ Software on PC has been set to a wrong instrument configuration. Choose **Edit > Configuration** from the menu bar to select the correct configuration of the used MACSQuant® Instrument.
- The Express Mode version of the MACSQuantify Software PC version is a different one as of the MACSQuant Instrument. Always use the same Express Mode version on PC and instrument.
- If **“No result !”** appears as special marking of cytokine concentration result: Calculation of the cytokine concentration for the relevant analysis was not possible as the standard APC median values do not show a proper increasing behavior. Other analysis within the experiment may also not have the expected behavior due to premixed cytokine standards. Please check all other results.

Refer to **www.miltenyibiotec.com** for all data sheets and protocols.

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