MTXQCvX2 documentation

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Contents

Chapter 1

Welcome

Let's get started .... this part is going to be written in the proximate 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 instructions in chapter @**??**maui) and @**??**metmax).

Subsequently each MTXQCvX2 module (chapter 6 - 10) is introduced in greater detail including a input / output files and processing parameter.

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 including further substances. How to do so and what files can be customizes is shown in chapter **??**.

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 (chapter14) 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 16 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 17.6).

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.

Chapter 3

Tutorial: Maui-annotation projects

3.1   Read this in case

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

Copy the dataset `tutorial_single_maui` somewhere local on your computer or laptop. The dataset represents the data of the first psirm metabolomics experiment in the Kempa Lab. HEK293 cells have been cultivated at 21% oxygen and a range of glucose levels (0.3 - 4.5 g/L Glc) and constant glutamine concentration (4 mM) following standard cell culture procedures. During the cell harvest cells have been labeled for two minutes with uniformly labeled 13C-glucose, three replicates per condition.

3.4   In detail

3.4.1   Setup 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 here
- Open and extract the zip-file
- Copy all folders and files into your newly created R-project 'tutorial_single_maui'

3.4.3   Process `MTXQC_init.Rmd`

The module `MTXQC_init.Rmd` provides you two important things: (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
- 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 if you see the new folder and browse through it.

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 copied into the corresponding subfolders of the `input`-folder.

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`.

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

The *sample extracts* file specify the nature and volume / weight of the sample extracts. The columns **Extract_vol** and **Unit** are mandatory here. This file here 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

We have to define the internal extraction standard for each experiment in the file `config_mtx/conversion_metabolite.csv`. Classical pSIRM experiments like the one we are using for this tutorial used cinnamic acid for tracking variations in experimental sample handling.

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

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[1]

Define the following parameter:

- Define project-folder: psirm_glucose
- Define input-format: maui
- 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

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

- Quantification standards: Quant1_v3
- Volume polar phase of Quants: 500
- Technical replicates[2]: 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 (1) successfull data imports, (2) fatal errors causing an automatic stop of data processing and (3) warning 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 document 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[3]

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

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 in this file.

`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 any processing of the data in the sense of analysis or statistics.[4]

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.

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[2]Also called backups
[3]Explained in detail in chapter 9 and section **??**
[4]Quality metrices are a different pair of shoe here and are not data per se

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
- Tpye of analysis: stringent
- Internal standard: QC[7]
- Quantitative value: InstStd_Conc_pmio[8]
- par1: time
- par2: substrate
- par3: whatelse

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. 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.

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[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)

Chapter 4

Workflow for Metmax-extracted projects

4.1 You want to follow this ...

- in case you have measured samples and quantification standards by GC-MS
- performed the annotation of intermediates in ChromaToF or vendor software
- exported all information into `.txt` files
- used metmax to extract peak areas / mass isotopomer distributions (MIDs)

4.2 Introduction

This document describes how to use MTXQCvX2 in combination with metmax[1].

Historically, MTXQCvX2 has been developed and optimized for Maui-derived input files. The `MTXQCvX2-part4.Rmd` functions as a converter of metmax-derived files in order to create suitable input formats for `MTXQCvX-part1.Rmd`.

This module could also be used to convert tables derived from other programs as long as they are stick with the herein described table formats. Mandatory columns are referenced in the text for each kind of input file.

The general workflow of the NMTXQCvX2 project is briefly shown below in quick view. More detailed instructions are summarised in the following paragraphs.

For more detailed explanations about the individual input parameter for each module of MTXQCvX2 please proceed to read the documentation about the individual modules and their knitting parameter. The relation of knitting parameter, input and output files are described in each section.

4.3 Quick view

1. Generate input files: run `MTXQC_part4.Rmd`[2]
2. Setup R-project and copy MTXQC-files
3. Knit with parameter: `MTXQC_init.Rmd`
4. Copy input files into corresponding folders

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[1]http://gmd.mpimp-golm.mpg.de/apps/metmax/default.htm
[2]read here the instructions

5. Create annotation.csv and sample_extracts.csv files[3]
6. Update metabolite names in `conversion_metabolite.csv`[4]
7. Define the internal standard and/or alkanes[5]
8. Knit with parameter: `MTXQC_ExperimentalSetup.Rmd`
9. Knit with parameter: `MTXQC_part1.Rmd`
10. Knit with parameter: `MTXQC_part2.Rmd`
11. If required - proceed with `MTXQC_part3.Rmd` for ManualValidation

4.4   Input files

If you need an introduction about how to use metmax - have a look at the separate documentation `Metmax_intro`.

The chapter **??** `MTXQCvX_part4` explains in detail how to use this module to generate suitable input files.

4.5   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 all file names from a file of your choice
2. Paste & transpose the content into a new Excel-File into column A
3. Call column A -> File
4. Optional: Remove any non-file name entry in this column
5. Add the column Type and specify each file either as **sample**, **Q1_diluation**, **,addQ1_dilution**[6]
6. Add more columns specifying your experimental conditions, e.g., Cellline and Treatment[7]
7. Save the content as `csv-file` in the `psirm_glucose/input/...`

4.6   Sample_extracts-file

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**[8]. 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/...`.

If the defined experimental conditions do not match up with the annotation `MTXQCvX2_part1.Rmd` exit data processing. A template file is saved for review and usage at `inst/template_files/...`.

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[3]Details further down this document

[4]Column: Metabolite_manual

[5]Also in conversion_metabolite.csv; see below paragraph Standards

[6]see for further details additionalQuant

[7]optimal: two-three parameter, max: four parameter. Consider possible combinations, e.g., HCT116-control, HCT116-BPTES

[8]Define: count, mg or ul

4.7 Update metabolite names in `conversion_metabolite.csv`

The file `conversion_metabolite.csv`, saved in `config_mtx/`, serves as a kind of translational table. It defines alternative version of metabolite library names that come in handy to plot data using shorter metabolite names. This file is also used to define settings and standard classifications. Detailed information for each column of the file are shown here: REF

4.7.1 Match your annotation with library names

Prior the analysis you need to match the names of your intermediates with the conversion_metabolite.csv file. You need to update or add the corresponding name for each intermediate in the column **Metabolite_manual**.

General suggestion for naming conventions in ChromaToF: Metabolite_Derivate, e.g., Lactic acid_(2TMS). In case of the presence of main- (MP) and byproducts (BP) use: Metabolite_Derivate_MP/BP, e.g., Glucose_(1MEOX)(5TMS)_MP.

If you have annotated intermediates that are not included so far in this table please follow the instructions how to extend `conversion_metabolite.csv`.REF

4.7.2 Define your internal standards and alkanes

MTXQCvX2 allows the specification of project-specific internal standards. Corresponding compounds have to be marked as an internal standard in `conversion_metabolite.csv` by adding the tag **InternalStandard** in the column Standard.

If you check the box - InternalStandard in the parameter selection for `MTXQCvX2_part4.Rmd` the module searches in your input file for peak areas of the defined standard and extracts the information. It also generates the file `InternalStandard.csv` and stores it at `psirm_glucose/input/gc/...`.

In the same way alkanes are defined in `conversion_metabolite.csv`. Each alkane has to be flag tagged with **Alk** in the column Standard. This gives you the opportunity to implement customized mixtures of alkanes in order to determine the retention index. `MTXQCvX_part4.Rmd` recognises the flag tag and generates `Alcane_intensities.csv` based on your input file containing peak areas and saves it in `psirm_glucose/input/gc/...`[9].

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

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[9]It should be al**k**ane, I know, but Maui doesn't, unfortunately…

Chapter 5

MTXQCvX2_init

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

```
## 'data.frame':    10 obs. of  3 variables:
##  $ Folder        : Factor w/ 4 levels "","figure","input",..: 2 3 1 1 1 1 4 1 1 1
##  $ subfolder     : Factor w/ 7 levels "","add_quant",..: 1 2 4 5 7 6 4 5 7 3
##  $ Used.in.module: Factor w/ 5 levels "Figure export of MTXQC_part1",..: 1 2 2 2 2 3 4 4 4 5
```

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

Chapter 7

MTXQCvX_part1.Rmd

Chapter 8

MTXQCvX_part2.Rmd

Chapter 9

MTXQCvX_part3.Rmd

Chapter 10

MTXQCvX_part4.Rmd - Metmax parser

10.1 This section explains ...

- what MTXQCvX_part4.Rmd does
- how do input files need to look like
- which files are generated
- what the distinct checkboxes mean

This module provides the generation of suitable input files for MTXQCvX2 based on spreadsheet exported information by tools like metmax.

10.2 Input files

10.2.1 Quantification - PeakAreas.csv

In order to perform absolute quantification of

You need a file containing all extracted peak areas for each metabolite and file[1]. 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[2] or using statistical compare. pSIRM experiments require the definition of pTop5 masses[3] instead of top5 masses in the reference in order to take into account the shift of intensities induced by the application of stable isotopes[4]

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

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[1]Tools/Options/Retention analysis, Parameter: Area
[2]See `vignette/ReferenceSearch`
[3]Extended list of quant masses considering isotope incorporation
[4]Mandatory columns: name, mass, files

MTXQCvX_part4 takes care of the formatting and correct column names of the peak areas file and saves it[5]. MTXQCvX_part4 generates also the file PeakDensities-Chroma.csv[6], in case you have selected the option to include sum of area normalisation while knitting this module.

10.2.2   Isotope incorporation - MIDs.csv[7]

In order to determine the incorporation of stable isotopes MTXQCvX2 requires as an input the mass isotopomer distributions (MIDs) for each intermediate and measurement[8]. Fragments for each intermediate have to be pre-defined in metmax at Tools/Options/metabolite masses. They can be imported[9] or manually specified each by each. 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[10].

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

MTXQCvX_part4 calculates the stable isotope incorporation and exports DataMatrix.csv as well as pSIRM_SpectraData.csv[11]. 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[12]. Please refer for more details to `vignettes/config_mtx-files`.

10.2.3   Derivatisation efficiency - mz73.csv[13]

The extraction of intensities for the ion m/z 73 works analogous to the extraction of MIDs[14]. Mass ranges have to be defined for each intermediate for the mass 73 by defining starting and end mass with 73. MTXQCvX_part4 generates the file MassSum-73.csv[15]. Check `inst\template_files\` for reference. Hopefully soon a new metmax button extracting specific intensities across the batch.

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[5] `input/quant/quantMassAreasMatrix.csv`
[6] `input/gc/PeakDensities-Chroma.csv`
[7] Required for `calculation isotope incorporation`
[8] Tools/Options/Isotope concentrator; Parameter: IntensityOfMass
[9] `inst/template_files/MetMax_MIDs.txt`
[10] Mandatory columns: name, mass, files
[11] `input/inc/DataMatrix` & `pSIRM_SpectraData.csv`
[12] `config_mtx/incorpo_calc_masses.csv`
[13] Required for: `sum of area normalisation`
[14] Tools/Options/Isotope concentrator; Parameter: IntensityOfMass
[15] `input/gc/MassSum-73.csv`

Chapter 11

Configuration of MTXQCvX2 - config_mtx/...

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` & `mid_backups.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

Column.name	Description	Value
Metabolite	Library name of metabolite	Alanine_ beta-_(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

12.1 Processing In ChromaToF

Create a new folder in ChromaToF Pegasus Acquired Samples and import your files. The processing of files for Maui-assisted annotation is a two step process. Therefore two data processing methods have to be set up and applied to all files.

12.1.1 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 `Export of ....` Subsequently, you are asked to define an output folder and the following paramter:

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

12.1.2 Combo-export (`.cdf` & `.csv`)

Re-import the generated `.peg`-files into a subfolder and apply the following data processing method.

Activate the box `asddasd` and define for both file types the following parameter.

`.cdf`-file:

- export directory

-

`.csv`-file:

- export directory

-

12.2   Maui pSIRM workflow

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

Chapter 13

Data Processing - Metmax

13.1   Resampling

13.2   1D-basic

13.3   Reference search

13.4   Export for Metmax

13.5   Data extraction with Metmax

13.5.1   Peak areas

13.5.2   MIDs

Chapter 14

Frequently Asked Questions

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**[1] 6. Add more columns specifying your experimental conditions, e.g., Cellline and Treatment[2] 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**[3]. 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/...`.

If the defined experimental conditions do not match up with the annotation `MTXQCvX2_part1.Rmd` exit data processing. A template file is saved for review and usage at `inst/template_files/...`

---

[1]see for further details additionalQuant

[2]optimal: two to three parameter, at maimumx: four parameter. Consider combinations of parameter, e.g., HCT116-control, HCT116-BPTES

[3]Define: count, mg or ul

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[4] 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[5]. 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`[6] 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`[7] 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`
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

---

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

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

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 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_mtx` tables

15.1.1.1 conv_filenames.csv

```
## 'data.frame':    10 obs. of  2 variables:
##  $ AssociatedFile: Factor w/ 10 levels "addQ","alkane_int",..: 4 2 7 8 10 9 5 1 6 3
##  $ Filename      : Factor w/ 10 levels " incorp_calc_masses.csv",..: 6 4 7 8 10 9 5 3 1 2
```

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

```
## 'data.frame':    3 obs. of  2 variables:
##  $ AssociatedFile: Factor w/ 3 levels "inc","pSIRM_se",..: 3 2 1
##  $ Filename      : Factor w/ 3 levels "DataMatrix_manVal.csv",..: 3 2 1
```

	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

15.1.1.3 DEPRICATED FILE: MQ_correction.csv

Not in use anymore since fluffy adventure!

```
## 'data.frame':    7 obs. of  3 variables:
## $ Metabolite : Factor w/ 7 levels "Alanine_(2TMS)_BP_RI:1097_IDENT:B+C",..: 1 2 3 6 7 4 5
##  $ Cor_factor  : num  12 12 6.33 11.82 2.41 ...
##  $ Target_value: num  134695 134695 83259 388544 65154 ...
```

Metabolite	Cor_factor	Target_value
Alanine_(2TMS)_BP_RI:1097_I	12.00	134695.25
Alanine_(3TMS)_MP_RI:1367_I	12.00	134695.25
Fructose_(1MEOX)(5TMS)_BP_	6.33	83259.33
Glucose_(1MEOX)(5TMS)_MP_	11.82	388543.52
Glycerol_(3TMS)_MP_RI:1280_	2.41	65153.65
Fructose_(1MEOX)(5TMS)_MP_	6.33	83259.33
Glucose_(1MEOX)(5TMS)_BP_]	11.82	388543.52

15.1.2 config_files tables

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

15.1.2.1 conversion_metabolite.csv

```
## 'data.frame':    10 obs. of  3 variables:
##  $ Column.name: Factor w/ 10 levels "Lettercode","Mass_Pos",..: 4 3 5 1 8 2 9 7 6 10
##  $ Description: Factor w/ 10 levels "Checked if quant1:1 value available",..: 9 7 10 6 1 8 4 3 5 2
##  $ Value      : Factor w/ 8 levels "","#Alanine (2TMS)",..: 2 6 5 4 8 3 8 8 1 7
```

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

```
## 'data.frame':    100 obs. of  7 variables:
## $ Letter_Derivate: Factor w/ 69 levels "2HG","2OG","3PGA",..: 1 2 3 4 5 5 6 6 7 8 ...
## $ Lettercode     : Factor w/ 100 levels "2HG","2OG","3PGA",..: 1 2 3 4 6 5 8 7 9 10 ...
## $ Pathway        : Factor w/ 9 levels "aa","glut","glyc",..: 2 9 3 4 5 5 1 1 1 1 ...
## $ Pathway.1      : Factor w/ 9 levels "1-glyc","2-tca",..: 3 2 1 7 9 9 5 5 5 5 ...
## $ Met_pathway    : Factor w/ 97 levels "1-glyc_DHAP_BP",..: 28 21 17 91 96 96 35 34 36 37 ...
## $ Subs_class     : Factor w/ 11 levels "aa","amino sugar",..: 6 6 8 3 4 4 1 1 1 1 ...
## $ Met_class      : Factor w/ 97 levels "aa_Ala_2TMS",..: 55 56 83 43 46 46 2 1 3 4 ...
```

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

```
## 'data.frame':    90 obs. of  3 variables:
## $ Metabolite: Factor w/ 45 levels "Alanine_(2TMS)_BP_RI:1097_IDENT:B+C",..: 1 1 2 2 3 3 4 4 5 5 ...
## $ Mass_mz   : int  116 118 188 190 245 249 273 275 273 276 ...
## $ LI_MID    : Factor w/ 2 levels "m0","minc": 1 2 1 2 1 2 1 2 1 2 ...
```

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

```
## 'data.frame':    70 obs. of  3 variables:
##  $ Letter_Derivate: Factor w/ 70 levels "2HG","2OG","3PGA",..: 1 2 3 4 5 6 7 8 9 10 ...
##  $ Quant1_v4      : int  57270 34220 43480 7400 18710 134700 11480 22710 15030 11220 ...
##  $ Quant1_v3      : int  57270 34220 43480 7400 18710 134700 11480 22710 15030 11220 ...
```

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

```
## 'data.frame':    224 obs. of  4 variables:
##  $ Metabolite   : Factor w/ 38 levels "Alanine_ beta-_(3TMS)_MP_RI:1435_IDENT:A+D",..: 1 1 1 1 2 2 2 2 3 3
##  $ Mass.m.z.    : int  188 189 190 191 116 117 118 119 188 189 ...
##  $ BackupPeakArea: int  4960 876 307 53 2616179 323019 99834 19759 4960 876 ...
##  $ BackupMID    : num  0.8005 0.1414 0.0495 0.0086 0.8553 ...
```

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_R	188	4960	0.8005000
Alanine_(3TMS)_MP_R	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`.

```
## 'data.frame':    3 obs. of  3 variables:
##  $ Column.name: Factor w/ 3 levels "Batch_Id","qc_metric",..: 1 2 3
##  $ Description: Factor w/ 3 levels "Batch-Id","Class of QC metric",..: 1 3 2
##  $ Value      : Factor w/ 3 levels "0.937254457",..: 3 1 2
```

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

```
## 'data.frame':    3 obs. of  3 variables:
##  $ Column.name: Factor w/ 3 levels "Batch_Id","qc_metric",..: 1 2 3
##  $ Description: Factor w/ 3 levels "Batch-Id","Class of QC metric",..: 1 3 2
##  $ Value      : Factor w/ 3 levels "0.937254457",..: 3 1 2
```

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`

```
## 'data.frame':    5 obs. of  3 variables:
##  $ Column.name: Factor w/ 5 levels "Batch_Id","File",..: 2 5 1 4 3
##  $ Description: Factor w/ 5 levels "Bacth-Id","Determined normalisation factor",..: 4 5 1 2 3
##  $ Value      : Factor w/ 5 levels "0.837457514",..: 4 2 3 1 5
```

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

```
## 'data.frame':    8 obs. of  3 variables:
##  $ Column.name: Factor w/ 8 levels "Batch_Id","File",..: 2 7 1 4 3 6 5 8
##  $ Description: Factor w/ 8 levels "Batch-Id","Evaluation regarding QC",..: 3 7 1 5 2 6 4 8
##  $ Value      : Factor w/ 8 levels "0.837457514",..: 7 5 6 1 8 4 2 3
```

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

```
## 'data.frame':    9 obs. of  3 variables:
##  $ Column.name: Factor w/ 9 levels "area_fac","Batch_Id",..: 3 2 6 9 7 4 8 1 5
##  $ Description: Factor w/ 9 levels "Extracted Batch-Id derived from file name",..: 2 1 6 8 9 3 7 4 5
##  $ Value      : Factor w/ 9 levels "1.296568521",..: 9 8 2 6 3 5 4 1 7
```

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

```
## 'data.frame':    2 obs. of  3 variables:
##  $ Column.name: Factor w/ 2 levels "Batch_Id","n_50": 1 2
##  $ Description: Factor w/ 2 levels "Batch-Id","Number corresponding to fifty percent of the maximum number
##  $ Value      : Factor w/ 2 levels "53.5","e18274ba": 2 1
```

	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`

```
## 'data.frame':    7 obs. of  3 variables:
##  $ Column.name: Factor w/ 7 levels "Batch_Id","File",..: 2 1 3 6 4 7 5
##  $ Description: Factor w/ 7 levels "Batch-ID","File name",..: 2 1 3 6 4 7 5
##  $ Value      : Factor w/ 7 levels "0.002407244",..: 7 6 3 2 4 5 1
```

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`

```
## 'data.frame':    21 obs. of  3 variables:
##  $ Column.name: Factor w/ 21 levels "adj_r_squared",..: 9 2 4 17 14 21 10 11 7 16 ...
##  $ Description: Factor w/ 21 levels "","Adjusted Rsquare value of linear regression of the calibration cur
##  $ Value      : Factor w/ 18 levels "","-898.3400476",..: 11 12 13 6 7 16 3 10 9 17 ...
```

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`.

```
## 'data.frame':    43 obs. of  4 variables:
##  $ Column.name: Factor w/ 43 levels "absconc","adj_r_squared",..: 4 6 9 13 36 41 42 12 43 23 ...
##  $ Class      : Factor w/ 9 levels "AnnExp","AnnExtract",..: 1 1 1 1 1 1 1 1 2 2 3 ...
##  $ Description: Factor w/ 41 levels "Absolute quantity in pmol",..: 3 14 14 16 6 14 40 7 9 18 ...
##  $ Value      : Factor w/ 37 levels "","#Glycerol-3-phosphate (4TMS)",..: 27 24 25 28 1 3 34 18 26 29 ...
```

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`

```
## 'data.frame':    5 obs. of  3 variables:
##  $ Column.name: Factor w/ 5 levels "Batch_Id","Lettercode",..: 2 1 3 4 5
##  $ Description: Factor w/ 5 levels "Batch-Id","Library name of metabolite",..: 4 1 2 3 5
##  $ Value      : Factor w/ 5 levels "0.996053496",..: 2 4 3 5 1
```

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`

```
## 'data.frame':    7 obs. of  3 variables:
##  $ Column.name: Factor w/ 7 levels "Batch_Id","count",..: 4 3 1 5 2 7 6
##  $ Description: Factor w/ 7 levels "Batch-Id","Evaluation of peak area in relation to calibration curve if
##  $ Value      : Factor w/ 5 levels "1","3PGA","51",..: 2 5 4 NA 3 3 1
```

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

15.3.2.2.5 `top5_CalibrationInfo_unique.csv`

```
## 'data.frame':    8 obs. of  3 variables:
##  $ Column.name: Factor w/ 8 levels "adj_r_squared",..: 6 5 2 7 1 4 8 3
##  $ Description: Factor w/ 8 levels "Adjsuted Rsquare of calibration curve",..: 6 5 2 7 1 4 8 3
##  $ Value      : Factor w/ 8 levels "0.000194064",..: 6 5 7 8 2 4 1 3
```

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`

```
## 'data.frame':    15 obs. of  3 variables:
##  $ Column.name: Factor w/ 15 levels "adj_r_squared",..: 8 7 14 10 2 5 3 4 13 11 ...
##  $ Description: Factor w/ 15 levels "Adjusted Rsquare of calibration curve",..: 8 5 13 9 2 6 4 3 12 14 ...
##  $ Value      : Factor w/ 13 levels "0.000194064",..: 9 9 7 11 12 2 6 4 13 10 ...
```

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`

```
## 'data.frame':    4 obs. of  3 variables:
##  $ Column.name: Factor w/ 4 levels "Batch_Id","Lettercode",..: 2 1 3 4
##  $ Description: Factor w/ 4 levels "Batch-Id","Lettercode name of metabolite",..: 2 1 3 4
##  $ Value      : Factor w/ 4 levels "0.740740741",..: 2 3 4 1
```

	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`

```
## 'data.frame':    5 obs. of  3 variables:
##  $ Column.name: Factor w/ 5 levels "Batch_Id","fracr_prop",..: 3 1 5 4 2
##  $ Description: Factor w/ 5 levels "Batch-Id","Class of NA-value",..: 4 1 2 5 3
##  $ Value      : Factor w/ 5 levels "0","0.851851852",..: 4 5 1 3 2
```

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

```
## 'data.frame':    9 obs. of  3 variables:
##  $ Column.name: Factor w/ 9 levels "Batch_Id","count_score",..: 4 1 3 8 7 5 6 9 2
##  $ Description: Factor w/ 6 levels "_expl_","Batch-Id",..: 4 2 3 1 1 1 1 6 5
##  $ Value      : Factor w/ 9 levels "1861","2.30897367",..: 4 6 7 3 1 2 9 5 8
```

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

```
## 'data.frame':    6 obs. of  3 variables:
##  $ Column.name: Factor w/ 6 levels "Batch_Id","count_score",..: 3 1 2 4 6 5
##  $ Description: Factor w/ 6 levels "Batch-Id","Evaluation of MID",..: 3 1 2 5 4 6
##  $ Value      : Factor w/ 5 levels "1","54","Cit",..: 3 4 5 2 2 1
```

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

Chapter 16

Appendix - 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.

16.1   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.
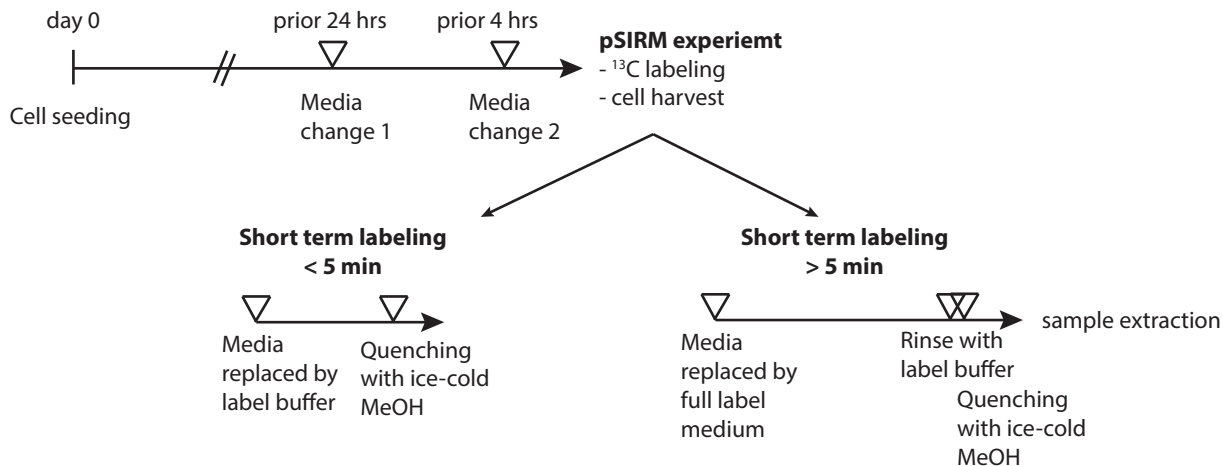
Figure 16.1: Experimental design of a pSIRM experiment distuingishing short and long labeling with stable isotopes.

Table 16.1: 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)	

16.2   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 16.1). 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.

---

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

16.3 Protocol pSIRM

16.3.1 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:

- 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.

16.3.2 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

---

[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

- 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. 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.

16.4   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 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.

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