# MTXQCvX2 documentation

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# Contents

# Chapter 1

# Preface

Let's get started .... this part is going to be written in the near future.

# Chapter 2

# Introduction MTXQCvX documentation

This documentation introduces to you how to use MTXQCvX2 in order to run a first straight-forward data analysis of your metabolomics experiment. The underling experimental and mathematical concepts have been introduced for the pulsed stable isotope resolved metabolomics (pSIRM) approach published in (Pietzke et al., 2014).

This documentation shows you how to use MTXQCvX2 in order to assess the quality of your GC-MS derived data, perform the determination of calibration curves and absolute quantification. It furthermore provides you two normalisation strategies and the calculation of quantities in, e.g., pmol/1e+6 cells or pmol/mg tissue.

MTXQCvX2 does also enable the calculation of stable isotopic incorporation and the evaluation of the underlying data, the mass isotopomer distributions (MIDs).

The tool has been set up to support the in-lab developed workflow for quantitative metabolomics experiments using the in-house developed software Maui for the annotation of data. MTXQCvX2 bridges the gap between quality control and first data post-processing / analysis of GC-MS derived data (MTXQCvX2_part1, MTXQCvX2_part2).

Nevertheless MTXQCvX2 includes a module in order to integrate all kind of data provided in spreadsheet-format, e.g., derived from metmax, extracting required information and creating corresponding files (MTXQCvX2_part4).

## 2.1 Structure

MTXQCvX2 contains a suite of modules is optimized to process GC-MS derived data and processed either in Maui or Chromatof/Metmax. Workflows for both approaches are introduced with step-by-step tutorials in chapter 3 and 4.

Subsequently each MTXQCvX2 module is introduced in greater detail including the list of processing parameter (chapter 6 - 10).

The configuration of MTXQCvX2 has been split into two categories - (1) a general configuration `config_files` and (2) metabolomics specific parameters `config_mtx`. Latter one is meant to provide flexibility to adapt MTXQCvX2 to your needs. How to do so and what files can be customizes is shown in chapter 11.

Workflow-specific processing methods applied in Chromatof are introduced separately in the chapter 12 and 13 including all parameter as well as a short introduction.

The remaining chapter cover a chapter for frequently asked questions (chapter16) that might give you a hint where to search for the information you are looking for.

The appendix summarises technical reference material in the form of a dictionary of tables (chapter 15), protocols for the pSIRM workflow 18 and a complete distinct chapter listing protocols (chapter 17) including standard and sample preparation and derivatisation (chapter 17.4 and 17.5) and the current setting of the GC-ToF-MS machine (chapter 19).

## 2.2 How to use this documentation

This documentation provides for each level the right starting point. Complete newbies are highly suggested to start with one of the two tutorials and following the step-by-step descriptions using the example datasets. Also if you are using for the first time please go through to the chapter once to be aware of all the steps that are required to succeed.

Experienced users might be referred to the explanation of the individual modules or to the how-to-guides for data processing and the FAQs section.

## 2.3 Example datasets and files

introduce inst/template folder

## 2.4 What do I do if I don't find the answer?

If you are familiar with github and the create an issue - please head on to the gihub repository (github.com/ChrisZasa/MTXQC_documentation) of this documentation and create one or write me a message (christin.zasada[at]mdc-berlin.de).

# Chapter 3

# Tutorial: MTXQCvX2 for Maui-projects

## 3.1 Prerequisites

- You have successfully finished the annotation using Maui-SILVIA
- You have exported all required MAUI container (see section 12.3)
- You have a list of your GC-MS sequence and related experimental conditions
- You know the extraction procedure of your standards and samples

## 3.2 In a nutshell

1. Setup a new R-project
2. Copy all MTXQC template files and folders
3. *Knit with parameter*: `MTXQC_init.Rmd` and create project folder (e.g., `psirm_glucose`)
4. Copy input files and rename `ManualQuantTable.tsv` (e18205cz.tsv)
5. Create your `annotation.csv` and `sample_extracts.csv` files
6. Define the internal extraction standard
7. *Knit with parameter*: `MTXQC_ExperimentalSetup.Rmd`
8. *Knit with parameter*: `MTXQC_part1.Rmd`
9. *Knit with parameter*: `MTXQC_part2.Rmd`
10. If required, proceed with `MTXQC_part3.Rmd` for manual validation of your data

## 3.3 Dataset of the tutorial - `tutorial_single_maui`

## 3.4 In detail

### 3.4.1 Create a R-project

R-projects provide a secure environment to handle your data from the processing in MTXQCvX2 until the final reports and analysis. Think about it as a big bubble containing and carrying all your data and analysis savely from one place to the other undisturbed of the outside changes.

- Open R-studio and create a new project following: `File -> New project -> New Project -> New Directory` and call the directory `tutorial_single_maui` and the preferred subdirectory.
- I recommend to start each project in a new session (tick the box at the bottom of the dialogue box).

### 3.4.2 Copy MTXQCvX2 template files

- Download the current version of MTXQCvX2 called fluffy adventure[1]
- Open and extract the zip-folder
- Copy all folders and files into your R-project 'tutorial_single_maui'

### 3.4.3 Process `MTXQC_init.Rmd`

The module `MTXQC_init.Rmd` performs two important steps: 1. Check-up package installation 2. Creation of project-folder

The project folder is supposed to provide a tidy structure while data processing and analysis and contains several pre-defined folder. Besides the following folders: 'input', 'output' and 'figures' you see a detailed subfolder structure. You find more details about each folder and additional suggestion how to use project-folder in chapter 5.

All you need to do is to process the `MTXQC_init.Rmd`. The following procedure is how you process all `.Rmd`-files of MTXQCvX2:

- Click on the small black triangle next to the ball of yarn in the R-Studio toolbar
- Choose `Knit with Parameters...`

If the `.Rmd`-file contains defined parameters a shiny dialogue pops up and provides an interactive selection based on the document. In the case of `MTXQC_init.Rmd` you are asked to define a so-called project-folder, e.g., `psirm_glucose`.

- **Define the name of the project-folder:** psirm_glucose

Check in the files tab of R-Studio, browse through it and get familiar with the structure. This is the home of your metabolomics projects data analysis.

### 3.4.4 Prepare and copy input files

Several files have to be exported from Maui and copied into the folders `input/gc`, `input/quant` and `input/inc` for the evaluation of the GC-MS performance, absolute quantification and isotope incorporation.

Please follow the detailed instructions in section 12.3 if you need further information how to perform the export in Maui. For the purpose of the tutorial the exported files are already processed and provided in tutorial dataset `input`-folder. Select each file and copy it to the correct location inside your R-project.

### 3.4.5 Copy files: `annotation.csv` and `Sample_extracts.csv`

Both files contain important information about your metabolomics experiment and since these information are highly specific you need to create them on your own and save them in `project-folder/input/`.

Browse to both files in the tutorial dataset, have a look at the content and copy both files into `psirm_glucose/input`.

[1] github.com/ChrisZasada/fluffy_adventure

**The annotation file**

The herein shown *annotation file* contains information about the eperimental conditions of each sample and measurement file. The columns **File** and **Type** are mandatory and the minimum content of an annotation file. Additional columns defining further conditions, like cell line or glucose level, are totally customizable to your preferences.

Though, I suggest to set up a maximum of four parameter. If your project requires more try to create combinations. Later on this helps to visualise your data using MTXQC_part2. The number of parameters is limited by the availabily of diagram axes, facets and paper size. Subsequently written individual reports are independent of this and conditions could be split and extended without any limitations.

**The sample extrats file**

The *sample extracts* file specify the nature and volume / weight of the sample extracts. The columns **Extract_vol** and **Unit** (count, mg or ul) are mandatory here.

The minimal file contains these two columns with at least one row / entry, e.g. $1.5e+6$ & count. This would result into the association of $1.5e+6$ cells to each file.

This file here in this tutorial shows cell extracts defined with the unit *count* for specific conditions. Please be aware that names of experimental conditions have to match between annotation and sample extracts file.

Nevertheless it is not necessarily required to define for each combination of conditions a cell count. Please refer FAQs section 14.1 and 14.2 for further information how to create and set up both files from the scretch.

### 3.4.6 Define an internal standard - cinnamic acid

The tutorials dataset contains data derived from a classical pSIRM experiment. This includes that the experimentalist supplemented the extraction solvent with an internal extraction standard - cinnamic acid. All that we have to do is to check if this compound has been defined as `InternalStandard` in the file `config_mtx/conversion_metabolite.csv`.

To do so:

- Open the file: 'config_mtx/conversion_metabolite.csv'
- Scroll down to the entry `Cinnamic acid trans-, 1TMS`
- Verify that `InternalStandard` is defined in the column `Standard`
- Save the file and close it

Read the section **??** how to proceed in case you want to define a different compound as internal standard.

### 3.4.7 Process `MTXQC_ExperimentalSetup.csv`

We are ready now to start processing the second module of the MTXQCvX2 called the Experimental Setup. In order to faciliate automated data processing throughout MTXQC files and parameters have to be defined beforehand for each project-folder.

Defined parameters are saved in the file `MTXQC_params.csv` inside the project-folder for future reference enabling to move the project-folder independently of the remaining MTXQC-files. A huge advantage for debugging and sharing your project[2]

Define the following parameter:

- **Define project-folder:** psirm_glucose
- **Define input-format:** maui
- **Name of annotation file:** annotation.csv

[2] Just zip-file the project-folder and send it via mail.

- **Name of sample extracts file:** sample_extracts.csv
- **Type of experiment:** pSIRM
- **Applied 13C-substrates:** glc
- **Internal standard:** check
- **Type of samples:** cell extracts
- **Additional calibration curves:** no
- **Integrate add. calibration curves:** no
- **Quantification standards:** Quant1_v3
- **Volume polar phase of Quants:** 500
- **Technical replicates:**[3] no

Confirm the parameter and file definition and wait a moment. Check if you can find the file `MTXQC_params.csv` and also `Maui_params.csv`. Latter one is automatically generated by selecting maui project as input format. `Maui_params.csv` contains the information of the pre-defined file names containing all required input data.

Have a look at the produced pdf-file and check the comments and messages. Throughout MTXQCvX2 messages inform about: * Successfull data imports * Fatal errors causing an automatic stop of data processing and * Warnings pointing towards things that should be checked before moving to the next step.

### 3.4.8 Process `MTXQC_part1.Rmd`

Since we have defined all required input parameter the main module is ready to run after the definition of only three parameter:

- **Definition of the project-folder:** psirm_glucose
- **Type of data:** maui
- **Manual validated data:** none

Also here, scroll carefully through the generated pdf-file and have a look at the messages. If all works out it doesn't matter, but it might help to know what should be written if you end up with an error message instead of.

Each generated graphic is going to be explained in detail in chapter 7. For now please refer to my phd thesis (Zasada, 2017).

All quality metrices are visualised on the last page of the report split into two heatmaps:

- GC-performance (orange): across all measurements
- Quantification & isotope incorporation (blue): for each intermediate individually, but across all exp. conditions

Besides the generation of the MTXQC_part1 report several `csv-files` are exported into the output folder summarising statistics of all quality control metrices. The content of each file is summarised in the dictionary of tables (chapter 15).

The most important file `CalculationFileData.csv` summarises all experimental conditions, normalisation factors as well as information about calibration curve parameter and derived quantities. You find this file here - `project-folder/output/quant/`.

It is worth to open this file in a program of your choice and check for a selected intermediate the content. Wrong assignments of conditions in the annotation or sample extracts-file might cause duplication of values, easily to identify if exactly the same peak areas, so the same file, appears multiple times.

`CalculationFileData.csv` is all about the absolute quantities and data normalisation. Why does it not contain information about isotope incorporation you might ask. Well, depending on the experimental setup you might follow different ways how to combine both entities. Furthermore `MTXQC_part1` is not about doing

[3] Also called backups

any processing of the data in the sense of analysis or statistics[4]. It is supposed to assess the quality meaning the evaluation of the mass isotopomer distributions (MIDs) that are the basis for the calculation of the isotope incorporation.

The next module has been set up to give you a first quick report about the content of your data performing basic post processing steps inclduing the statistics part.

### 3.4.9 Process `MTXQC_part2.Rmd`

This module aims to provide you a quick overview of your data. It generates, based on your parameter definition while processing this file, statistics of quantities for each intermediate as well as isotope incorporation and 13C-labeled quantities.

A senseful association of your experimental parameter might provide you even ready-to-show plots for a presentation or report. Different color schemes are in place to meet your personal taste - play a bit and find your favorite ;).

All kind of outputs (graphics, statistics) are saved at `output/quant/...` into a day-specific folder[5], meaning if you repeat the same analysis several times a day the content is replaced with each run[6]. Simply modify the folder name to avoid this behaviour. If you go on with your analysis the next or another day a new folder is created.

Go and process this module with the following parameter:

- **Project-folder:** psirm_glucose
- **Type of analysis:** stringent
- **Internal standard:** QC[7]
- **Quantitative value:** InstStd_Conc_pmio[8]
- **par1:** time
- **par2:** substrate
- **par3:** whatelse
- **par4:** none
- **updated:** none
- **pre-defined color scheme:** choose yourself

Feel free to add further plots at the end of this report if required or define your project specific color code at the beginning of the document.

I recommend to use the output of `MTXQC_part2.Rmd` as a kind of overall visualisation of the data. Specific questions might be addressed in smaller reports using the output-files of `MTXQC_part2.Rmd`.

I furthermore suggest to process both type of analysis - stringent and less stringent - to get an impression at what points the manual validation of distinct intermediates might be useful.

## 3.5 At the end...

So far you have used MTXQCvX2 to generate two reports: 1. one summarising the quality of the generated data and 2. a first statistical overview about the content of your data.

[4] Quality metrices are a different pair of shoes here and are not data per se

[5] Called: Date_PostProcessing_AnalysisParam_QuantitativeValue

[6] Same thing for the generated pdf-report so I recommend to move it into the post processing folder for future reference

[7] meaning, that all the internal standard has to be within a defined range of variation, files with higher variation are excluded from the analysis

[8] Translated: Normalised (regarding the internal standard) quantities in (pmol/1e+6 cells)

With this the tutorial ends and refers to the plenty of detailed information scattered in a number of additional chapters of this documentation. The FAQ section tries to guide you through more specific settings and introduces you to the flexibility of MTXQCvX2.

Since every metabolomics experiment has it's own purpose and reasoning I want to point out, that MTXQCvX2 does not want to provide a magical tool giving the perfect solution for everyone. It rather tries to provide a common platform for discussions, information exchange and inspiration by reducing the time that has to be spend on "the boring part of research". There is no limit to make MTXQC your own perfect tool writing your own reports and extensions. Feel free and hoepfully motivated to share your report as a template with your colleagues.

# Chapter 4

# Tutorial: MTXQCvX2 for for Metmax-extracted projects

## 4.1 Prerequisites

- You have processed and annotated your intermediates in ChromaToF
- You have defined the correct quantification masses in the reference search
- You have exported the data in `.txt` files
- You have used metmax[1] to extract the data (see chapter 13)

## 4.2 In a nutshell

1. Setup a new R-project
2. Copy MTXQCvX2 files
3. *Knit with parameter:* `MTXQC_init.Rmd`
4. Copy input files extracted with Metmax into `input/metmax/`
5. Generate annotation and sample extracts file (see section 14.1 & 14.2)
6. Modify metabolite names in `conversion_metabolite.csv` column **Metabolite_manual**
7. Define the internal standard and/or alkanes in conversion_metabolite.csv (see section 14.3 & **??**defalkanes))
8. *Knit with parameter:* `MTXQC_ExperimentalSetup`
9. *Knit with parameter:* `MTXQC_part4.Rmd`
10. *Knit with parameter:* `MTXQC_part1.Rmd`
11. *Knit with parameter:* `MTXQC_part2.Rmd`
12. If required - proceed with `MTXQC_part3.Rmd` for ManualValidation

## 4.3 Tutorial dataset

Birte test measurement

[1] http://gmd.mpimp-golm.mpg.de/apps/metmax/default.htm

## 4.4 In detail

### 4.4.1 Setup a new R-project

R-projects provide a secure environment to handle your data from the processing in MTXQCvX2 until the final reports and analysis. Think about it as a big bubble containing and carrying all your data and analysis savely from one place to the other undisturbed of the outside changes.

- Open R-studio and create a new project following: `File -> New project -> New Project -> New Directory`
- Call the directory `tutorial_metmax` and the preferred subdirectory.
- I recommend to start each project in a new session (tick the box at the bottom of the dialogue box).

### 4.4.2 Copy MTXQCvX2 template files

- Download the current version of MTXQCvX2 called fluffy adventure[2]
- Open and extract the zip-folder
- Copy all folders and files into your R-project `tutorial`

### 4.4.3 Process `MTXQC_init.Rmd`

The module `MTXQC_init.Rmd` performs two important steps: 1. Check-up package installation 2. Creation of project-folder

The project folder is supposed to provide a tidy structure while data processing and analysis and contains several pre-defined folder. Besides the following folders: `input`, `output` and 'figures´ you see a detailed subfolder structure. You find more details about each folder and additional suggestion how to use project-folder in chapter 5.

All you need to do is to process the `MTXQC_init.Rmd`. The following procedure is how you process all `.Rmd`-files of MTXQCvX2:

- Click on the small black triangle next to the ball of yarn in the R-Studio toolbar
- Choose `Knit with Parameters...`

If the `.Rmd`-file contains defined parameters a shiny dialogue pops up and provides an interactive selection based on the document. In the case of `MTXQC_init.Rmd` you are asked to define a so-called project-folder.

- **Define the name of the project-folder:** `t_metmax`

Check in the files tab of R-Studio, browse through it and get familiar with the structure. This is the home of your metabolomics projects data analysis.

### 4.4.4 Copy input files fom tutorial dataset

`MTXQC_part4.Rmd` convienently transform spreadsheet kind of data extracted with Metmax into suitable input files in the style of Maui input files. You do not need necessarily provide all three kind of information (peak areas, MIDs and m/z 73 values). It depends on the scope and setup of your metabolomics experiment.

If you need detailed instructions how to use metmax have a look at the section 13. The input files of the tutorial dataset have to be copied from 'input/metmax/' into your R-project and project folder at the same location.

Copy the files into `tutorial/input/t_metmax/`:

[2] github.com/ChrisZasada/fluffy_adventure

- PeakAreas.csv and
- mz73.csv
- MIDs.csv

### 4.4.5 Copy files: `annotation.csv` and `Sample_extracts.csv`

Both files contain important information about your metabolomics experiment and since these information are highly specific you need to create them on your own and save them in `project-folder/input/`.

Browse to both files in the tutorial dataset, have a look at the content and copy both files into `t_metmax/input`.

**The annotation file**

The herein shown *annotation file* contains information about the eperimental conditions of each sample and measurement file. The columns **File** and **Type** are mandatory and the minimum content of an annotation file. Additional columns defining further conditions, like cell line or glucose level, are totally customizable to your preferences.

Though, I suggest to set up a maximum of four parameter. If your project requires more try to create combinations. Later on this helps to visualise your data using MTXQC_part2. The number of parameters is limited by the availabily of diagram axes, facets and paper size. Subsequently written individual reports are independent of this and conditions could be split and extended without any limitations.

**The sample extrats file**

The *sample extracts* file specify the nature and volume / weight of the sample extracts. The columns **Extract_vol** and **Unit** (count, mg or ul) are mandatory here.

The minimal file contains these two columns with at least one row / entry, e.g. $1.5e+6$ & count. This would result into the association of $1.5e+6$ cells to each file.

This file here in this tutorial shows cell extracts defined with the unit *count* for specific conditions. Please be aware that names of experimental conditions have to match between annotation and sample extracts file.

Nevertheless it is not necessarily required to define for each combination of conditions a cell count. Please refer FAQs section 14.1 and 14.2 for further information how to create and set up both files from the scretch.

### 4.4.6 Update `Metabolite_manual` in the file `config_mtx/conversion_metabolites.csv`

In order to automatically process through MTXQCvX2 you need to link the names of your intermediates given in ChromaToF with the corresponding metabolites in `conversion_metabolites.csv`

- Open the file `conversion_metabolite.csv` and your peak area matrix file
- Update the column 'Metabolite_manual' with the choosen names

I suggest to use always a similar format, e.g. `#Alanine_3TMS`. In case of the presence of main- (MP) and byproducts (BP) use: Metabolite_Derivate_MP/BP, e.g., Glucose_(1MEOX)(5TMS)_MP. In that way names keep short, easy to remember and effeciently setup in all other files like the metmax import list to extract the MIDs[3] for each intermediate.

### 4.4.7 Define the internal standard and alkanes in `config_mtx/conversion_metabolites.csv`

Both information are specified in the column `Standard` with either `InternalStandard` or `Alk`, respectively. Furhtermore, we need to define cinnamic acid as internal standard and nine alkanes (c10-c36).

[3] Mass isotopomer distribution

The in-lab protocol considers nine alkanes from c10 to c36. Standard annotation includes an hashtag, e.g., #c10. Small fun fact: if you use this annotation even Metmax would be able to determine the retention index.

To do so:

- Open the file: `config_mtx/conversion_metabolite.csv`
- Scroll down to the entry `Cinnamic acid trans-, 1TMS`
- Verify that `InternalStandard` is defined in the column `Standard`
- Check the annotation of alkanes in `Metabolite_manual`
- Define all nine with adding `Alk` in the column `Standard`

### 4.4.8 Process `MTXQC_ExperimentalSetup.csv`

We are ready now to start processing the second module of the MTXQCvX2 called the Experimental Setup. In order to faciliate automated data processing throughout MTXQC files and parameters have to be defined beforehand for each project-folder.

Defined parameters are saved in the file `MTXQC_params.csv` inside the project-folder for future reference enabling to move the project-folder independently of the remaining MTXQC-files. A huge advantage for debugging and sharing your project[4]

Define the following parameter:

- **Define project-folder:** t_metmax
- **Define input-format:** metmax
- **Name of annotation file:** annotation.csv
- **Name of sample extracts file:** sample_extracts.csv
- **Type of experiment:** pSIRM
- **Applied 13C-substrates:** glc
- **Internal standard:** check
- **Type of samples:** cell extracts
- **Additional calibration curves:** no
- **Integrate add. calibration curves:** no
- **Quantification standards:** Quant1_v4
- **Volume polar phase of Quants:** 500
- **Technical replicates:**[5] 2

Confirm the parameter and file definition and wait a moment. Check if you can find the file `MTXQC_params.csv`.

Have a look at the produced pdf-file and check the comments and messages. Throughout MTXQCvX2 messages inform about: * Successfull data imports * Fatal errors causing an automatic stop of data processing and * Warnings pointing towards things that should be checked before moving to the next step.

### 4.4.9 Process `MTXQC_part4.Rmd`

Now everything is prepared to transform our spreadsheet data into proper MTXQCvX2 input files. Please go ahead and define the following parameter to run `MTXQC_part4.Rmd`:

- **Project folder:** t_metmax
- **File containing peak areas:** PeakAreas.csv
- **File containing mz 73 values:** mz73.csv
- **File containing MIDs:** MIDs.csv

[4] Just zip-file the project-folder and send it via mail.
[5] Also called backups

- **Generate file for internal standard:** (check)
- **Generate file for alkanes:** (check)
- **Generate file for sum of area normalisation:** (check)
- **Generate ManualQuantTable and samples file:** (check)
- **Determine isotope incorporation:** (check)

After the successfull processing you should find the parameter file `Metmax_params.csv` inside your project folder, as well as all files in their correct `input-folders`.

Have a look and check if you got all of them and have a look at the messages in the `MTXQC_part4.Rmd` report.

### 4.4.10 Process `MTXQC_part1.Rmd`

Since we have defined all required input parameter the main module is ready to run after the definition of only three parameter:

- **Definition of the project-folder:** t_metmax
- **Type of data:** metmax
- **Manual validated data:** none

Also here, scroll carefully through the generated pdf-file and have a look at the messages. If all works out it doesn't matter, but it might help to know what should be written if you end up with an error message instead of.

Each generated graphic is going to be explained in detail in chapter 7. For now please refer to my phd thesis (Zasada, 2017).

All quality metrices are visualised on the last page of the report split into two heatmaps:

- GC-performance (orange): across all measurements
- Quantification & isotope incorporation (blue): for each intermediate individually, but across all exp. conditions

Besides the generation of the MTXQC_part1 report several `csv-files` are exported into the output folder summarising statistics of all quality control metrices. The content of each file is summarised in the dictionary of tables (chapter 15).

The most important file `CalculationFileData.csv` summarises all experimental conditions, normalisation factors as well as information about calibration curve parameter and derived quantities. You find this file here - `project-folder/output/quant/`.

It is worth to open this file in a program of your choice and check for a selected intermediate the content. Wrong assignments of conditions in the annotation or sample extracts-file might cause duplication of values, easily to identify if exactly the same peak areas, so the same file, appears multiple times.

`CalculationFileData.csv` is all about the absolute quantities and data normalisation. Why does it not contain information about isotope incorporation you might ask. Well, depending on the experimental setup you might follow different ways how to combine both entities. Furthermore `MTXQC_part1.Rmd` is not about doing any processing of the data in the sense of analysis or statistics[6]. It is supposed to assess the quality meaning the evaluation of the mass isotopomer distributions (MIDs) that are the basis for the calculation of the isotope incorporation.

The next module has been set up to give you a first quick report about the content of your data performing basic post processing steps incldluing the statistics part.

[6] Quality metrices are a different pair of shoes here and are not data per se

### 4.4.11 Process `MTXQC_part2.Rmd`

This module aims to provide you a quick overview of your data. It generates, based on your parameter definition while processing this file, statistics of quantities for each intermediate as well as isotope incorporation and 13C-labeled quantities.

A senseful association of your experimental parameter might provide you even ready-to-show plots for a presentation or report. Different color schemes are in place to meet your personal taste - play a bit and find your favorite ;).

All kind of outputs (graphics, statistics) are saved at `output/quant/...` into a day-specific folder[7], meaning if you repeat the same analysis several times a day the content is replaced with each run[8]. Simply modify the folder name to avoid this behaviour. If you go on with your analysis the next or another day a new folder is created.

Go and process this module with the following parameter:

- **Project-folder:** t_metmax
- **Type of analysis:** less_stringent
- **Internal standard:** QC[9]
- **Quantitative value:** InstStd_Conc_pmio[10]
- **par1:** Time
- **par2:** CL
- **par3:** none
- **par4:** none
- **updated:** none
- **pre-defined color scheme:** choose yourself

Feel free to add further plots at the end of this report if required or define your project specific color code at the beginning of the document.

I recommend to use the output of `MTXQC_part2.Rmd` as a kind of overall visualisation of the data. Specific questions might be addressed in smaller reports using the output-files of `MTXQC_part2.Rmd`.

I furthermore suggest to process both type of analysis - stringent and less stringent - to get an impression at what points the manual validation of distinct intermediates might be useful.

## 4.5 At the end...

So far you have used MTXQCvX2 to generate two reports: 1. one summarising the quality of the generated data and 2. a first statistical overview about the content of your data.

With this the tutorial ends and refers to the plenty of detailed information scattered in a number of additional chapters of this documentation. The FAQ section tries to guide you through more specific settings and introduces you to the flexibility of MTXQCvX2.

Since every metabolomics experiment has it's own purpose and reasoning I want to point out, that MTXQCvX2 does not want to provide a magical tool giving the perfect solution for everyone. It rather tries to provide a common platform for discussions, information exchange and inspiration by reducing the time that has to be spend on "the boring part of research". There is no limit to make MTXQC your own perfect tool writing your own reports and extensions. Feel free and hoepfully motivated to share your report as a template with your colleagues.

[7] Called: Date_PostProcessing_AnalysisParam_QuantitativeValue

[8] Same thing for the generated pdf-report so I recommend to move it into the post processing folder for future reference

[9] meaning, that all the internal standard has to be within a defined range of variation, files with higher variation are excluded from the analysis

[10] Translated: Normalised (regarding the internal standard) quantities in (pmol/1e+6 cells)

# Chapter 5

# MTXQCvX2_init

## 5.1 Knit with parameter

List of parameter:

- **Definition of the project-folder:** psirm_glucose (text)

## 5.2 Text

`MTXQCvX2_init.Rmd` - why and how to use it. Advantages of the project folder.

| Folder | subfolder | Used.in.module |
|---|---|---|
| figure | | Figure export of MTXQC_part1 |
| input | add_quant | Input for MTXQC_part1 |
| | gc | Input for MTXQC_part1 |
| | inc | Input for MTXQC_part1 |
| | quant | Input for MTXQC_part1 |
| | metmax | Input for MTXQC_part4 |
| output | gc | Output for MTXQC_part1 |
| | inc | Output for MTXQC_part1 |
| | quant | Output for MTXQC_part1 |
| | Date_PostProcessing_xy | Output for MTXQC_part2 |

# Chapter 6

# MTXQCvX_ExperimentalSetup.Rmd

## 6.1 Knit with parameter

List of parameter:

- **Define project-folder:** psirm_glucose (text)
- **Define input-format:** maui, metmax
- **Name of annotation file :** annotation.csv (text)
- **Name of sample extracts file :** sample_extracts.csv (text)
- **Type of experiment:** qMTX[1], pSIRM, pSIRM time series]
- **Applied 13C-substrates:** glc, gln, pyr, other, multiple, none
- **Internal standard:** check
- **Type of samples:** cell extracts, blood/serum, tissue, supernatant, mixed
- **Additional calibration curves:** no
- **Integrate add. calibration curves:** no
- **Quantification standards:** Quant1_v3, Quant1_v4, Quant1_indv[2]
- **Volume polar phase of Quants:** 500 (numeric, in uL)
- **Technical replicates:** 1 (numeric, range 1-5)

[1] quantitative metabolomics, no evaluation of stable isotopes
[2] Refer to section 14.9

# Chapter 7

# MTXQCvX_part1.Rmd

## 7.1 Knit with parameter

List of parameter:

- **Definition of the project-folder:** psirm_glucose (text)
- **Type of data :** maui, metmax
- **Manual validated data :** none, PeakArea, Incorporation, both

## 7.2 Text

# Chapter 8

# MTXQCvX_part2.Rmd

## 8.1 Knit with parameter

List of parameter:

- **Project-folder:** psirm_glucose (text)
- **Tpye of analysis:** stringent, less stringent
- **Internal standard:** QC, all, none
- **Quantitative value:**[1]
  - PeakArea
  - absconc: applied calibration curve
  - corr_absconc: technical replicates, quant-volume
  - Conc_pmio: pmol per defined unit defined in sample extracts
  - Conc_microM[2]
  - sumA_Conc: norm (sum of area) pmol
  - sumA_Conc_pmio: norm. (sum of area) pmol / defined unit
  - sumA_Conc_microM
  - IntStd_Conc: norm (internal standard) pmol
  - IntStd_Conc_pmio
  - IntStd_Conc_microM
  - IntStd_sumA_Conc
  - IntStd_sumA_Conc_pmio
- **par1:** parmater[3], visualised as x-axis parameter
- **par2:** subgrouping
- **par3:** sub-figure, extra panel/page
- **par4:**
- **updated:** none, PeakArea, Incorporation, both
- **pre-defined color schemes:**
  - main
  - cheerful
  - hot
  - greens
  - sleek
  - clean
  - cool

[1] Or visualised as y-axis parameter
[2] only calculated for unit = ul in sample extracts
[3] accordingly to annotation file

– art

## 8.2 In more detail - processing parameter

## 8.3 Notes

# Chapter 9

# MTXQCvX_part3.Rmd

## 9.1 Knit with parameter

List of parameter:

- **Project folder:** (text)
- **Output folder for MTXQC_part3:** (text)
- **Prepare data for man. validation:** none, PeakArea, Incorporation
- **Evaluate data from man. validation:** none, PeakArea, Incorporation
- **Input format:** maui, metmax

## 9.2 Text

# Chapter 10

# MTXQCvX_part4.Rmd - Metmax parser

## 10.1 Knit with parameter

List of parameter:

- **Project folder:** (text)
- **File name for peak areas** (text)
- **File name for m/z 73 values:** (text)
- **File name for MIDs:** (text)
- **Generate input file for internal standard**
- **Generate input file for alkane standards**
- **Generate input file for sum of area normalisation**
- **Generate ManualQuantTables and samples peak area file**
- **Calculate stable isotope incorporation and generate input files**

## 10.2 Text

# Chapter 11

# Customization of MTXQCvX2

Herein explained are the customizable tables of the MTXQCvX2 universe.

## 11.1 conversion_metabolite.csv

| Column.name | Description | Value |
|---|---|---|
| Metabolite_manual | Manual defined metabolite name | #Alanine (2TMS) |
| Metabolite | Library name of the metabolite | Alanine_(2TMS)_BP_RI:1097_IDENT:B+C |
| Metabolite_short | Short version of library name of the metabolite | Alanine_(2TMS) |
| Lettercode | Lettercode version of metabolite name | Ala_2TMS |
| Q1_value | Checked if quant1:1 value available | x |
| Mass_Pos | m/z-value corresponding to m inc | 118 |
| SE_sel | Evaluation of the MIDs | x |
| Q_sel | Evaluation for absolute quantification | x |
| nopsirm | Exclusively for absolute quantification | |
| Standards | Defined as standard | InternalStandard, Alk |

## 11.2 letter_pathway_complete.csv

| Column.name | Description | Value |
|---|---|---|
| Letter_Derivate | Derivate definition | Ala |
| Lettercode | Lettercode name of metabolite | Ala_3TMS |
| Pathway | Ass.pathway | aa |
| Pathway.1 | Ass. pathway - ordered for heatmap | 5-aa |
| Met_pathway | Ass. pathway - ordered for heatmap incl. Lettercode | 5-aa_Ala_3TMS |
| Subs_class | Substance class | aa |
| Met_class | Substance class incl. Lettercode | aa_Ala_3TMS |

## 11.3 quant1-values.csv

| Column.name | Description | Value |
|---|---|---|
| Letter_Derivate | Derivate name of metabolite | 3PGA |
| Quant1_v4 | Quantity in (pmol) | 43480 |
| Quant1_v3 | Quantity in (pmol) | 43480 |

## 11.4 incorporation_calc.csv

| Column.name | Description | Value |
|---|---|---|
| Metabolite | Library name of metabolite | Alanine_(2TMS)_BP_RI:1097_IDENT:B+C |
| Mass_mz | m/z-value | 116, 118 |
| LI_MID | Definition of mass level | m0, minc |

## 11.5 mid_backups.csv

| Column.name | Description | Value |
|---|---|---|
| Metabolite | Library name of metabolite | Alanine_ beta-<br>_(3TMS)_MP_RI:1435_IDENT:A+D |
| Mass.m.z. | m/z value | 188 |
| BackupPeakArea | Peak area of Backup MID | 4960 |
| BackupMID | MID value for corresponding Mass.m.z. | 0.8005 |

# Chapter 12

# Data processing - MAUI

*Please refer to the OneNote summary how to process GC-MS files for a pSIRM experiment with Maui. This part of the documentation is still under construction.*

## 12.1 Processing In ChromaToF

## 12.2 Maui pSIRM workflow

*see note abote*

## 12.3 Maui exports

### 12.3.1 Subfolder: `input/gc`

This folder contains all input files that are required to assess the quality of the GC-MS perfomance for the complete GC-MS run. In order to validate all four parameter we need to export the following information from the Maui-project.

You can perform exports via right click on either (1) the project name and the functions provided in the menue `Diagnostics` or (2) with a direct right click on data container, e.g.,

- Open your Maui-project and export:

1. `Alcane_intensities.csv`: Diagnostics/Export Alcane intensities
2. `MassSum-73.csv`: Diagnostics/QC Mass Sum Export and define 73 for m/z 73
3. `PeakDensities-Chroma.csv`: Diagnostics/Export PeakDensities

- Export the information of the cinnamic acid container with right-click and Export quantification, follow the pop-up dialogues
- Use the explorer and open `.../mauiproject-name/export/Diagnostics` and copy the csv.files into `input/gc`
- Navigate to `.../mauiproject-name/export/ExportQuantification/defined-folder`
- Copy the file `quantMassAreasMatrix.csv` without applied normalisation and rename it `InternalStandard.csv`

### 12.3.2 Subfolder: `input/quant`

Two files have to be copied into this folder in order to perform the quantification of metabolites: (1) peak areas of the calibration curves and (2) of all samples.

The first file `ManualQuantTable.tsv` is automatically generated during the processing of the absolute quantification in Maui.

- Navigate in the explorer to `.../mauiproject-name/export/QM-ManualQuantification/`
- Copy the file `ManualQuantTable.tsv`
- Rename it with the corresponding batch-id of the GC-MS run (e.g., e17205cz)

Peak areas for each measurement and metabolite have to be exported in Maui:

- Open your Maui-project and export:
- Export the information of the samplePeakGroups container with right-click and Export quantification, follow the instructions
- Navigate in the explorer to `.../mauiproject-name/export/ExportQuantification/defined-folder`
- Copy the file `quantMassAreasMatrix.csv` without applied normalisation

### 12.3.3 Subfolder `input/inc`

The evaluation of stable isotope incorporation requires two input files exported from the container pSIRM-samplesPeakGroups, right click and corresponding export function:

- `DataMatrix.csv`: Export % Label
- `pSIRM_SpectraData.csv`: pSIRM Spectra Export
- Navigate in the explorer to `.../mauiproject-name/export/SpectraExport/defined-folder` and `xy`
- Copy both files, no renaming required

```
##
## Attaching package: 'dplyr'

## The following objects are masked from 'package:stats':
##
##     filter, lag

## The following objects are masked from 'package:base':
##
##     intersect, setdiff, setequal, union
```

# Chapter 13

# Data Processing for Metmax

The following steps are done in ChromaToF on the raw data exported from the machine. I highly recommend to create a local copy of your data and then import it into ChromaToF. ChromaToF is not the fastest software and the server doesn't help for sure with that.

For each data processing (DP) method you need to define tasks by checking boxes at the top of the window. With each ticked box the related parameter that can be modified are listed below that list of tasks. So always remeber to scroll down, and after setting parameters, scroll up and check your ticks another time.

In general you first adjust the choice of DP method first and apply it subsequently - choose one file, right click and choose *Process data*. Select all files and the DP method.

## 13.1 Import data into ChromaToF

Create a new project folder in the menu `Acquired Samples` and rename it, e.g., with the date and project name. Click on the folder to activate it.

Now you can move down a window, just right click somewhere in this window and choose *Import data*. Select your `.SMP` files and wait until ChromaToF is done.

## 13.2 DP - Resampling

Resampling is commonly applied and results into a data transformation enabling an improved detection of low abundant peaks and a reduction of noise. (Maybe include an example?)

The processing methods requires to tick the task `Export data file`. Subsequently, you are asked to define an output folder and the following paramter:

- Reduction rate: 4
- From the beginning to end of the file
- `.peg`-files

In the next step the generated files have to be re-imported into a subfolder in `Acquired Samples` in ChromaToF.

- Create subfolder called `resample` in ChromaToF
- Right-click file window below
- Import `.peg`-files

## 13.3 DP - 1D-basic

Please check the following task in ChromaToF `Data Processing (DP) Methods`:

- Baseline
- Peak Find
- Library Search
- Calculate Area / Height
- Retention Index Method

**Baseline**

- enter baseline tracking from `start of the run` until `end of run`
- baseline offset 1
- number of data points for smoothing: auto

**Peak Find**

- peak width: 4
- maximum number of unknown peaks to find: 600
- S/N: 50.0
- library identity: normal
- library search mode: forward
- number of library hits returned: 10
- min. molecular weigth allowed: 0
- max. molecular weight allowed: 1500
- mass threshold: 0
- min. similarity match: 0
- Add libraries: gmd-merged-kempa

**Calculate Area / Height**

- enter mass to use: U

**Retention Index Method**

- choose your retention index method

### 13.3.1 Create the Retention Index Method

Before proceeding with applying processing method you need to prepare the Retention Index Method.

Create a new Retention Index Method:

- Scroll down in the main menu and click on Retention Index Methods
- Right click on the white in the window below
- Select new
- Rename it

This creates an empty table in the main window. Use the blue plus sign and add nine rows. If you cannot click on the plus, make sure you clicked the name of the retention indey method before.

Adjust the entries the following way:

- Change names: #c10, #c12, #c15, #c17, #c19, #c22, #c28, #c32, #c36
- Retention Index is equal to the product of number of carbons and 100 (RI of #c10 is 1000)
- Verification Mass: 85

Go back to your measurements and select a wash run. Select the alkane masses, normally saved in shortcut B, and collect the retention times. Add these values in the retention index method. All done, select the retention index method in data processing methods.

Run the processing method `1D-basic` over all your files.

## 13.4 Reference search

### 13.4.1 Create a reference in ChromaToF

### 13.4.2 Process your reference search

Check the task and following parameter:

- Apply Reference(s):
  - S/N: 10 - 50
  - Mass Threshold: 0
  - Add the references

**Very important** Always scroll up and check the ticked tasks again. **Always... and really always** when you change the reference ChromaToF checks the PeakFind option for you. But this we definitely do not want since the basic processing just pre-annotated all peaks that were detected and identifiable. If you forget to uncheck the box only the compounds listed in your reference are annotated, all other annotations are replaced with unknown and a number.

## 13.5 Export for Metmax

Check tasks in 'data processing methods:

- Export peak information ASCII CSV format
  - define the directory where files should be saved
  - field separator: TAB
  - filter: choose all but not **Not Founds**
  - sort by: Name, ascending
  - exportet information:
  - Name
  - Concerns
  - Quant Masses
  - Area
  - Expected Ion Ratio 1
  - Calibration
  - Match
  - Quant S/N
  - Similarity
  - Type
  - Concentration
  - Sample Concentration
  - 1st Dimension Time (s)
  - Retention Index
  - R.T. (s)
  - Reverse
  - Conc. Units

– 2nd Dimension Time (s)

## 13.6 Data extraction with Metmax

### 13.6.1 Quantification - PeakAreas.csv[1]

In order to perform absolute quantification you need a file containing all extracted peak areas for each metabolite and file[2].

The header of metmax-extracted files looks like shown below (see table 1). Please, remember to delete the second header row, representing the column loads for each file before saving as csv-file. Otherwise you end up with weird imported dataframes in R.

Quantification masses have to be updated while processing in ChromaToF prior the export of the data e.g., with a reference search (see section **??**] or using statistical compare. pSIRM experiments require the definition of pTop5 masses; an extended list of quant masses considering isotope incorporation instead of top5 masses in the reference in order to take into account the shift of intensities induced by the application of stable isotopes[3] (see *somewhere*).

| name | mass | ri | row.load | file_1 | file_2 | file_x |
|---|---|---|---|---|---|---|
| Lac | 219 | 1051 | 0.76 | 15423 | 135444 | 465486 |
| Pyr | 174 | 1042 | 0.65 | 56978 | 46888 | 4354544 |
| Cit | 273 | 1805 | 0.99 | 1326 | 23321 | 132121 |

MTXQC_part4 takes care of the formatting and correct column names of the peak areas file and saves it in `input/quant/quantMassAreasMatrix.csv`.
MTXQC_part4 generates also the file PeakDensities-Chroma.csv and saves it in `input/gc/PeakDensities-Chroma.csv` in case you have selected the option to include sum of area normalisation while knitting this module.

### 13.6.2 Isotope incorporation - MIDs.csv[4]

In order to determine the incorporation of stable isotopes MTXQCvX2 requires as an input the mass isotopomer distributions (MIDs) for each intermediate and measurement[5]. Fragments for each intermediate have to be pre-defined in metmax in the menue Tools/Options/metabolite masses.

You can import a prepared list (have a look at `inst/template_files/MetMax_MIDs.txt`) or specify each entry manually. An example of the metmax output is shown in table 2. The output has to be saved as csv-file, including the deletion of the partial row `column.load`, respectively[6].

| name | mass | ri | row.load | file_1 | file_2 | file_x |
|---|---|---|---|---|---|---|
| Lac | 219 | 1051 | 0.85 | 31026 | 5165829 | 5829 |
| Lac | 220 | 1051 | 0.85 | 3607 | 662277 | 277 |
| Lac | 221 | 1051 | 0.85 | 1222 | 111481 | 81 |
| Lac | 222 | 1051 | 0.85 | 188 | 1003494 | 10023 |
| Lac | 223 | 1051 | 0.85 | 0 | 33542 | 342 |

[1] Required for: all parameter, just not `calculation stable isotope incorporation`
[2] Tools/Options/Retention analysis, Parameter: Area
[3] Mandatory columns: name, mass, files
[4] Required for `calculation isotope incorporation`
[5] Metmax: Tools/Options/Isotope concentrator; Parameter: IntensityOfMass
[6] Mandatory columns: name, mass, files

MTXQC_part4 calculates the stable isotope incorporation and exports DataMatrix.csv as well as pSIRM_SpectraData.csv saved in `input/inc/DataMatrix` & `pSIRM_SpectraData.csv`, respectively. The mathematics behind are outlined in (Pietzke et al., 2014)

**Important:** Extracted MIDs have to match with defined mass couples for each metabolite in MTXQCvX2 defined in `config_mtx/incorpo_calc_masses.csv`. Please refer for more details in chapter 11.

### 13.6.3 Derivatisation efficiency - mz73.csv[7]

The extraction of intensities for the ion $m/z73$ works analogous to the extraction of MIDs[8].

Mass ranges have to be defined for each intermediate for the mass 73 by defining starting and end mass with 73. MTXQC_part4 generates the file and saves it in `input/gc/MassSum-73.csv`. Check `inst\template_files\` for reference. Hopefully soon a new metmax button extracting specific intensities across the batch.

[7]Required for: `sum of area normalisation`
[8]Tools/Options/Isotope concentrator; Parameter: IntensityOfMass

# Chapter 14

# Frequently Asked Questions

Please keep in mind that thorughout this section the project-folder is referred as 'psirm_glucose'.

If you do not find an answer to your question - please create an issue directly in the github repository[1] or write an e-mail.

## 14.1 How do I create my annotation-file?

The annotation file relate file names with experimental conditions or specify quantification standards in your batch. Two columns - **File and Type** - are obligatory and have to be present in the annotation file. In the case of their absence `MTXQCvX_part1.Rmd` stops processing and shows an error message.

A quick way to generate an annotation file is described below:

1. Copy the first row / header of `quantMassAreaMatrix.csv` file
2. Paste & transpose the content into a new Excel-File into column A
3. Change the first entry: Metabolite -> File
4. Remove the entry QuantMasses at the very end of the column A
5. Add the column Type and specify each file either as **sample**, **Q1_dilution** or **addQ1_dilution**[2]
6. Add more columns specifying your experimental conditions, e.g., Cellline and Treatment[3]
7. Save the content as `csv-file` in, e.g., `psirm_glucose/input/...`

## 14.2 How do I create the file `sample_extracts.csv`?

The `sample_extracts.csv` file is required in order to determine automatically absolute quantities in the manner of pmol/1e+6 cells or pmol/mg tissue in the `CalculationFileData.csv`.

This file requires two obligatory columns - **Extract_vol** and **Unit**[4]. Please specify for each experimental condition the amount of extracted cells (count), tissue (mg) or volume of blood/plasme (ul) in the unit shown in the brackets.
The names of the columns of the experimental conditions have to match up with the annotation file. Save the file in the folder `psirm_glucose/input/...`.

[1] check https://www.github.com/ChrisZasa/MTXQC_documentation/
[2] see for further details additionalQuant
[3] optimal: two to three parameter, at maimumx: four parameter. Consider combinations of parameter, e.g., HCT116-control, HCT116-BPTES
[4] Define: count, mg or ul

If the defined experimental conditions do not match up with the annotation `MTXQC_part1.Rmd` exit data processing. A template file is saved for review and usage at `inst/template_files/...`

## 14.3 How do I define an internal extraction standard other than cinnamic acid?

MTXQCvX2 allows the specification of project-specific internal extraction standards. The only thing you need to do is to define the corresponding compounds as an internal standard in the `config_mtx/conversion_metabolite.csv` file. To do so, add to the compound in last column `Standard` the entry `InternalStandard`.

The definition of an internal standard requires the file 'InternalStandard.csv' in the folder `input/gc` since it is used for the evaluation of normalisation factors in MTXQC_part1.

**Scenario 1** Maui-project and Cinnamic acid

For an classical pSIRM experiment in the Kempa lab we are using cinnamic acid. The evaluation of this compound has been integrated into Maui. Peak areas of cinnamic acid are exported from the container called `cinAcid`. The exported file[5] has to be renamed to `InternalStandard.csv` though and moved to `psirm_glucose/input/gc/...`.

**Scenario 2** Maui-project and different internal standard

If you have used a different compound as an internal extraction standard you might need to extract the peak areas of this compound from the file `quantPeakAreasMatrix.csv` and save it in the folder `psirm_glucose/input/gc/InternalStandard.csv`, respectively. Prerequisite here - you have annotated the compound in your Maui-project.

**Scenario 3** Metmax-project

In the case you used metmax the module `MTXQC_part4.Rmd` takes care for you of the generation of the `InternalStandard.csv` based on the definition in `conversion_metabolite.csv` and provided peak areas[6]. This procedure is independent what standard you have defined. It only requires the annotation of your compound in chromtof and successful export with metmax.

## 14.4 How do I combine multiple MAUI-projects into a single MTXQC-project?

Certain circumstances might wish you to combine *multiple MAUI-projects* into one MTXQC-project. This might be the case when you run the same samples in split and splitless mode on the machine or your experimental setup has been measured in multiple batches in order to avoid derivatisation effects.

It is highly recommended to combine the input files derived from a different Maui projects beforehand the analysis. In that way you have only to work with a single file `CalculationFileData.csv`[7] containing all data of the your experiment.

The herein described process provides a quick way how to combine the exported files from different Maui projects. The script `combine-sets.R`[8] automatically saves all combined files into the correct `input` folder. Just update the folder and subfolder names. All the rest has been taken care of for you.

1. Create in the MTXQC-project folder (e.g., `psirm_glucose/`) a new folder called `raw-data`

[5] see section **??**
[6] see chapter 10
[7] stored in `psirm_glucose/output/quant/...`
[8] inst/template_files/...

2. Create a subfolder for each Maui-project in `psirm_glucose/raw_data/...`
3. Copy into this folder all your Maui-derived input files altogether
4. Update the parameter of `combine-sets.R`, meaning folder name definitions, file
5. Execute the R script
6. Merged files have been generated and copied into the corresponding folder: `psirm_glucose/input-folder/gc/...` or `psirm_glucose/input-folder/inc/...`
7. Copy the renamed `ManualQuantTable.tsv` files of each Maui project into `psirm_glucose/input/quant/...`

## 14.5 How do I extend conversion_metabolite.csv

At a certain point you might want to add a compound or derivate that is not part of the current setup of metabolites. For simplicity we assume you want to add an intermediate for quantitative and isotope incorporation[9].

In order to include this new compound you need to add information to the following files in `config_mtx`:

- `conversion_metabolite.csv`
- `letter_pathway_complete.csv`
- `incorp_calc_masses.csv`
- `mid_backups.csv`

If you desire the absolute quantification of this new compound you most probably need to either: (1) include the compound in quant-standards or (2) measure additional quantification standards. Please read sections 14.8, @ref(#quantind) and 14.10 how to proceed.

**`conversion_metabolite.csv`**

- Define the three variations - Metabolite, Metaboite_short, Lettercode - of the compound name by analogy with the present list (text)
- Q1_value: check only if this compound is part of a quantification mix (x)
- Mass_Pos: define the m/z-value reflecting the shift of intensity in case of the incorporation of stable isotopes (numeric)
- SE_sel: check only if MID exports are available (x)
- Q_sel:
- nopsirm: check if this compound should not considered for isotope inc. (x)
- Standards: define as *InternalStandard* if applicable

** `letter_pathway_complete.csv`

** `incorp_calc_masses.csv`

Define the couple of m/z-values for your compound: - m0 - mono-isotopic mass/charge value and - minc - mass/charge value reflecting shift of intensities

In case you want to define more than one pair of m/z-values have a look at the defined TCA-cycle intermediates.

** `mid_backups.csv`

[9] Just skip this if this is not required

## 14.6 How do I prepare my data in ChromaToF for manual data validation

## 14.7 How do I perform the manual validation with MTXQC_part3?

## 14.8 How do I distinguish between standard and additional quantification standards?

## 14.9 How do I define my own quantification standards?

## 14.10 How do I integrate additional quantification standards into MTXQCvX2

# Chapter 15

# Appendix - Dictionary Tables

This chapter shows the structure of all input or output csv-files that are referenced throughout the documentation. Please refer to the chapters for more detailed explanations.

## 15.1 MTXQC base tables

### 15.1.1 `config_files` tables

#### 15.1.1.1 conv_filenames.csv

| AssociatedFile | Filename |
|---|---|
| cin_acid | InternalStandard.csv |
| alkane_int | Alcane_intensities.csv |
| mz_73 | MassSum-73.csv |
| peak_densities | PeakDensities-Chroma.csv |
| sample_area | quantMassAreasMatrix.csv |
| pSIRM_se | pSIRM_SpectraData.csv |
| inc | DataMatrix.csv |
| addQ | additional_quant1_values.csv |
| mass_li | incorp_calc_masses.csv |
| backups_mid | mid_backups.csv |

#### 15.1.1.2 conv_filesnames_manVal.csv

| | AssociatedFile | Filename |
|---|---|---|
| 1 | sample_area | quantMassAreasMatrix_manVal.csv |
| 2 | pSIRM_se | pSIRM_SpectraData_manVal.csv |
| 3 | inc | DataMatrix_manVal.csv |
| NA | NA | NA |
| NA.1 | NA | NA |

### 15.1.2 `config_mtx` tables

These tables are supposed to be modified in relation to the individual needs of a project.

#### 15.1.2.1 conversion_metabolite.csv

| Column.name | Description | Value |
|---|---|---|
| Metabolite_manual | Manual defined metabolite name | #Alanine (2TMS) |
| Metabolite | Library name of the metabolite | Alanine_(2TMS)_BP_RI:1097_IDENT:B+C |
| Metabolite_short | Short version of library name of the metabolite | Alanine_(2TMS) |
| Lettercode | Lettercode version of metabolite name | Ala_2TMS |
| Q1_value | Checked if quant1:1 value available | x |
| Mass_Pos | m/z-value corresponding to m_inc | 118 |
| SE_sel | Evaluation of the MIDs | x |
| Q_sel | Evaluation for absolute quantification | x |
| nopsirm | Exclusively for absolute quantification | |
| Standards | Defined as standard | InternalStandard, Alk |

#### 15.1.2.2 letter_pathway_complete.csv

| Letter_Derivate | Lettercode | Pathway | Pathway.1 | Met_pathway | Subs_class | Met_class |
|---|---|---|---|---|---|---|
| 2HG | 2HG | glut | 3-glut | 3-glut_Glut_2h | organic acid | organic acid_Glut_2hydroxy |
| 2OG | 2OG | tca | 2-tca | 2-tca_Glut_2ox | organic acid | organic acid_Glut_2oxo |
| 3PGA | 3PGA | glyc | 1-glyc | 1-glyc_PGA | phosphate | phosphate_PGA |
| A | A | nucleobase | 7-nucleobase | 7-nucleobase_A | nucleobase | nucleobase_Adenosine |

#### 15.1.2.3 incorp_calc_masses.csv

| Metabolite | Mass_mz | LI_MID |
|---|---|---|
| Alanine_(2TMS)_BP_RI:1097_I | 116 | m0 |
| Alanine_(2TMS)_BP_RI:1097_I | 118 | minc |
| Alanine_(3TMS)_MP_RI:1367_I | 188 | m0 |
| Alanine_(3TMS)_MP_RI:1367_I | 190 | minc |
| Aspartic acid_(2TMS)_BP_RI:1433_IDEI | 245 | m0 |

#### 15.1.2.4 quant1_values.csv

| Letter_Derivate | Quant1_v4 | Quant1_v3 |
|---|---|---|
| 2HG | 57270 | 57270 |
| 2OG | 34220 | 34220 |
| 3PGA | 43480 | 43480 |
| A | 7400 | 7400 |
| Adenosine | 18710 | 18710 |

#### 15.1.2.5 mid_backups.csv

| Metabolite | Mass.m.z. | BackupPeakArea | BackupMID |
|---|---|---|---|
| Alanine_ beta-_(3TMS)_MP_RI:1435_ | 188 | 4960 | 0.8005000 |
| Alanine_ beta-_(3TMS)_MP_RI:1435_ | 189 | 876 | 0.1414000 |
| Alanine_ beta-_(3TMS)_MP_RI:1435_ | 190 | 307 | 0.0495000 |
| Alanine_ beta-_(3TMS)_MP_RI:1435_ | 191 | 53 | 0.0086000 |
| Alanine_(2TMS)_BP_R | 116 | 2616179 | 0.8552984 |
| Alanine_(2TMS)_BP_R | 117 | 323019 | 0.1056035 |
| Alanine_(2TMS)_BP_R | 118 | 99834 | 0.0326384 |
| Alanine_(2TMS)_BP_R | 119 | 19759 | 0.0064597 |
| Alanine_(3TMS)_MP_F | 188 | 4960 | 0.8005000 |
| Alanine_(3TMS)_MP_F | 189 | 876 | 0.1414000 |

## 15.2 Input data

### 15.2.1 MAUI derived tables

### 15.2.2 Metmax derived tables

## 15.3 Output data

### 15.3.1 Experimental Setup

### 15.3.2 MTXQCvX2 part1

#### 15.3.2.1 `output/gc/...`

##### 15.3.2.1.1 `HM_GC_values.csv` & `qcmetric_xy.csv`

MTXQC exports a file summarising quality factors for each of the four parameter evaluating the GC performance. A summary representing the values illustrated in the heatmap are shown in table `HM_GC_values.csv`, individual exports for each metric in table `qcmetric_xy.csv`.

| Column.name | Description | Value |
|---|---|---|
| Batch_Id | Batch-Id | e18274ba |
| qc_metric | QC metric factor corresponding with 1 - very good and 0 - very low | 0.937254457 |
| title | Class of QC metric | alkanes |


| Column.name | Description | Value |
|---|---|---|
| Batch_Id | Batch-Id | e18274ba |
| qc_metric | QC metric factor corresponding with 1 - very good and 0 - very low | 0.937254457 |
| title | Class of QC metric | alkanes |


#### 15.3.2.1.2 `IntStandard_normfactors.csv` & `IntStandard_stats.csv`

| Column.name | Description | Value |
|---|---|---|
| File | File name | e18274ba_17.cdf |
| PeakArea | Peak area of internal extraction standard | 89308492 |
| Batch_Id | Bacth-Id | e18274ba |
| IntStd_fac | Determined normalisation factor | 0.837457514 |
| IntStd_eval | Evaluation of normalisation factor in relation to defined range plus/minus one standard deviation | within |

| Column.name | Description | Value |
|---|---|---|
| File | File name | e18274ba_17.cdf |
| PeakArea | Peak area of internal extraction standard | 89308492 |
| Batch_Id | Batch-Id | e18274ba |
| IntStd_fac | Normalisation factor | 0.837457514 |
| IntStd_eval | Evaluation regarding QC | within |

#### 15.3.2.1.3 `Min_Annotation.csv` & `SumArea_stats.csv`

| Column.name | Description | Value |
|---|---|---|
| File | File name | e18274ba_17.cdf |
| Batch_Id | Extracted Batch-Id derived from file name | e18274ba |
| n_area | Number of peak areas per file | 101 |
| sum_area | Sum of all peak areas | 44614610885 |
| n_total | Total number of entries (including NA) | 107 |

| | Column.name | Description | Value |
|---|---|---|---|
| 1 | Batch_Id | Batch-Id | e18274ba |
| 2 | n_50 | Number corresponding to fifty percent of the maximum number of annotated peaks per file | 53.5 |
| NA | NA | NA | NA |
| NA.1 | NA | NA | NA |
| NA.2 | NA | NA | NA |

#### 15.3.2.1.4 `mz73_data.csv`

| Column.name | Description | Value |
|---|---|---|
| File | File name | e18274ba_17.cdf |
| Batch_Id | Batch-ID | e18274ba |
| mean_73 | Mean value of the sum of m/z 73 intensities per file | 16314646.1 |
| sd_73 | Standard deviation of the mean of the sum of m/z 73 intensities per file | 143890119.5 |
| n_peaks | Number of intensities used for statistics | 600 |

### 15.3.2.2 `output/quant/...`

#### 15.3.2.2.1 `calcheck_linearity.csv`

| Column.name | Description | Value |
|---|---|---|
| Metabolite | Full library name of the metabolite | Alanine_(3TMS)_MP_RI:1367_IDENT:B+C |
| Batch_Id | Batch-Id | e18274ba |
| File | File name | e18274ba_53.cdf |
| QuantMasses | Defined quantification masses | 110.0 133.0 114.0 100.0 188.0 190.0 |
| PeakArea | Sum of peak areas based on defined QuantMasses | 12710956 |

#### 15.3.2.2.2 `CalculationFileData.csv`

This is porbably the most important file that is generated by running `MTXQCvX2_part1.Rmd`. It summarises all quality factors, experimental data and determined quantities of your experiment. This file provides the input for `MTXQCvX2_part2-PostProcessing.Rmd`.

| Column.name | Class | Description | Value |
|---|---|---|---|
| Batch_Id | AnnExp | Batch-Id extracted from file name | e18274ba |
| CL | AnnExp | Experimental parameter | BE(2)-C |
| Cond | AnnExp | Experimental parameter | Control |
| File | AnnExp | File name | e18274ba_25.cdf |
| Standards | AnnExp | Defined as standard (InternalStandard, Alk) | |
| Time | AnnExp | Experimental parameter | 0 |
| Type | AnnExp | Type of measurement | sample |
| Extract_vol | AnnExtract | Defined extractes in count, mg or uL defined in Unit | 3290000 |
| Unit | AnnExtract | Defined unit for corresponding Extract_vol | count |
| Lettercode | AnnMet | Lettercode version of metabolite name | Glyc3P |

#### 15.3.2.2.3 `HeatMap_Quant_pTop5.csv`

| Column.name | Description | Value |
|---|---|---|
| Lettercode | Short name of metabolite | Cit |
| Batch_Id | Batch-Id | e18274ba |
| Metabolite | Library name of metabolite | Citric acid_(4TMS)_MP_RI:1814_IDENT:B+D |
| Par | Parameter | R2_cal |
| Val | Value of the parameter for corresponding metabolite | 0.996053496 |

#### 15.3.2.2.4 `pTop5_Calibration_Samples_lincheck.csv`

| Column.name | Description | Value |
|---|---|---|
| Lettercode | Short name of metabolite | 3PGA |

#### 15.3.2.2.5 `top5_CalibrationInfo_unique.csv`

| Column.name | Description | Value |
|---|---|---|
| Metabolite | Library name of metabolite | Citric acid_(4TMS)_MP_RI:1814_IDENT:B+D |
| Lettercode | Lettercode name of metabolite | Cit |
| Batch_Id | Batch-Id | e18274ba |
| Origin | Origin of quant1:1 value | Qstd |
| adj_r_squared | Adjsuted Rsquare of calibration curve | 0.996053496 |

#### 15.3.2.2.6 `top5_QMQcurveInfo.csv`

| Column.name | Description | Value |
|---|---|---|
| Lettercode | Lettercode of metabolite name | Cit |
| Letter_Derivate | Derivate name | Cit |
| Quant1_v4 | Quant1:1 value in (pmol) | 52050 |
| Metabolite | Library name of metabolite | Citric acid_(4TMS)_MP_RI:1814_IDENT:B+D |
| Batch_Id | Batch-Id | e18274ba |
| Dilution | Dilution factor | 0.2 |
| ChromIntensities | Corresponding peak areas | 45074572 |
| Concentration | Concentration in (pmol) | 10410 |
| Origin | Origin of quantification standard | Qstd |
| Metabolite_short | Short name of metabolite | Citric acid 275_(4TMS) |
| adj_r_squared | Adjusted Rsquare of calibration curve | 0.996053496 |
| intercept | Intercept of calibration curve | 564.549288 |
| slope | Slope of calibration curve | 0.000194064 |
| max | Max. value of calibration curve | 52050 |
| min | Min. value of calibration curve | 260.25 |

#### 15.3.2.3 `output/inc/...`

##### 15.3.2.3.1 `HeatMap_Incorporation.csv`

| | Column.name | Description | Value |
|---|---|---|---|
| 1 | Lettercode | Lettercode name of metabolite | 3PGA |
| 2 | Batch_Id | Batch-Id | e18274ba |
| 3 | Par | Parameter | NA_count |
| 4 | Val | Value of the parameter shown in heatmap | 0.740740741 |
| NA | NA | NA | NA |

##### 15.3.2.3.2 `SE_calculation_NAscore.csv`

| Column.name | Description | Value |
|---|---|---|
| Lettercode | Lettercode name of metabolite | Cit |
| Batch_Id | Batch-Id | e18274ba |
| na_frac_r | Class of NA-value | 0 |
| N | Number of MIDs | 46 |
| fracr_prop | Fraction of MIDs with x NA-values in relation to total number of evaluated MIDs | 0.851851852 |

##### 15.3.2.3.3 `SE_classification.csv`

| Column.name | Description | Value |
|---|---|---|
| Lettercode | Lettercode name of metabolite | Cit |
| Batch_Id | Batch-Id | e18274ba |
| File | File name | e18274ba_26.cdf |
| sum_spa | _expl_ | 4297 |
| sum_bpa | _expl_ | 1861 |

#### 15.3.2.3.4 `SE_validation.csv`

| Column.name | Description | Value |
|---|---|---|
| Lettercode | Lettercode name of metabolite | Cit |
| Batch_Id | Batch-Id | e18274ba |
| count_score | Evaluation of MID | goodQ |
| N_count | Number of evaluated MIDs | 54 |
| sum_files | Number of detected MIDs | 54 |

#### 15.3.2.4 MTXQCvX2 part3

#### 15.3.2.5 MTXQCvX2 part4

## 15.4 Files for Maui-processing

## 15.5 Files for Metmax-processing

# Chapter 16

# Frequently Asked Questions

## 16.1 What are additional quantification standards

## 16.2 How do I extend conversion_metabolite.csv

# Chapter 17

# Appendix - Protocols

## 17.1 Materials

## 17.2 Solutions

### 17.2.1 Label buffer

Materials:

- ddH2O (500 ml)
- 140 mM NaCl (4.1 g)
- 5 mM Hepes (0.569 g)
- pH calibration 7.4

Procedure:

- Weigh the correct amounts of Hepes and NaCl
- Resolve in a glas bottle with 450 ml of water
- Stir carefully
- Check and adjust pH
- Adjust volumes to 500 ml

### 17.2.2 MCW

Materials:

- Methanol
- Chloroform
- ddH2O
- Cinnamic acid stock in MeOH (2 mg/ml): final conc. 2 ug/ml

Procedure:

- Mix the solvents in the ratio of volumes - Methanol:Chlorofom:Water – 5:2:1
- Supplement cinnamic acid stock 1:1000
- Store at -25°C

### 17.2.3 Alkane-Mix

Materials:

- Hexane
- Alkanes: c10, c12, c15, c17, c19, c22, c28, c32, c36
- Thermo mixer
- Glass vials and caps

Procedure:

- Prepare stock solutions in hexane:
- c10 - c17 (liquid): 25 ul/ml
- c19 - c32: 20 mg/ml
- c36: two-times 15 mg/1.5 ml
- Warm up alkane stocks in thermo mixer 40°C
- Prepare a text mixture in equal amounts, e.g., 50 ul each, but use twice the volume of c36
- Mix test mixture with MSTFA: 10 ul / 1 ml MSTFA
- Check alkane profile by GC-MS
- If required: adjust volumes and re-test or create larger volume of zour mixture for aliquots
- Store aiquots in glass vials, close well and store at 4°C
- For usage: gently warm up glass vials at 30 C on thermo mixer for 10 min and vortex before adding it to the MSTFA

Adjust the volumes of the alkane stocks in order to create a curve shaped distribution of all alkanes in the chromatogram: lower intensities for c10 and c32-36, slowly increasing intensities for the alkanes in between.

## 17.3 Idents & Quant-Standards

has to be written

## 17.4 Sample Extraction

### 17.4.1 Cell extracts

Materials:

- cell culture dishes (10 cm), max. confluency 75%
- washing buffer (Hepes, NaCl, ph 7.4)
- 50% MeOH, ice-cold
- 2 mg/ml cinnamic acid
- chloroform
- 15 ml falcon tubes
- cell lifter

Procedure:

- prepare cell culture dishes accordingly to your experimental conditions
- discard cell culture media
- add quickly 5 ml of washing buffer, discard it
- add very immediately 5 ml ice-cold 50% MeOH suppl. 2 ug/ul cinnamic acid
- detach cells using cell lifter
- collect and transfer cell extract into 15 ml falcon
- store falcons until further processing on ice

- add 1 ml chloroform
- incube for 60 min at cold temperatures (4 C) on rotary or thermo shaker
- centrifuge at max speed for 10 min, cold temperatures
- collect polar and lipid phases into fresh falcons / tubes
- dry under vacuum

In order to generate technical backups:

- resuspend dried extracts in 600 ul 20% MeOH
- shake at cold temperature on thermo shaker for 30 min
- split volumes into equal parts in fresh eppendorf tubes
- dry under vacuum

Suggested cell density: $2 - 3e + 6$ cells / extract.

### 17.4.2 Tissue samples

Materials:

- Methanol:Chloroform:Water (MCW) in ratio 5:2:1
- 2 mg/mg cinnamic acid in MeOH
- ddH20
- eppendorf tubes
- tissue lyzer / pulverizer

Procedure:

- snap-freeze tissue samples
- pulverize samples
- aliquote 50 mg of tissue powder
- add 1.5 ml of MCW (suppl. with cinnamic acid final conc. 2 ug/ul)
- shake for 60 min on rotary shaker at cold temperature (4 C)
- add 0.5 ml ddH20 for phase separation
- centrifuge maximum speed, 10 min, cold temperatures
- collect polar and lipid phases in fresh vessels
- dry under vacuum

### 17.4.3 Blood samples

Material:

- Methanol:Chloroform:Water (MCW) in ratio 5:2:1
- 2 mg/mg cinnamic acid in MeOH
- ddH20
- eppendorf tubes

Procedure:

- give 20 ul blood / sera directly into 1 ml MCW to avoid lumps
- in case of lumps sonicate samples
- shake samples at 4 C for 800 rpm for 60 min
- add 500 ul ddH20 and vortex shortly
- spin down at 4 C at max speed for 10 min
- aliquote polar phase into 2-3 times 500 ul in 1.5 ml tubes
- aliquote lipid phase 2 times in 100 ul lower in 1.5 ml eppi
- dry in SpeedVac (35 C)

## 17.5 Derivatisation for GC-MS

Materials:

- Methoxamine (MeOx)
- Pyridine (open under the hood only!)
- MSTFA
- Alkane mix (c10-c36) in Hexane
- chromacol vials and caps (big, small)
- samples: extracted and speed-vac dry for min 30 min prior procedure
- quant-standards: extracted and speed-vac dry
- ident-standards: extraction not required, speed-vac dry

Mixtures:

- Solvent 1: 40 mg MeOx in 1 ml Pyridine
- Solvent 2: 10 ul Alkane mix in 1 ml MSTFA

Volumens of both solvents are shown for standard (small vol.) procedures.

Procedure:

- make sure samples are completly dry (1 h speed vac)
- add 20 ul (10 ul) of solvent 1 / sample
- incubate on rotary shaker, 30 C, for 60 min
- add 80 ul (25 ul) of solvent 2 / sample
- incubaate on rotary shaker, 37 C, for 90 min
- centrifuge to spin down insoluble materials
- prepare aliquotes three times 28 ul or two times 15 ul (small glass vials)
- keep on room temperature until measurement (max. 10 days)

## 17.6 GC-MS settings

In the following paragraphs details of GC-MS settings are described in detail. The herein described settings have been optimized for cell extracts measured in split-mode 1:5 on the instrument Pegasus 4D-C GC-ToF-MS in 1D mode equiped with an autosampler Gerstel MPS.

# Chapter 18

# pSIRM experiments

The application of stable isotopes provides a powerful tool to track the activity of metabolic pathways. the time-dependent and atom-specific routing along a metabolic pathway resolved how substrates like glucose or glutamine are used in order to maintain a certain phenotype and energetic homeostatsis.

We developed an approach called pulsed stable isotope resolved metabolomics (pSIRM) enabling the quantitative evaluation of metabolite pool sizes and incorporation of stable isotopes, e.g., $^{13}C_6$-glucose. A thoughtful setup of the experimental design including the applied substrates and carefull experimental handling are prerequisites for a successful pSIRM experiment. Essential aspects are collected in the below paragraphs along with a number of usefull tweaks.

## 18.1 Experimental design

An *in vitro* pSIRM experiment lasts in total up to three days starting from the cell seeding at day zero. Further along the way up to two media changes should be included until the application of stable isotopes and harvesting the cells maintaining the continouse availability of nutrients and avoiding the accumulation of waste products (Figure **??**fig:psirm)). The media change four hours prior the harvest is set up in order to give cells time to recover from the mechanical stress of the media change. At the time point of harvest cells should be in a perfect happily state regarding metabolic environment and stress.

Choose carefully the *seeding density of you cells* in the first place. High confluency inducing contact inhibition of cell growth has a strong impact on several cellular processes including the uptake of nutrients. Try to aim for petri dishes with a maximum confluency of 75-80 %. A pre-experiment including different cell densities for seeding at a number of experimental conditions helps you to get a feeling for the cell growth in general and an expected output of cells at the time point of the harvest. Later one is useful to plan sample extraction and measurement subsequently.

For adherent cell cultures only: Include for each experimental condition an additional petri dish that is solely used to determine the cell count at the time point of your harvest. This additional plate ensures a correct determination of absolute quantities and might reduce variation of pool sizes in the statistical analysis[1]. Think carefully about control conditions and include cell culture dishes that are not labeled. These dishes function as a control for your labeling procedure and the natural abundance of isotopes.

[1] Pelleting these cells and snap-freezing might give usefull additional samples for western blotting.

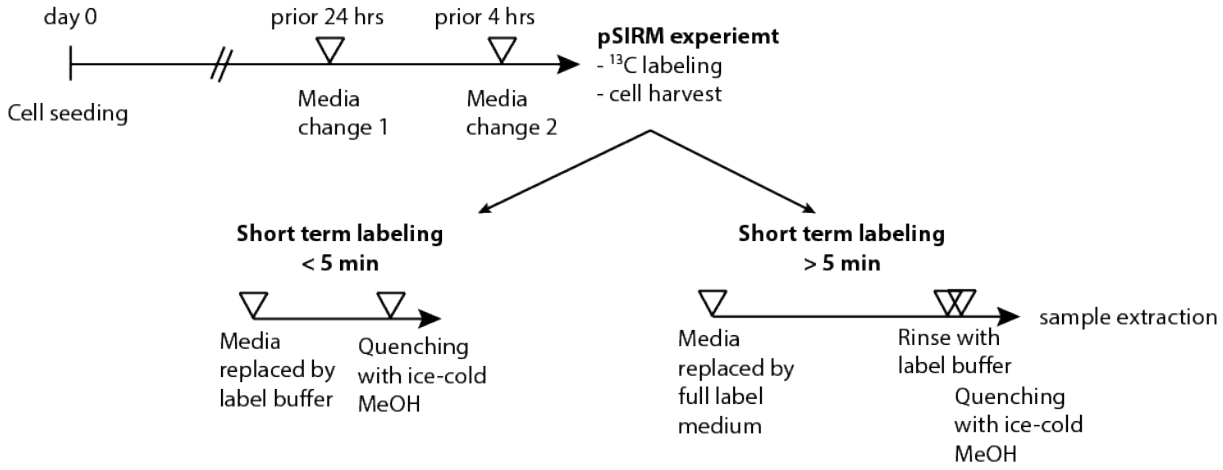


Figure 18.1: Experimental design of a pSIRM experiment distuingishing short and long labeling with stable isotopes.

Table 18.1: Solvent composition of Full label medium (LM) and label buffer (BF) for a pSIRM experiment labeling with 13C-glucose.

| Solvent | Base | Carbon.source | Supplem |
|---|---|---|---|
| Full label medium (LM) | DMEM, without glucose, glutamine, pyruvate | 13C-Glc (2.5 g/L) 12C-Gln (2 mM) | small mo |
| Label buffer (LB) | HEPES (5 mM), NaCl (140 mM), pH 7.4 | 13C-Glc (2.5 g/L) 12C-Gln (2 mM) | |

## 18.2 Experimental procedures

There is a slight difference in the protocols for adherent and suspension cells. Please read instructions and footnotes carefully.

Short[2] and long term labeling procured differ only in the applied solvents during the labeling - either label buffer (LB) only or a combination of full label medium (LM) and label buffer (LB). Latter one is applied in order to remove extracellular metabolites of the media. Both label buffer and media contain the major nutrients / stable isotopes to keep the main substrates at constant supply at all times (Table 18.1). During the application of stable isotopes longer than a few minutes cells might sense the absence of further intermediates provided during standard cell culture procedure and and adjust their metabolic program accordingly.

The quality of your data later heavily relies on the exact handling of the cells and a *consistent timing* throughout the pSIRM experiment. Especially the step removing the LB and quenching the cells should be a matter of a tenth of seconds rather than seconds. It is of great value to perform the cell harvest with a second person.

## 18.3 Protocol pSIRM

### 18.3.1 Adherent cell cultures

The herein described protocols are detailed explanations how to perform a pSIRM cell harvest for long term label application. If you want to label for less than 2 minutes omit solely *omit steps 6-8*.

Materials:

[2]I would rather recommend this up to 2 min of labeling

- Cell culture dishes, max. confluency 80 %
- Labeling media (LM) supplemented with substrates (5 ml / dish)[3]
- Label buffer (LB) supplemented with substrates (5 ml / dish)
- Ice-cold 50 % MeOH supplemented with 2 ug/ul cinnamic acid
- 2x 5 ml pipette and tips[4]
- Beaker
- Ice
- 15 ml falcons (chloroform resistant)
- Cell lifter
- Biological waste bin next to your bench

Procedure:

1. Pre-warm LB and LM in the water bath
2. Take a number of petri dishes (condition-wise including all biol. replicates)
3. Discard cell culture media
4. Carefully add *long term labeling* LM OR *short term labeling*: LB
5. Incubate cells on the bench or in an incubator
6. Discard LM (beaker)
7. Add immediatly 5 ml of LB
8. Rotate dish once in order to cover complete surface
9. Meanwhile 2nd person get prepared with 5 ml ice-cold MeOH
10. Discard LB into beaker and *immediatly* 2nd person quenches with ice-cold MeOH
11. Collect cell extracts using cell lifter
12. Transfer cell extracts into 15 ml falcons
13. Store falcons on ice until further processing

Repeat this procedure (step 6-10) for all dishes of a single condition first. Once MeOH is added metabolic processes are interrupted and cell extracts can be collected with the help of cell lifter without rush and subsequently transfered to 15 ml falcon and stored on ice until further processing (see chapter Cell extraction methanolic extracts).

Determine the cell count using your additional petri dishes for each condition.

### 18.3.2 Supension cell cultures

Materials:

- Cell culture flasks
- Labeling media (LM) supplemented with substrates
- 5 ml pipette and tips[5]
- 1 ml pipette
- Beaker
- paper tissues
- Liquid nitrogen
- 15 ml falcons
- 1.5 ml eppendorf tubes
- Biological waste bin next to your bench

Procedure:

1. Pre-warm LM in the water bath
2. Determine the cell count of your cell suspension(s)

[3]Not required for short term labeling
[4]Highly recommended, makes labeling and harvest super quick
[5]Highly recommended, makes labeling and harvest super quick

3. Take aliquots of $10 - 15e + 6$ cells and transfer into 15 ml falcon
4. Spin down cells very gently 300 g, 2 min at room temperature
5. Discard media into beaker
6. Resuspend cells gently in three-times 1 ml[6]
7. Incubate and keep warm
8. Fractionate cell supension in three eppendorf tubes (3x 1 ml each)
9. Spin down quickly in top-bench centrifuge[7]
10. Discard media blandtly on paper tissues
11. Snap-freeze immediatly in liquid nitrogen
12. Store cells until further processing (see chapter Cell extraction suspension cells)

The important step here to be quick is the alquotation of the cell suspension and subsequent spin down in the table centrifuge. Suspension cells are rather small, nevertheless $3e + 6$ cells per extract are a good starting point for GC-MS measurements.

## 18.4 Hints & notes

- The only way to be reproducible and fast is to team up with a second person.
- Keep timing consistently through the experiment.
- Keep substrate concentrations constant throughout the experiment in all solutions.
- Supplement one stable isotopic labeled substrate with all remaining substrates in non-labeled form.
- Think about nutrient levels in your cell culture and your experimental conditions. Maybe you want to change things to physiological levels.
- Add additional plate to each condition in order to have material for western blotting and others.
- Check carefully the confluency of your dishes and determine seeding densities for different conditions.
- In case of small molecule inhibitors: Try to avoid to solve them in DMSO - strong impact on chromatography.

[6] To generate three replicates
[7] Most of the times 30 s are already enough

# Chapter 19

# GC-MS settings

In the following paragraphs details of GC-MS settings are described in detail. The herein described settings have been optimized for cell extracts measured in split-mode 1:5 on the instrument Pegasus 4D-C GC-ToF-MS in 1D mode equiped with an autosampler Gerstel MPS.

### 19.0.1 Autosampler settings

The table **??** summarises the defined parameter for method settings of the Gerstel MPS.

The injector itself is a temperature-regulated system providing the advantage of a focused injection of the sample. Gradient

### 19.0.2 Gas chromatography

The most important settings of the GC method are listed below. A graphical representation of the gradient in the oven is shown in figure 19.2 and corresponding values in table 19.1.

Flow path:

1. Inlet: Front
2. Capillary: GC Oven 50 m, 250 u int. diameter, 0.25 u film thickness, RTX-5 phase
3. Capillarty: Detector 0.21 m, 250 u int. diameter, 0.25 u film thickness, RTX-5 phase
4. Detector: TOF

### 19.0.3 Mass spectrometer settings

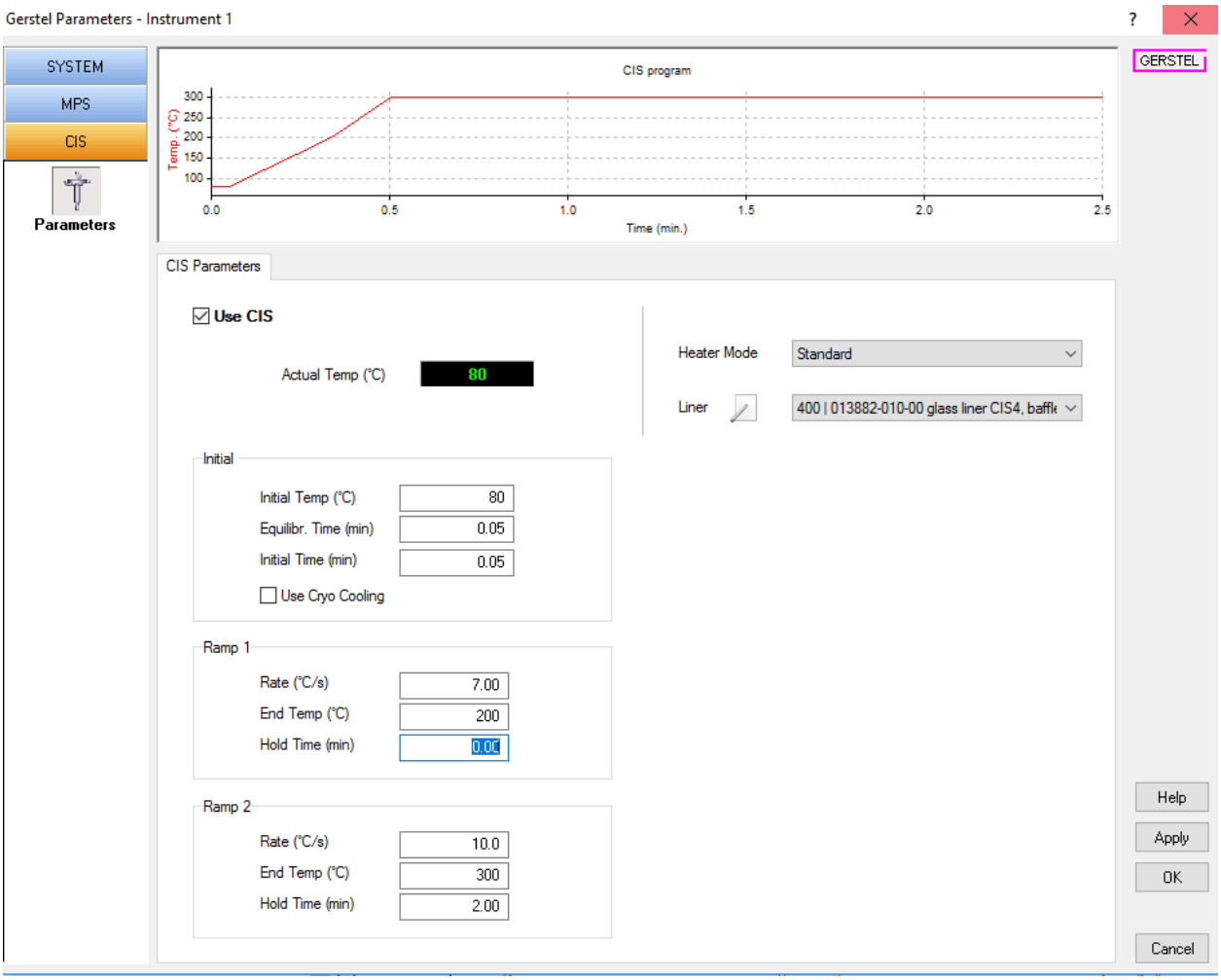


Figure 19.1: Settings temperature-regulated injection for Gerstel MPS

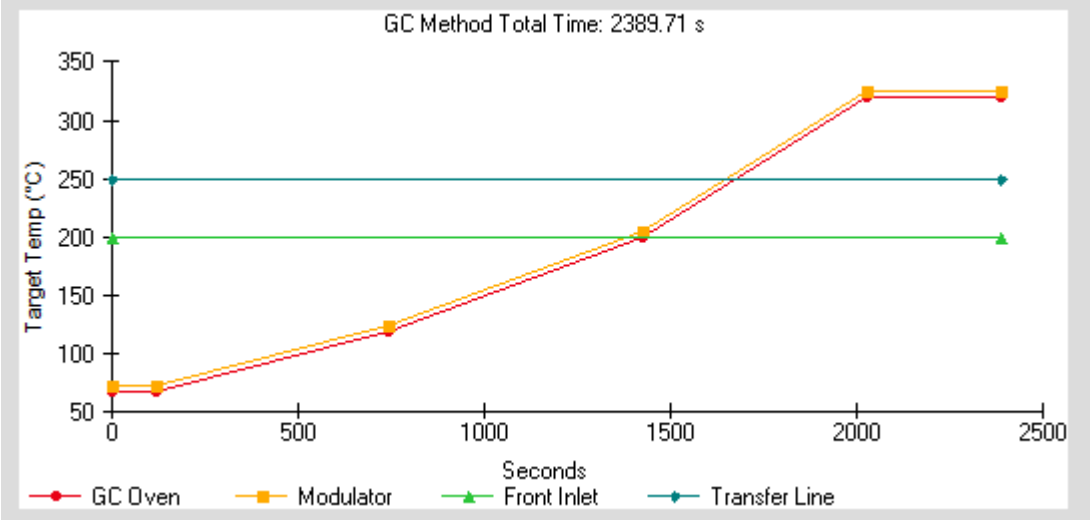


Figure 19.2: GC gradient - graphical representation. Rate in (°C/min), Target temperature in (°C), Duratin in (min).

Table 19.1: GC gradient profile - Rate shown in (K/min), Target Temp in (C), Duration in (min)

| Rate | Target_Temp | Duration |
|---|---|---|
| Initial | 68 | 2 |
| 5 | 120 | 0 |
| 7 | 200 | 0 |
| 12 | 320 | 6 |

# Chapter 20

# Solutions and Materials

## 20.1 Solutions

### 20.1.1 Label buffer

Materials:

- ddH2O (500 ml)
- 140 mM NaCl (4.1 g)
- 5 mM Hepes (0.569 g)
- pH calibration 7.4

Procedure:

- Weigh the correct amounts of Hepes and NaCl
- Resolve in a glas bottle with 450 ml of water
- Stir carefully
- Check and adjust pH
- Adjust volumes to 500 ml

### 20.1.2 MCW

Materials:

- Methanol
- Chloroform
- ddH2O
- Cinnamic acid stock in MeOH (2 mg/ml): final conc. 2 ug/ml

Procedure:

- Mix the solvents in the ratio of volumes - Methanol:Chlorofom:Water – 5:2:1
- Supplement cinnamic acid stock 1:1000
- Store at -25°C

### 20.1.3 Alkane-Mix

Materials:

Table 20.1: List of chemicals including company names and catalog number.

| Chemical | Catalog.Nb | Company | Storage |
|---|---|---|---|
| Chloroform | 132950-1L | Sigma | RT |
| Cinnamic acid | C80857 5g | Aldrich | RT |
| Hexane for HPLC > 97\% (GC) | 34859-1L | Sigma | RT |
| Methanol | 1060351000 | Merck | RT |
| Methoxyamine hydrochloride | 226904-5G | Aldrich | RT |
| MSTFA - Methyltrimethylsilyltrifluoracetamide | 701270201 | Macherey-Nagel | 4C |
| Pyridine | 270970-100ML | Sigma | RT |

- Hexane
- Alkanes: c10, c12, c15, c17, c19, c22, c28, c32, c36
- Thermo mixer
- Glass vials and caps

Procedure:

- Prepare stock solutions in hexane:
- c10 - c17 (liquid): 25 ul/ml
- c19 - c32: 20 mg/ml
- c36: two-times 15 mg/1.5 ml
- Warm up alkane stocks in thermo mixer 40°C
- Prepare a text mixture in equal amounts, e.g., 50 ul each, but use twice the volume of c36
- Mix test mixture with MSTFA: 10 ul / 1 ml MSTFA
- Check alkane profile by GC-MS
- If required: adjust volumes and re-test or create larger volume of zour mixture for aliquots
- Store aiquots in glass vials, close well and store at 4°C
- For usage: gently warm up glass vials at 30 C on thermo mixer for 10 min and vortex before adding it to the MSTFA

Adjust the volumes of the alkane stocks in order to create a curve shaped distribution of all alkanes in the chromatogram: lower intensities for c10 and c32-36, slowly increasing intensities for the alkanes in between.

## 20.2 Materials

# Bibliography

Pietzke, M., Zasada, C., Mudrich, S., and Kempa, S. (2014). Decoding the dynamics of cellular metabolism and the action of 3-bromopyruvate and 2-deoxyglucose using pulsed stable isotope-resolved metabolomics. *Cancer & metabolism*, 2(1):9.

Zasada, C. (2017). Experimental and mathematical analysis of the central carbon metabolism in cancer and stem cells. *Humboldt University*.