# Data acquisition and analysis of MACSPlex miRNA Kits using the MACSQuant® Analyzer Express Mode in MACSQuantify™ Software version 2.8

## Contents

## 1. Description

### 1.1 Background information

MACSPlex Assays have been designed for determining concentrations of miRNAs in a single sample. The analysis is based on MACSPlex (MPx) 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® Analyzers the MACSPlex miRNA 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 Mode **MACSPlex_miRNA** has been developed for use with the MACSPlex miRNA Kits. The measurement and analysis using this Express Mode can be performed with the MACSQuant Analyzer and the MACSQuant Analyzer 10. It is not suitable for use with the MACSQuant VYB.

**MACSPlex_miRNA** is used for the measurement of miRNA samples. With the MACSQuantify™ Software the samples can be analyzed all at once with a so-called "batch analysis". For this feature the samples must be grouped. The grouping can be performed before or after acquisition.

### 1.2 Reagent and instrument requirements

- MACSQuant Analyzer or MACSQuant Analyzer 10 (# 130-096-343) with MACSQuantify Software version 2.8 or higher and at least Express Mode version **installExpressModes_28.1.15443**.

  ▲ **Note:** The MACSQuant VYB cannot be used.

- Chill 96 Rack (# 130-094-459)

- MACSQuant Calibration Beads (# 130-093-607)
- MACSPlex miRNA Kit, for example, MACSPlex miRNA Kit – Cancer (# 130-106-194)

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

Estimation for acquisition with default settings of Express Modes (50 µL sample uptake with Medium flow rate and Standard Mode with active mixing):

|  | MACSQuant Running Buffer | Time |
|---|---|---|
| 1 sample | 15 mL | 1.5 minutes |
| 96 samples | 1500 mL | 3 hours |

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

▲ Please 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 analytes, median for B1, and median for B2. When using MACSPlex miRNA 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.

For a correct MACSPlex Round Bottom Plate calibration please contact your administrator.
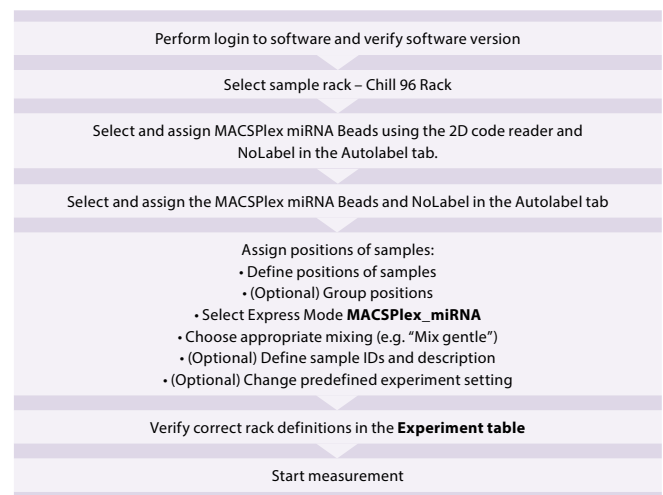
Perform login to software and verify software version

Select sample rack – Chill 96 Rack

Select and assign MACSPlex miRNA Beads using the 2D code reader and NoLabel in the Autolabel tab.

Select and assign the MACSPlex miRNA Beads and NoLabel in the Autolabel tab

Assign positions of samples:
• Define positions of samples
• (Optional) Group positions
• Select Express Mode **MACSPlex_miRNA**
• Choose appropriate mixing (e.g. "Mix gentle")
• (Optional) Define sample IDs and description
• (Optional) Change predefined experiment setting

Verify correct rack definitions in the **Experiment table**

Start measurement

**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 2.8 or higher and at least Express Mode version 28.1.15443 (installExpressModes_28.1.15443) are installed on the MACSQuant Instrument.
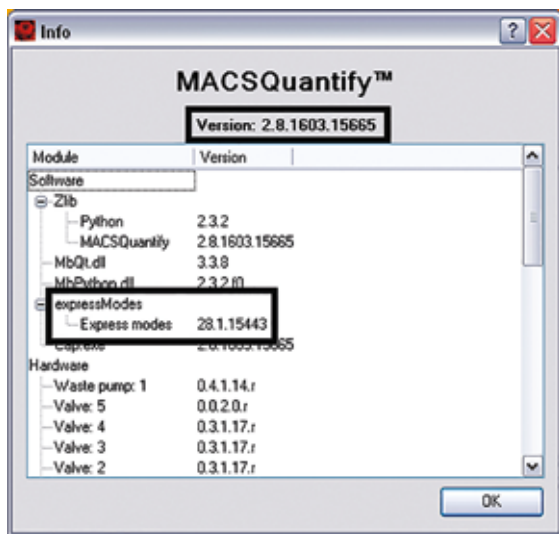


**Figure 2.2:** Information window for MACSQuantify Software and Express Mode version

## 2.3 Instrument settings

The instrument settings are automatically uploaded when using the MACSPlex Express Modes with MACSQuantify Softversion version 2.8 or higher after assigning the MACSPlex miRNA Beads and selecting Autolabel function MPx miR 39 CB. The loading of the settings will be performed when the measurement starts.

## 2.4 Select the sample rack

In the sidebar **Experiment** tab, select the **Chill 96 rack** from the **Rack** drop-down list.

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

## 2.5 Select and assign MACSPlex reagents

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.

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 "Troubleshooting".

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 miRNA Cancer 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. A dialog box informs where to place the reagent on the reagent rack.

2. Tick the respective MACSPlex miRNA Beads (e.g. MPx miR 39 CB) and NoLabel boxes in the **Autolabel** tab located in the **Experiment** tab. The red exclamation mark indicates the activated autolabel function.

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

   ▲ **Note:** In case of MACSPlex miRNA Kits scanning of the bar code is only used to identify the reagents and add required information to data files. The reagents have not be placed in the reagent rack.
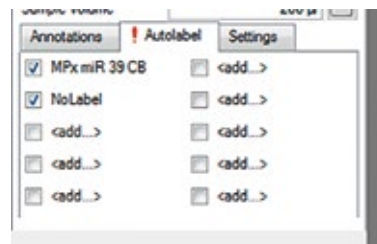


**Figure 2.3:** Control of MPx miR 39 CB and NoLabel boxes in the Autolabel tab.

## 2.6 Assignment of 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.

## 2.7 Select the Express Mode

1. Select the **Settings** tab.

2. Check the **Express** button.

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

4. Choose the Express Mode **MACSPlex_miRNA** from the **Mode** drop-down list.

5. A warning window appears in terms of sample mixing.

Choose an appropriate mixing from the **Mix sample** drop-down list.
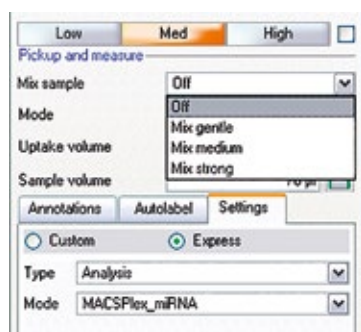


By selecting the Express Mode all experiment settings are loaded automatically except the **Mix sample** parameter. The loaded values are shown in the respective fields in the **Experiment** tab.

Check the following values:

Uptake volume: 50 µL
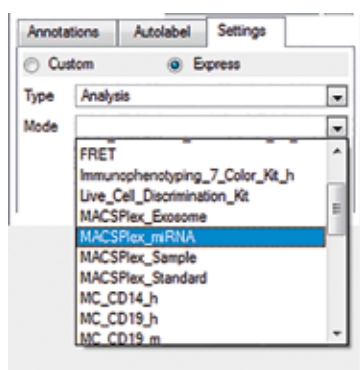
Sample volume: 70 µL

Measurement in Medium flow rate



**Figure 2.4:** Selection of the Express Mode MACSPlex_miRNA from the Settings tab.

**(Optional) Defining new sample IDs and a description for unknown samples**
Use the **Sample ID** and **Description** fields to enter relevant sample information.
Sample ID and Description can be allocated individually to each sample position within the group. Avoid special characters (e. g., "ö", "ë", "oe", "µ", etc.) in the data file names.

**(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.

### 2.8 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. Check the **Autolabel** and **Settings** tabs and make sure that for each selected position the correct **Express Mode** and **NoLabel function** are selected.



**Figure 2.5:** Control of rack definitions in the Autolabel tab of the Experiment table.



**Figure 2.6:** Control of rack definitions in the Settings tab of the Experiment table.

### 2.9 Control wells for liquid level

1. Place the MACSPlex Round Bottom Plate on a Chill 96 Rack.

2. Ensure that all used wells of the MACSPlex Round Bottom Plate contain the required amount of liquid, as described in the kit data sheet. If required, fill up wells with the relevant amount of buffer.

### 2.10 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, please 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.

### 3. Analysis of data files with the MACSPlex Express Mode

The analysis of the data files can be performed on the MACSQuant Instrument itself or on a PC with installed MACSQuantify Software version 2.8 or higer with at least Express Mode version **installExpressModes_28.1.15443.**

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

## 3.1 Analysis of data files using the Express Mode

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_miRNA 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. 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. MACSPlex_miRNA** 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 displayed medians are not related to the correct sample.
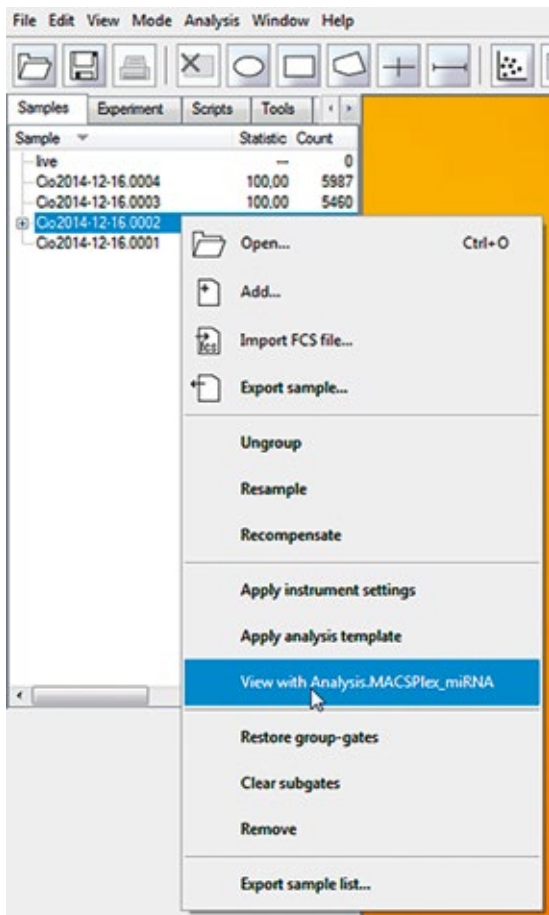


**Figure 3.1:** Selecting the Express Mode for analysis of the data files.

For the grouped MACSPlex_miRNA data during 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.
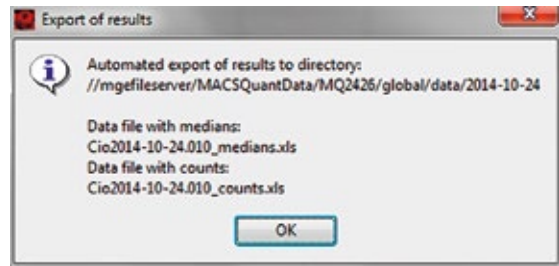


**Figure 3.2:** Information window about exported results.

▲ **Note:** The automatic export of results is only performed on the PC not on the MACSQuant Instrument.

4. The plot windows of the MACSPlex_miRNA analysis displays all relevant information, including the R1 median of each sample.

The FSC-A versus SSC-A density plot allows the identification of the singlet beads and the MACSPlex-B1 versus MACSPlex-B2 density plot for the identification of the different color-coded bead populations. The MACSPlex-B1 versus MACSPlex-R1 plot shows the increase in R1 signal intensites for detected miRNAs. 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 R1 median for each miRNA measured in the unknown sample (figure 3.3).
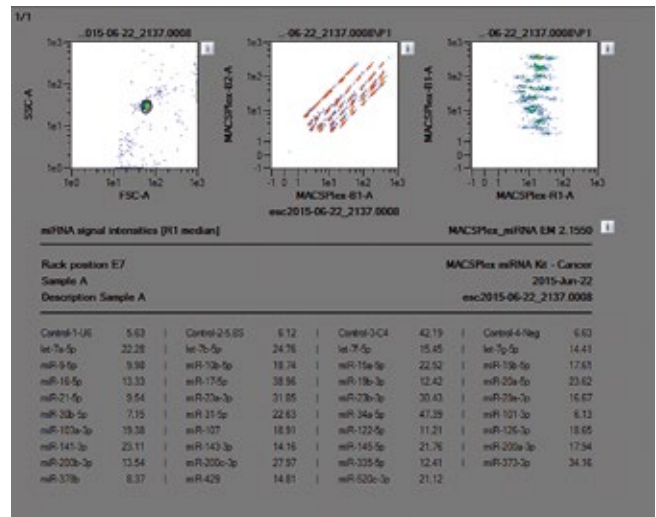


**Figure 3.3:** MACSPlex_miRNA analysis window.
Error: not all expected populations are detected! is displayed below results if less populations than expected are detected.

The automatic export contains a table with the sample identifying parameters well ID, sample ID, and description and the corresponding miRNA signal intensities or counts, respectively.



**Figure 3.4:** Example from automatically exported R1 medians of miRNA samples.

Please refer to the MACSPlex miRNA Kit data sheet for the calculation of relative miRNA expression levels.

### 3.2 Additional information about analysis

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.

## 4. 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 data file and select **Ungroup** to analyze manually.

2. Create an analysis template that can be applied to sample data files.

3. Set up the following gating strategy:
   Detection of singlet beads: forward scatter versus side scatter.
   Draw region P1 around the singlet bead population.

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

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

6. For R1 median determination display the R1 median of each 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.

## 5. Troubleshooting

The following section offers solutions for problems that might be encountered when using the MACSPlex Express Modes for the MACSPlex miRNA 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 miRNA** from the **Category** drop-down list.

2. Select the used MACSPlex miRNA beads from the **Reagent** drop-down list.

   ▲ **Note:** When clicking the **i** button in the Reagents window on the right hand side of the MPx 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. Click **Apply** to save the changes and close the window.



**Figure 5.1:** Manual selection of the MACSPlex miRNA Beads and noLabel settings.

5. Check the selected reagents in the **Autolabel** tab located in the **Experiment** tab. The red exclamation mark indicates autolabel activation.



**Figure 5.2:** Example of MACSPlex information displayed for MACSPlex miRNA Cancer Beads. 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 Data acquisition warning windows

A warning window appears when starting the MACSPlex_miRNA Express Mode, stating that no MACSPlex information is provided:



- MACSPlex reagents are not selected. Please 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. Please check the reagents in the **Autolabel** tab.

A warning window appears when starting the MACSPlex_miRNA Express Mode, stating that the opening of the instrument setting Setup-Default has failed:
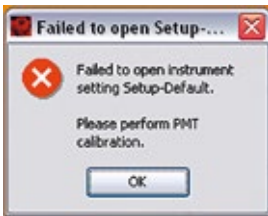


- Perform a PMT calibration of the instrument.

## 5.3 Data analysis warning windows

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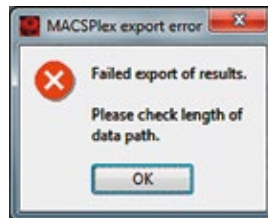


- Export failed as the limit of data path length is reached. Relocate data files to a shorter data path.

- Export failed due to use of special characters (e.g., "ö", "ë", "œ", "µ", etc.) in sample ID or description.

**Failure of analysis:**

Data files have to be analyzed manually if, for example, the warning window "Error during MACSPlex_miRNA analysis. Please contact MACSQuant Support." is displayed.

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 (see figure 2.3). Please, check the MACSPlex Reagents of the MACSPlex Kit.

- Wrong reagents have been selected in the **Autolabel** tab.

- 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.

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