Miltenyi Biotec

Data acquisition and analysis of the MACSPlex Cytokine Kits using the MACSQuant® Analyzer Express Modes in MACSQuantify™ Software version 2.13.1

Contents

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 specific 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. They also provide the possibility to manually correct the analyte identification for analysis if necessary.

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.

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 concentrations 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 2.13.1 (or higher) and the MACSPlex Express Mode package 213.2.20284 (or higher).
  - ▲ **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 Cytokine Reagent Kits, human or mouse, or MACSPlex Cytotoxic T/NK Cell Kit, human (# 130-125-800)
  - ▲ **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 on MACSQuant Analyzer 10 (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

SP0109.02

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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, name 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 the MACSPlex Cytotoxic 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.

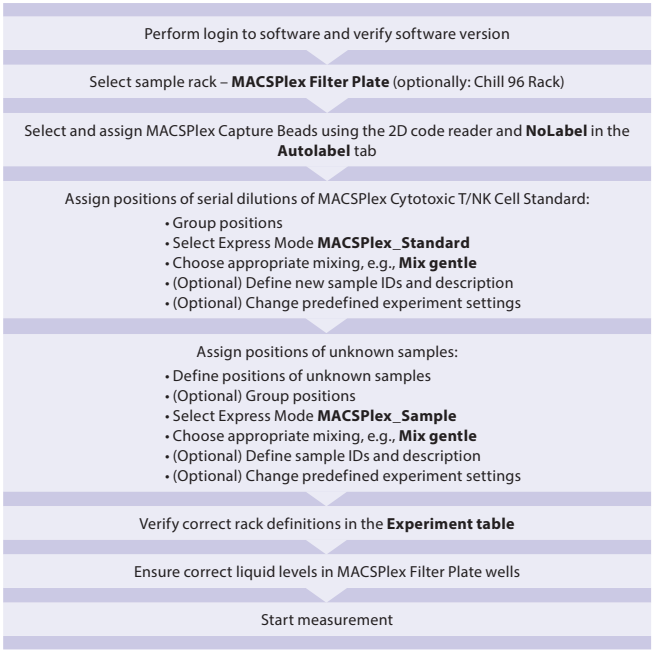

Figure 2.1: Overview of data acquisition with a manually defined experiment.

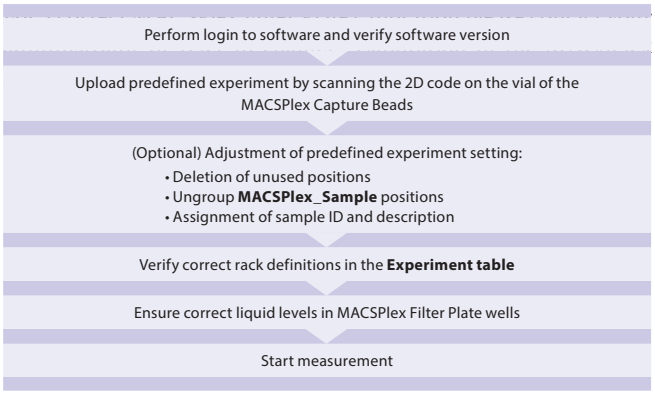

Figure 2.2: Overview of data acquisition with with predefined experiment settings.

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

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", "oe", "µ", etc.) for the data file name structure.

2.2 Verify software version

1. Select **Help > Info…** from the menu bar.
2. Verify that the MACSQuantify Software version 2.13.1 or higher (refer to figure 2.3) and the MACSPlex Express Mode package 213.2.20284 or higher are installed on the MACSQuant Instrument.

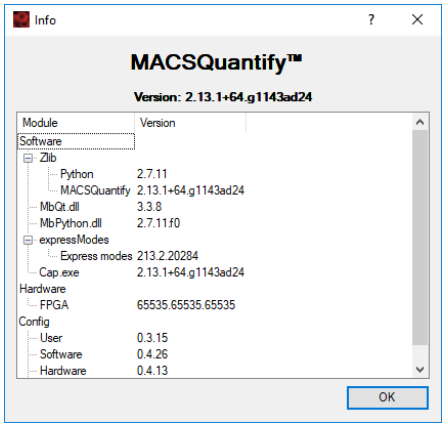

Figure 2.3: Information window for MACSQuantify Software.

2.3 Instrument settings

The instrument settings are automatically uploaded when using the MACSPlex Express Modes with MACSQuantify Software version 2.13.1 or higher after assigning the MACSPlex Capture Beads. The loading of the settings will be performed when the measurement starts.

2.4 Use predefined experiment settings or perform manual experiment definition

There are two options to set up the experiment. For the MACSPlex Cytokine 12 Kit, human, and MACSPlex Cytokine 10 Kit, mouse, predefined experiment settings are available in the software (please refer to chapter 2.4.1 and figure 2.2). For the other MACSPlex Cytokine kits and also for the individual combination of analytes (MACSPlex Custom or MACSPlex Mix) no predefined experiment settings are provided in the software. Here, the experiment settings have to be defined manually as described in chapter 2.4.2 and figure 2.1.

2.4.1 Use predefined experiment settings

2.4.1.1 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 2.13.1 a predefined experiment can be uploaded for the MACSPlex Cytokine 12 Kit, human, or for the MACSPlex Cytokine 10 Kit, mouse, by using the 2D barcode of the MACSPlex Capture Beads

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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.
When working with the MACSPlex Custom Cytokine Assays or other MACSPlex Cytokine Kits than mentioned above no predefined experiment settings are provided. For the manual creation of a MACSPlex Cytokine experiment, please proceed with chapter 2.4.2.

1. Click on the **Barcode button** of the toolbar and wait for the 2D 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 the **MACSPlex_Standard** positions (duplicate of serial dilution) with vertical rack processing order in column 1 and 2. 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 the plate are not positioned in column 1 and 2, please proceed with chapter 2.4.2.

2.4.1.2 (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 2.4.2.3 or chapter 2.4.2.4.

2.4.1.3 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.

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.

2.4.1.4 Assignment of Sample ID and Description

Additional user-defined information can be entered in the **Experiment tab**, 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”, “oe”, “µ”, etc.).

2.4.1.5 Further proceeding

Proceed with step 2.5.

2.4.2 Manual experiment definition

2.4.2.1 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.

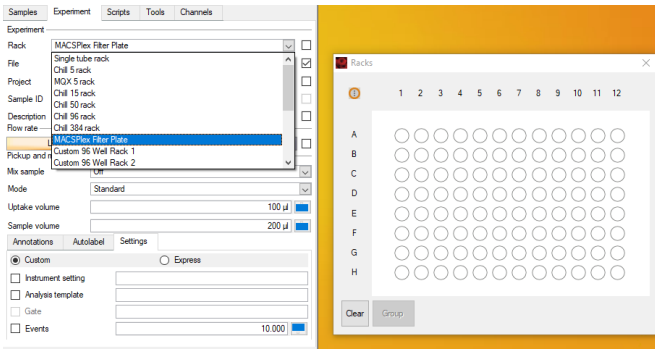

Figure 2.4: Selection of the rack type.

2.4.2.2 Select and assign MACSPlex Reagents

In order to use the Express Mode, reagent information of the MACSPlex Cytokine 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 scanning of the barcodes is not successful, the reagents can be manually selected (please refer to chapter 5.1).

When using MACSPlex Custom Cytokine reagents (also MACSPlex Mix) do not exceed the maximum number of seven analytes and do not mix human and mouse reagents in the same experiment. Do not mix different MACSPlex Mix categories.

1. Click on the **Barcode button** of the toolbar and wait for the 2D 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 (refer to chapter 2.4.1). If you need the rack “MACSPlex Filter Plate” for your manually defined experiment, select all positions on the rack, and clear them. If you need another rack type, select the appropriate rack accordingly and proceed with step 2 to check the reagents (refer to figure 2.5 and 2.6).

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 Cytokine 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.

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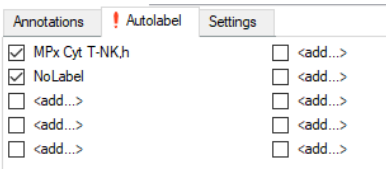

Figure 2.5: Tick **MACSPlex Capture Beads** and **NoLabel boxes** in the **Autolabel tab** (example given for MACSPlex Cytotoxic T/NK Cell Capture Beads).

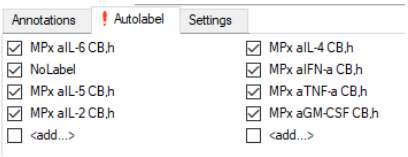

Figure 2.6: Tick **MACSPlex Capture Beads** and **NoLabel boxes** in the **Autolabel tab** (example given for MACSPlex Custom Cytokines with seven analytes).

2.4.2.3 Assignment of MACSPlex_Standard positions

▲ The first column(s) of your experiment on the plate have to contain the MACSPlex Cytokine Standards. Refer to the kit data sheet for more information.

▲ 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 individually for each serial dilution.

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

Define positions for serial dilutions of the cytokine standards

▲ Repeat steps for each of the two serial dilution individually.

1. Define the positions for the samples of one serial dilution of the 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.

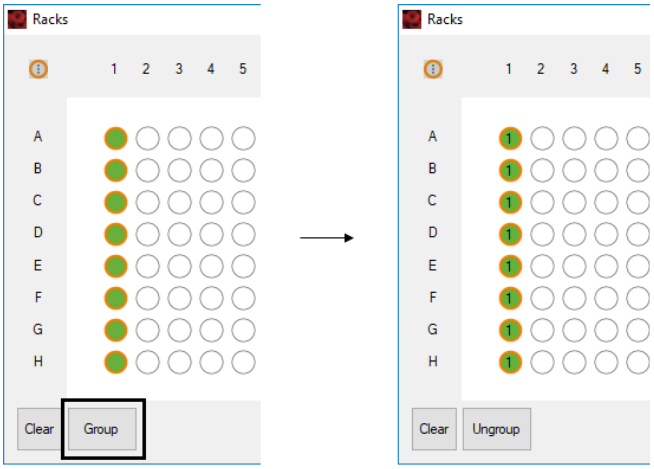

Figure 2.7: Select well positions for the samples of one serial dilution and use the **Group button**. Grouped well positions of each serial dilution of the MACSPlex Cytokine Standard will be assigned the same group number (example given for a kit with 8 standard positions allowed).

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 2.8).
5. A warn window appears in terms of sample mixing (refer to figure 2.9). Choose an appropriate mixing program from the **Mix sample drop-down list** (refer to figure 2.10).

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

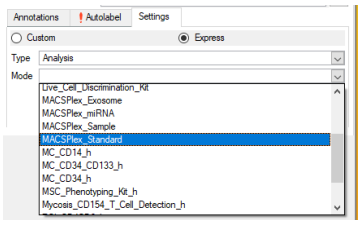

Figure 2.8: Selection of **MACSPlex_Standard** from the **Settings tab**.

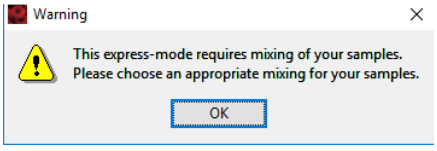

Figure 2.9: Warn window in terms of sample mixing.

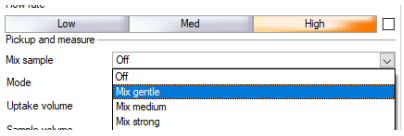

Figure 2.10: Selection of **Mix gentle** from the **Mix sample drop-down list**.

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(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", "oe", "µ", etc.) in the **Description** field.

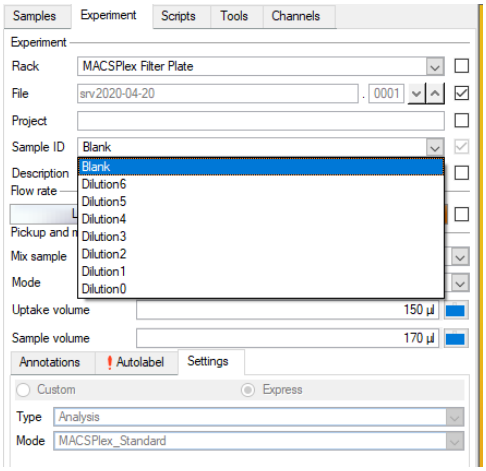

Figure 2.11: The sample IDs for the MACSPlex_Standard can be manually changed in the Sample ID drop-down list.

(Optional) Change predefined experiment settings
Logging in as a custom mode user allows 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.

2.4.2.4 Assignment of MACSPlex_Sample positions

Define and group positions for unknown samples

1. To define positions click on the wells that contain the 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 2.8).
6. A warn window appears in terms of sample mixing (refer to figure 2.9). Choose an appropriate mixing program from the **Mix sample drop-down list** (refer to figure 2.10).

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 2.4.2.3.

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.

2.4.2.5 Further proceeding

Proceed with step 2.5.

2.5 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 have been done.
4. Check the **Autolabel** and **Settings tabs** and make sure that for each selected position the correct MACSPlex Cytokine Capture Beads, **NoLabel** function, as well as the correct Express Mode are selected.

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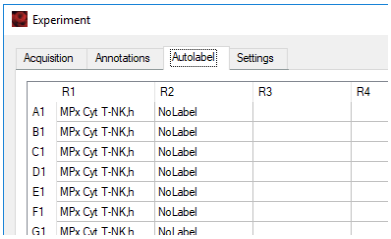

Figure 2.12: Control of rack definitions in the Autolabel tab of the Experiment table (example given for MACSPlex Cytotoxic T/NK Cell Kit, human).

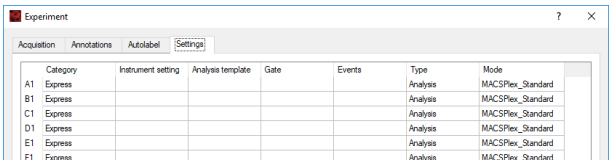

Figure 2.13: Control of rack definitions in the Settings tab of the Experiment table.

2.6 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.

2.7 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 2.13.1 (or higher) with the MACSPlex Express Mode package 213.2.20284 (or higher).

▲ **Note:** When the analysis of data files is performed on the MACSQuant Instrument, not all analysis functions are available. The functions of manual correction of analyte identification, the change of standard values, and the automatic export of results cannot be used.

▲ **Important!** The MACSQuantify Software version and 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).
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.

   ▲ **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.

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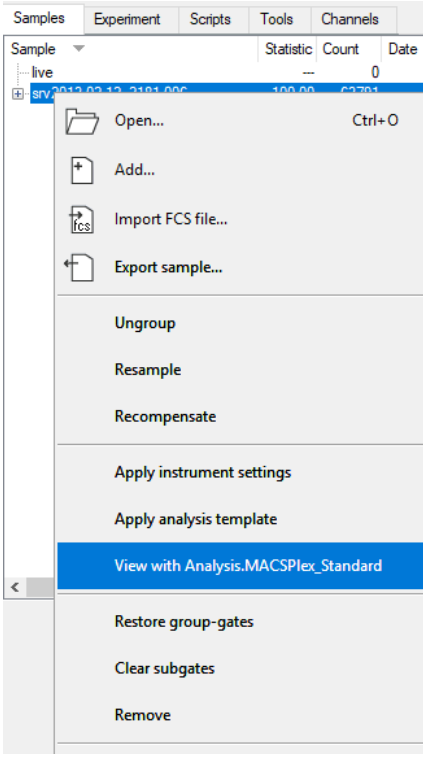

Figure 3.1: Selecting the Express Mode for analysis of the data files.

4. A control page is displayed in combination with the gate verification window (refer to figure 3.2) to allow a manual correction of the regions for the analyte identification. The gate verification window can be moved on the screen and allows access to the plots. Check if the analytes are assigned correctly to the bead populations (please compare with kit data sheet).

   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, which identify the analytes.

   When all gates are set correctly and all analytes are assigned correctly by the Express Mode, close the gate verification window by clicking the "x".

   If gates are not set correctly or if analytes are not assigned correctly, correct the regions manually. When done, close the gate verification window with the **Gating adjusted button**.

   The appropriate MACSPlex analysis page(s) will be displayed after analysis has been finished.

   ▲ **Note:** When analytes cannot be assigned at all, placeholder regions for the affected analytes are positioned in the upper left corner of the plot. This is done on the PC, but also on the MACSQuant® Instrument.

   ▲ **Note:** How the gate verification window is closed influences the finally displayed text in the information on the control page (refer to figures 3.4 and 3.5).

   ▲ **Note:** Only gate modification that is performed while the gate verification window is displayed, influences the Express Mode analysis and the Express Mode statistics. Gate modification that is performed after the Express Mode analysis is finished, does not influence the results.

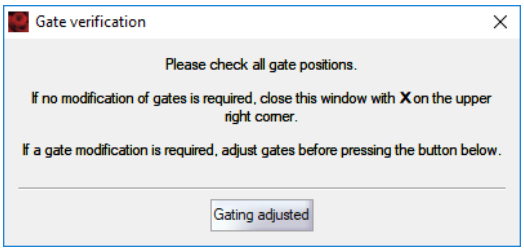

Figure 3.2: Gate verification window.

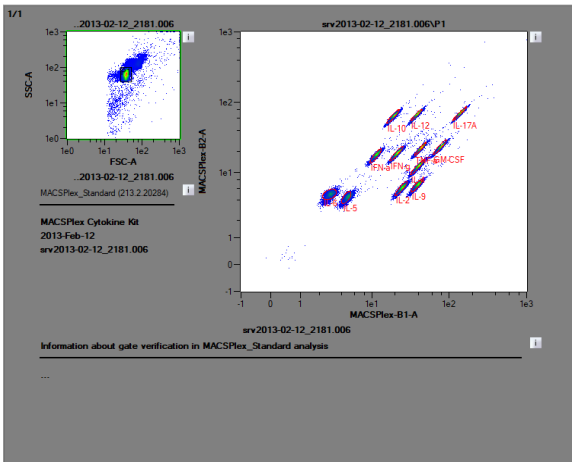

Figure 3.3: Control page for correction of analyte assignment (example given for Express Mode MACSPlex_Standard).

Information about gate verification in MACSPlex_Standard analysis

No manual modification of gates.

Figure 3.4: Information text on control page, when gate modification window is closed with "x" and no manual gate modification was performed.

Information about gate verification in MACSPlex_Standard analysis

Manual modification of gates performed in analysis.

Figure 3.5: Information text on control page, when gate modification window is closed with "Gating adjusted" and gates were adjusted manually.

The control page is part of the analysis of both Express Modes, MACSPlex_Standard and MACSPlex_Sample, and displayed as the first page in each case. In addition to that, for MACSPlex_Standard data files the data will be displayed in a single analysis window without further user interaction.

For the MACSPlex_Sample data during analysis, standard values or changed standard values can be chosen. For further details, please refer to chapter 3.3. At the end of the analysis an information window is displayed which shows the automatically exported results and their directory before the final analysis pages are displayed. By clicking **OK** in the info window, the display of the analysis pages will be finalized.

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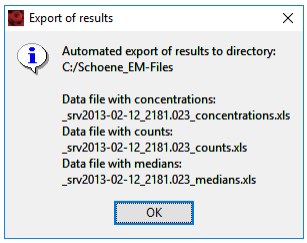

Figure 3.6: Information window about exported results.

▲ **Note:** The options to manually correct the analyte identification, to change standard values, and the automatic export of results are only performed in the software installed on an accessory computer and not on the MACSQuant Instrument itself. Also the control page is not displayed on the the MACSQuant Instrument.

▲ **Note:** When a manual correction of the analyte identification is done on the MACSPlex_Standard data file(s), the MACSPlex_Sample analysis has to be done with the changed standard values to use the corrected values for the analysis (please refer to chapter 3.3).

▲ **Note:** When the manual correction of the analyte identification is done on a grouped data file, the correction is automatically applied to all samples in the groupd data file.

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

The major plot of the MACSPlex_Standard analysis window shows the increase of the APC median values for increasing standard concentrations for the MACSPlex Cytokine Kit (figure 3.7). 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 3.8).

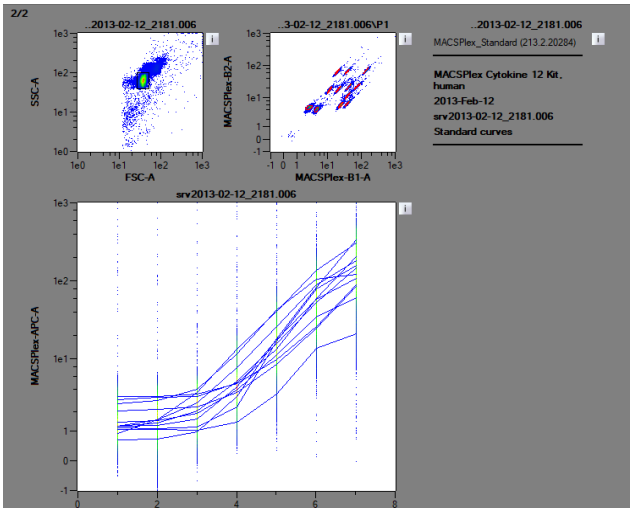

Figure 3.7: MACSPlex_Standard analysis window, displaying all relevant information. HDR-G abbreviates the group parameter in the MACSQuantify Software. In case of MACSPlex Assays, each position of HDR-G represents a measured standard cytokine concentration.

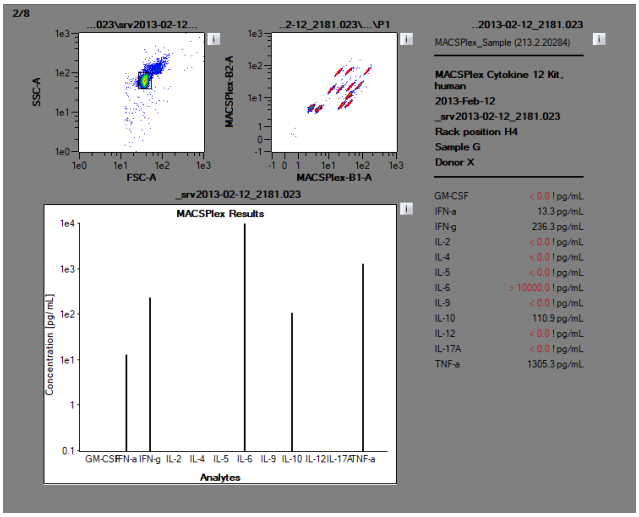

Figure 3.8: MACSPlex_Sample analysis window from the MACSPlex Cytokine 12 Kit. The statistics table displays the Express Mode name and version number, the kit name, the date of the measurement, the file name, the rack position of the sample, the sample ID, and the description of the data file and lists the final concentration of each cytokine measured in the unknown sample.

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	I
H4	Sample G	Donor X	<0,0!	13,3	236,3	<0,0!	<0,0!	<
A5	Sample H	Donor V	<0,0!	21,3	25	<0,0!	<0,0!	<
B5	Sample I	Donor W	0,6	28,4	86,8	2,7	2,3	<
C5	Sample J	Donor S	<0,0!	17,5	1,3	<0,0!	0,1	
D5	Sample K	Donor Z	3,9	58,4	62,8	0,6	22,1	
E5	Sample L	Donor T	37	97	6378,7	9144,9	17,3	
F5	Sample M	Donor U	3,9	81	53	0,4	20,9	

Figure 3.9: 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 concentration 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 analyses

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 (but on the control page). Click the **i button** next to the dot plot and select the **Region functions tab**. Select the

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functions **Name** or **Path** and confirm it by selecting **OK** or **Apply** to display the names of the regions.

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

During the analysis of the cytokine standard curves the range of monotonic increasing behavior is identified and sets the concentration range for the calculation (light grey area in figure 3.10). The upper and lower detection limits describe the APC median values that border the well-defined concentration-median assignment (dark grey area in figure 3.10). The calculation only takes place within the concentration range of monotonic increasing behavior. As shown as an example in figure 3.11, sample APC median values that are above the 2,000 pg/mL APC median value are marked with "> 2000.0 ! pg/mL".

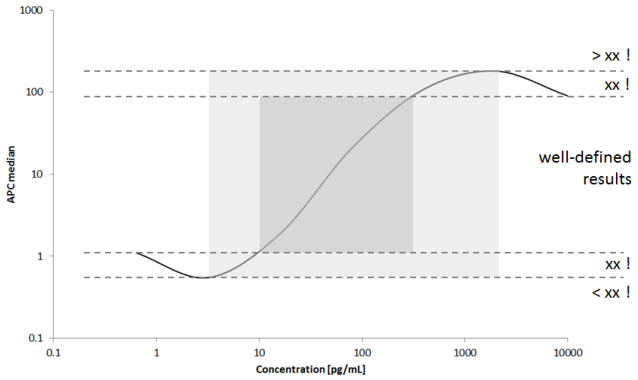

Figure 3.10: Demonstration of the different defined calculation areas within an exaggerated cytokine standard curve.

MACSPlex_Sample (213.2.20284)

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

GM-CSF	4.3 pg/mL
IFN-a	> 2000.0 ! pg/mL
IFN-g	5.1 pg/mL
IL-2	< 0.0 ! pg/mL
IL-4	4.2 pg/mL
IL-5	0.6 pg/mL
IL-6	996.1 pg/mL
IL-9	3.4 ! pg/mL
IL-10	44.2 pg/mL
IL-12	49.8 pg/mL

Figure 3.11: 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 3.10).

"<…!": 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.

The complete analysis of standard and sample data files is a self contained system. Later manual modifications of the gates, which are not done while the gate verification window is active in the analysis, 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 have 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 to change standard values.

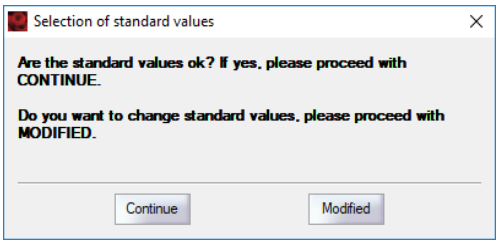

Figure 3.12: Question window if standard values are ok or not.

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 3.6).

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

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clicking on the up and down arrow buttons and click **OK**. A set of standard values is represented by the analyzed values of one MACSPlex_Standard data file. For each data file, these values are saved within a csv file.

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

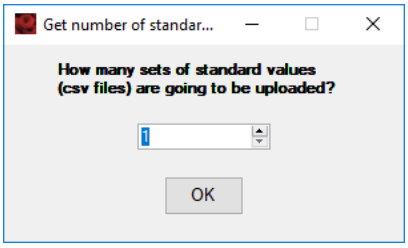

Figure 3.13: Question window how many standard sets are to be uploaded.

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 3.17).
When selecting more than one set of standard values the respective number of windows will be opened one after the other.

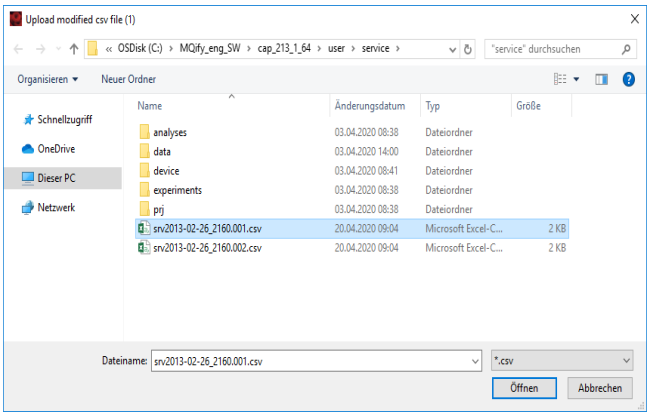

Figure 3.14: Opened explorer with user directory path to upload csv files.

Finally, the information window about the automatically exported results is displayed (refer to figure 3.6).

The modified standard values of the MACSPlex_Sample analysis are stated in the statistic on the analysis pages (refer to figures 3.15 and 3.16)

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

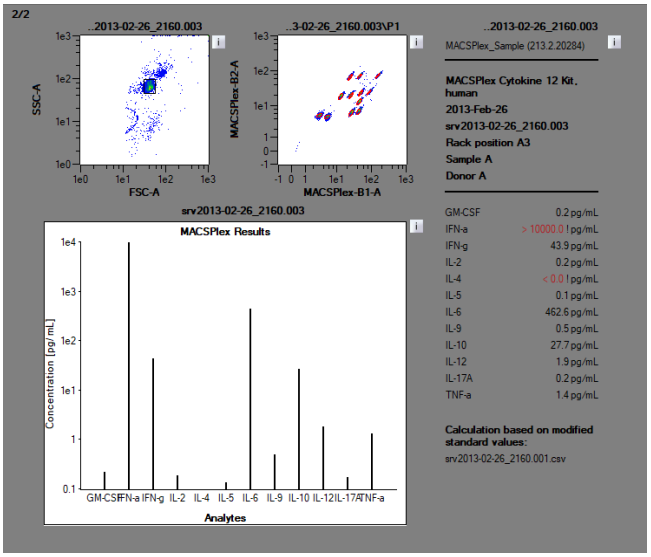

Figure 3.15: MACSPlex_Sample analysis from the MACSPlex Cytokine 12 Kit with changed standard values.

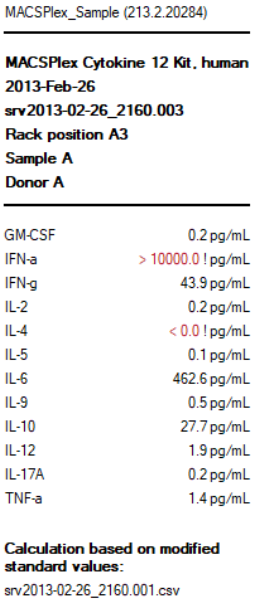

Figure 3.16: Statistic of MACSPlex_Sample analysis with changed standard values.

Directory mismatch

When trying to upload a csv file from another directory a directory mismatch warn window appears (refer to figure 3.17) and the upload of the chosen csv files is canceled (refer to figure 3.18).
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.

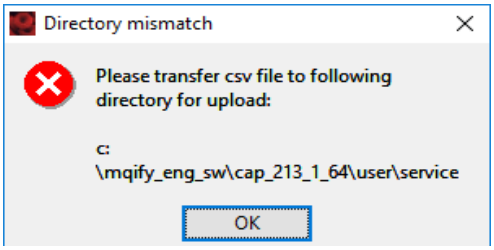

Figure 3.17: Directory mismatch warn window when trying to upload csv file from another directory than the user directory.

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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.

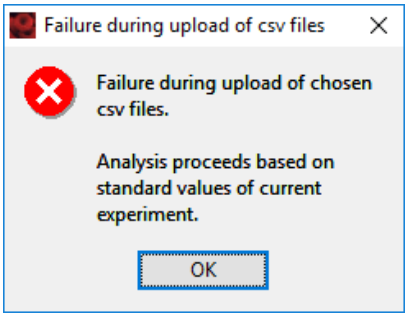

Figure 3.18: Warn window about the failure during upload of csv files.

4. Gating strategy for manual analysis without Express Mode

Data files can also be analyzed manually on any flow cytometer with a similar setup as MACSQuant® Analyzer 10 and MACSQuant X (equipped with blue (488 nm) and red (635 nm) lasers able to discriminate FITC, PE, and APC fluorescence. Please note that the MACSQuant VYB cannot be used.) 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 of 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).

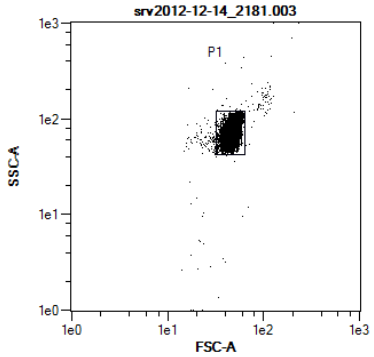

Figure 4.1: Detection of the singlet beads in a forward scatter versus side scatter plot.

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

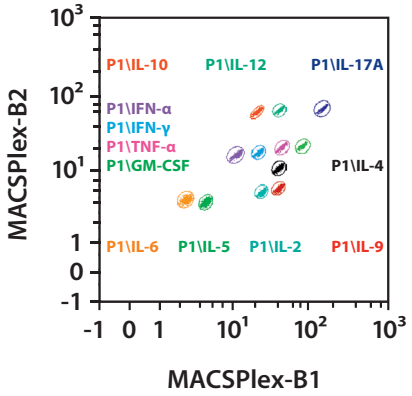

Figure 4.2: Detection of human MACSPlex Cytokine Capture Bead populations in a MACSPlex-FITC (B1) versus MACSPlex-PE (B2) dot plot (example given for MACSPlex Cytokine 12 Capture Beads, human).

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 4.3: Statistic format displaying the APC median of each MACSPlex Capture Bead population.

7. Further calculation:

   Calculate the cytokine standards: If using MACSPlex Cytokine Standard serial dilution replicates, average the APC median for each concentration level of 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.

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5. Troubleshooting

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

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 / MACSPlex Cytokine Kit from the **Reagent drop-down list**.

   ▲ **Note:** When clicking the **i button** in the Reagents window on the right hand side of the MACSPlex Cytokine reagent entry, an Info window will pop up, displaying MACSPlex information shown in figures 5.2 and 5.3.
3. Check reagent position R2. Select the category **Universal** and select **NoLabel** from the **Reagent drop-down list**.
4. Click **Apply** to save the changes and close the window.

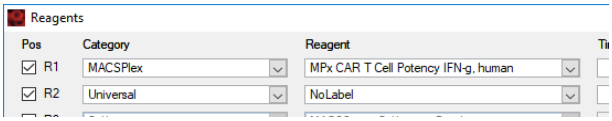


Figure 5.1: Manual selection of the MACSPlex Capture Beads and NoLabel settings (example given for MACSPlex Cytokine 12 Kit, human).

5. Check the selected reagents in the **Autolabel tab** located in the **Experiment tab**. The red exclamation mark indicates autolabel activation (refer to figure 2.5).

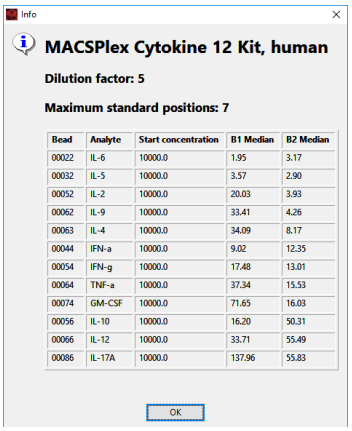


Figure 5.2: Example of MACSPlex information displayed for the MACSPlex Cytokine 12 Capture Beads, human. When clicking the **i button** in the **Reagents** window, an **Info** window will pop up, displaying MACSPlex information. This information will be provided when using the autolabel function.

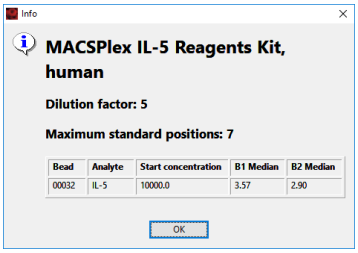


Figure 5.3: Example of MACSPlex information displayed for the MACSPlex IL-5 Capture Beads, human. When clicking the **i button** in the **Reagents** window, an **Info** window will popup, displaying MACSPlex information. This information will be provided when using the autolabel function.

5.2 Manual loading of predefined experiments

If the predefined experiments could not be uploaded automatically, they can be loaded manually.

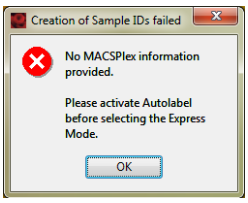
1. Click the **Open button** of the toolbar or select **File** > **Open…** from the menu bar.
2. Choose **Experiment** and **Public**.
3. Select the subfolder **MQ10** for correct instrument configuration.
4. Select the used kit to upload the predefined experiment.

   ▲ **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**.

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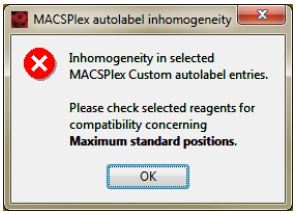
A warning window appears when scanning the MACSPlex Custom Cytokine Capture Beads, stating that placement is impossible.

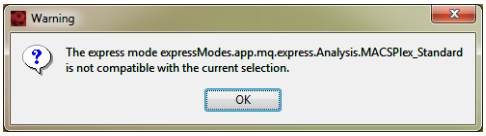
- Seven MACSPlex Custom Cytokine Capture Beads have been already scanned (including NoLabel), there are no open positions in the **Autolabel tab**. When working with the MACSPlex Express Modes and the MACSPlex Filter Plate the maximum number of seven analytes can be processed. Check the reagents in the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



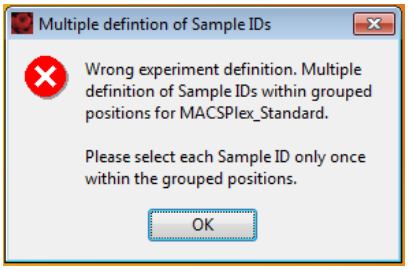
- MACSPlex reagents are not assigned correctly, e.g., MACSPlex Custom Cytokine reagents for human and mouse (also MACSPlex Mix) have been mixed. Check the reagents in the **Autolabel tab**.

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.



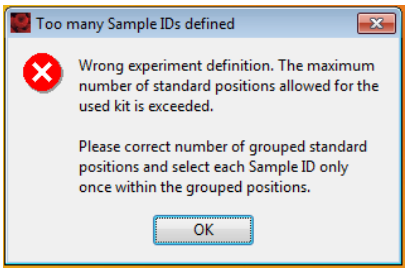
- No Chill Rack has been selected.
- Sample positions have not been grouped.

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 have 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 have been defined. Please refer to the kit data sheet for the maximum number of standard positions 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.

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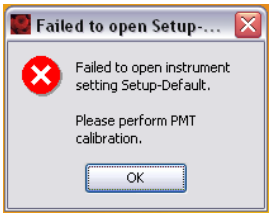
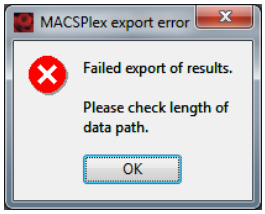
A warning window appears when starting the MACSPlex_Standard or MACSPlex_Sample Express Mode, stating that the opening of the instrument setting Setup-Default has failed.



- 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 “Gating strategy for manual analysis without Express Mode”.

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 2.5). 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.
- MACSPlex_Sample Analysis of older data files (with not all analytes assigned) is performed with the current MACSPlex Express Modes using the standard values of the experiment (likewise with not all analytes assigned). Repeat the MACSPlex_Sample analysis and use changed standard values (refer to chapter 3.3) to make sure that identically identified analytes are used for standard and sample analysis.

Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com for local Miltenyi Biotec Technical Support contact information.

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