MTXQCvX2 documentation

Christin Zasada

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Contents

Chapter 1

Welcome

This documentation introduced to 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).

Both workflows are introduced in the distinct chapters including their required input parameter (chapter Workflow Maui and Workflow Metmax).

Chapter 2

Introduction

Experimental and mathematical concepts have been introduced for the pulsed stable isotope resolved metabolomics (pSIRM) approach in (Pietzke et al., 2014).

```
knitr::include_graphics("images/now.png")
```

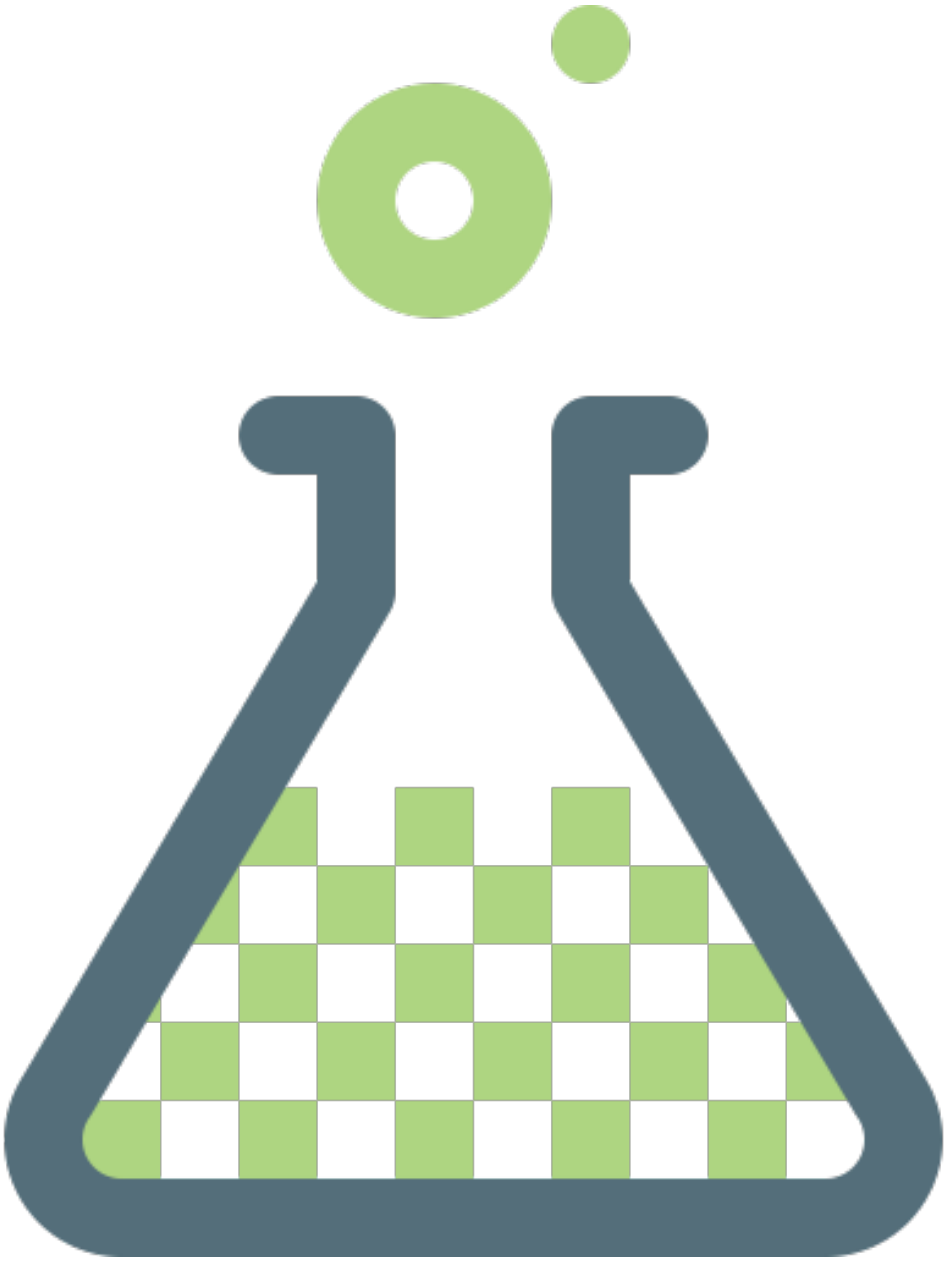

Figure 2.1: Test external figure and how to include it

Chapter 3

Workflow for Maui-annotation projects

3.1 Read this in case

- you have run a Maui project
- exported all required container (see **??**)
- you have a copy of sequence list and experimental conditions
- you know the extraction procedure

The following article describes briefly how to use MTXQCvX2 in case you used Maui for the annotation of your metabolomics project. It does not matter if you have performed an experiment including stable isotopes or if you just aim for the quantification of a few intermediates.

3.2 Quick view

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

3.3 Input files

Three different kind of export functions have been implemented in Maui. These functions provide the export of the actual data into `.csv` or `.tsv` files that are directly usable as input files for MTXQCvX2. Please refer to section 9.3 how you perform the export and which containers have to be exported using what export function and where to copy them in `psirm_glucose/input/`.

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`[1] 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`[2] 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
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/...`

3.4 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** or **addQ1__dilution**[3]
6. Add more columns specifying your experimental conditions, e.g., Cellline and Treatment[4]
7. Save the content as `csv-file` in the `psirm_glucose/input/...`

3.5 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**[5]. 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] stored in `psirm_glucose/output/quant/...`
[2] inst/template_files/...
[3] see for further details additionalQuant
[4] optimal: two-three parameter, max: four parameter. Consider possible combinations, e.g., HCT116-control, HCT116-BPTES
[5] Define: count, mg or ul

3.6 Internal Standard

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 `conversion_metabolite.csv` file. To do so, add `InternalStandard` to the compound in last column `Standard`.

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

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 Maui export file `quantPeakAreasMatrix.csv` file and save it in the folder `psirm_glucose/input/gc/InternalStandard.csv`, respectively. Prerequisite - you have annotated the compound in Maui.

The report of `MTXQCvX2_part1.Rmd` includes the defined internal standard for each project in a message.

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

[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/...`

[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.

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

Chapter 5

MTXQCvX2 Universe

Back then in 2015 I started to think about a way how to evaluate my own datasets regarding some quality parameters. From a scratch of a few quality metrices evaluated in the frame of a very, very long Rscript until now many different generations of the MTXQC existed.

Out of this evolution cycle MTXQC developed to a suit of modules that can be used complimentary in order to evaluate and process GC-MS derived metabolomics data. `MTXQC_part1.Rmd` represents the heart of this little universe. Besides that there are many ways how to use MTXQCvX2. Proposed workflows are illustrated in chapter Workflow Maui and chapter Workflow Metmax. The flow diagram introduced here might help you to decide how to get started.

The following sections give a short overview about the main parameters and functions of each module, its generated graphical illustrations and tables.

5.1 MTXQC_init.Rmd

5.2 MTXQC_ExperimentalSetup

5.3 MTXQC_part 1

5.4 MTXQC_part 2 - Post-Processing

5.5 MTXQC_part 3 - Manual Validation

5.6 MTXQC_part 4 - Metmax integration

Chapter 6

Configuration of MTXQCvX2 - `config_mtx/...`

Herein explained are the customizable tables of the MTXQCvX2 universe.

6.1 `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

6.2 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

6.3 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

6.4 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 7

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.

7.1 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.

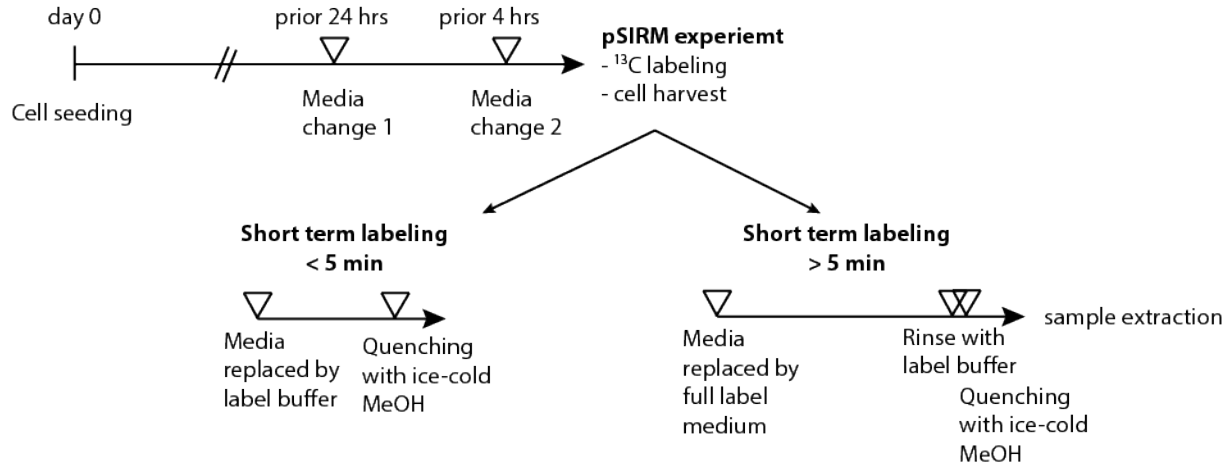

Figure 7.1: Experimental design of a pSIRM experiment distuingishing short and long labeling with stable isotopes.

7.2 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 7.2). 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.

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

Solvent

Base

Carbon.source

Supplements

Full label medium (LM)

DMEM, without glucose, glutamine, pyruvate

13C-Glc (2.5 g/L) 12C-Gln (2 mM)

small molecules (inhibitor, antibiotics)

Label buffer (LB)

HEPES (5 mM), NaCl (140 mM), pH 7.4

13C-Glc (2.5 g/L) 12C-Gln (2 mM)

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

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

a matter of a tenth of seconds rather than seconds. It is of great value to perform the cell harvest with a second person.

7.3 Protocol pSIRM

7.3.1 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.

7.3.2 Supension cell cultures

Materials:

- Cell culture flasks
- Labeling media (LM) supplemented with substrates

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

- 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. 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.

7.4 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.

[5] Highly recommended, makes labeling and harvest super quick
[6] To generate three replicates
[7] Most of the times 30 s are already enough

Chapter 8

Experimental Protocols

8.1 Metabolomics sample extraction

8.1.1 Cell culture experiments

8.1.1.1 Cell extracts in MeOH

Materials:

- Chloroform
- 15 ml falcon tubes
- thermo or rotary shaker

Procedure:

- Add 1 ml chloroform to 5 ml methanolic cell extract
- Incube for 60 min at 4°C on rotary or thermo shaker
- Centrifuge at max speed for 10 min at 4°C
- 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 4°C 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.

8.1.1.2 Cell pellets

Materials:

- MCW suppl. cinnamic acid
- 15 ml falcon tubes
- thermo or rotary shaker

Procedure:

- Resuspend cell pellets in 1 ml MCW

- Incube for 60 min at 4°C on rotary or thermo shaker
- Add 0.5 ml of H2O to induce phase separation
- 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.

8.1.2 Tissue samples

Materials:

- MCW suppl. cinnamic acid
- 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 4°C
- Add 0.5 ml ddH20 for phase separation
- Centrifuge maximum speed, 10 min at 4°C
- Collect polar and lipid phases in fresh vessels
- Dry under vacuum

8.1.3 Blood samples

Material:

- Minivette for capillary blood samples
- MCW suppl. cinnamic acid
- 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)

8.2 Sample derivatisation

8.2.1 Standard protocol

Materials:

- Methoxamine (MeOx)
- Pyridine (open under the hood only!)
- MSTFA
- Alkane mix (c10-c36) in Hexane
- Chromacol vials and caps (big, small)

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 volumes) 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)

8.2.2 Specific protocols

8.2.2.1 Andrash

8.2.2.2 Fabian

8.2.2.3 Henning

Chapter 9

Data processing - MAUI

9.1 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.

9.1.1 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

9.1.2 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
-

9.2 Maui notes

9.3 Maui exports

With initiation of a project folder via `MTXQCvX2_init.Rmd` you created an `input`-folder containing three subfolders: `gc`, `inc`, `quant`. In the following all files that should be exported and copied into these folders are described in detail.

9.3.1 `input/gc/...`

Four input files are exported in order to assess the quality of the GC-MS performance of the run. The menue `Diagnostics` is selectable via right click on your Maui project name. Only the cinnamic acid peak areas are exported via the function `Export Quantification` with right click on the actual container.

Exported Files:

- `Alcane_intensities.csv` - Diagnostics/Export Alcane intensities
- `InternalStandard.csv` - cinAcid container, Export quantification, rename!
- `MassSum-73.csv` - Diagnostics/QC Mass Sum Export; enter: 73 for m/z 73
- `PeakDensities-Chroma.csv` - Diagnostics/ExportPeakDensities

9.3.2 `input/quant/...`

Only one container has to be exported and contains the peak areas of each metabolite and measurement. Keep in mind that you should have uploaded `pTop5 mass list` for the correct determination of peak areas in case of labeling with stable isotopes.

A further note - Maui performs absolute quantification and stores values in the `samplePeakGroups-QMQ` container. These quantities are determined by polynominal regression, and not linear regression.

The file `ManualQuantTable.tsv` is automatically generated by Maui during processing the absolute quantification.

Exported Files:

- `ManualQuantTable.tsv` - location: `Maui-project/export/QM-AbsoluteQuantification/...`[1]
- `quantMassAreasMatrix.csv` - Quantification export of the container `samplesPeakGroups`

9.3.3 `input/inc/...`

This exports are only required in case of an experiment including the application of stable isotopes. It´s this the case you should have performed two things:

1. Used the optional upload of `pTop5 mass list`
2. Go through the pSIRM workflow in Maui

Exported Files:

- `DataMatrix.csv` - Export % Label of container `pSIRM-samplesPeakGroups`
- `pSIRM_SpectraData.csv` - pSIRM Spectra Export of container `pSIRM-samplesPeakGroups`[2]

[1] Don´t forget to rename it - e.g., e17123cz
[2] Requires the selection of Natural_MIDs.txt

Chapter 10

Data Processing - Metmax

10.1 Resampling

10.2 1D-basic

10.3 Reference search

10.4 Export for Metmax

10.5 Data extraction with Metmax

10.5.1 Peak areas

10.5.2 MIDs

Chapter 11

Solutions and Materials

11.1 Solutions

11.1.1 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

11.1.2 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

11.1.3 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.

11.2 Materials

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

4°C

Pyridine

270970-100ML

Sigma

RT

Chapter 12

Output tables

12.1 Project-files

12.1.1 `MTXQC_params.csv`

Parameter

Description

Value

inputformat

Format of input files

maui

ann

File name of the annotation file

annotation.csv

sample_ext

File name of the file containing cell counts or extracted tissue weight, volumes of blood

Sample_extracts.csv

instd

Internal extraction standard present

TRUE

addQ

Additional quantification standards measured?

no

addQ_Int

Integration of additional quantification standards in all batches of the MTXQC project

no

substr

Applied stable isotopes

glc

quant_vol

Dried volume of polar phase of quantification standards in uL

500

backups

Preparation of technical aliquots

2

samples

Origin of samples

cell extracts

subf

subfolder or project folder

psirm_glucose/

data

Nature of the experiment

pSIRM

quant

Version of quantification standards

Quant1_v4

12.1.2 `Maui_params.csv`

Parameter

Description

Value

spath

subfolder or project folder

psirm_glucose/

matrix

Input file containing all peak areas

quantMassAreasMatrix.csv

mz

Input file containing m/z intensities

MassSum-73.csv

mid

Input file containing MIDs for each intermediate and file

pSIRM_SpectraData.csv

inc_data

Input file containing calculated stable isotope incorporation in the format 100 percent = 1

DataMatrix.csv

inputformat

Input format type

maui

intstd

If MTXQC project contains an internal extraction standard

TRUE

alkanes

If MTXQC project contains evaluation of alkane profile

TRUE

peakchroma

If MTXQC project contains evaluation of derivatisation efficiency

TRUE

mqt

If MTXQC project includes absolute quantification

TRUE

inc

If MTXQC project includes labeling with stable isotopes

TRUE

12.1.3 Metmax_params.csv

12.2 output/gc/...

12.2.1 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

12.2.2 `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

n_batch

Number of annotated intermediates

54

mean_batch

Mean value of internal standard peak area across the batch

106642415.3

sd_batch

Standard deviation of the peak area across the batch of the internal extraction standard

36710894.86

12.2.3 `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

mean_batch

Mean value of peak areas across the batch

34409759414

sd_batch

Standard deviation of peak areas across the batch

12913422445

area_fac

Normalisation factor of the file

1.296568521

n_50

Number of annotated peak areas per file corresponding to fifty percent of maximum number of annotated peaks in the batch

53.5

Column.name

Description

Value

Batch_Id

Batch-Id

e18274ba

n_50

Number corresponding to fifty percent of the maximum number of annotated peaks per file

53.5

12.2.4 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

sum_area

Sum of all m/z intensities

6777312761

ratio_total

Ratio of sum_area in relation to the sum of all annotated peak areas

0.002407244

12.3 output/quant/...{o_quant}

12.3.1 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

Type

Kind of sample

sample

Metabolite_manual

Manual defined metabolite name

#Alanine (3TMS)

Metabolite_short

Short version of metabolite name

Alanine_(3TMS)

Lettercode

Lettercode version of metabolite name

Ala_3TMS

Q1_value

Quantity in the quantification standard Q1:1 if included in pmol

x

Mass_Pos

m/z value that should be evaluated in case of the application of isotopes

190

SE_sel

Intermediate used for evaluation of MIDs if present

x

Q_sel

Intermediate used for absolute quantification if Q1:1 present

x

nopsirm

If exclusivly used for quantification purposes only

Standards

Origin

Quantification standard

Qstd

adj_r_squared

Adjusted Rsquare value of linear regression of the calibration curve

0.997002799

slope

Slope of the calibration curve determined by linear regression

0.000578604

intercept

Intercept of the calibration curve determined by linear regression

-898.3400476

calc_curve

Calibration curve available for absolute quantification

yes_cal

islinear

Evaluation of peak area in relation to linear range of the calibration curve

linear

12.3.2 CalculationFileData.csv

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

Metabolite

AnnMet

Library name

Glycerol-3-phosphate_(4TMS)_MP_RI:1756_IDENT:B+C

Metabolite_manual

AnnMet

Manual defined metabolite name

#Glycerol-3-phosphate (4TMS)

Metabolite_short

AnnMet

Short version of library name

Glycerol-3-phosphate_(4TMS)

absconc

CalCurve

Absolute quantity in pmol

24061.77157

adj_r_squared

CalCurve

Adjusted Rsquare value of calibration curve

0.999667641

calc_curve

CalCurve

Relation of peak area to calibration curve

yes_cal

intercept

CalCurve

Intercept of calibration curve

234.0083706

islinear

CalCurve

Classification of peak area

linear

Origin

CalCurve

Source of Quant1:1 value

Qstd

Q1_value

CalCurve

Quantity defined in Q1:1 standard

x

slope

CalCurve

Slope of calibration curve

0.000821689

corr_absconc

ExtrFactor

Corrected absolute quantities by extraction factor

16041.18105

extr_fac

ExtrFactor

Extraction factor derived from MTXQC_params

0.666666667

Mass_Pos

MTXQCsettings

m/z-value (m_inc) used for determination of stable isotope incorporation

359

nopsirm

MTXQCsettings

Exclusivly used for absolute quantification

Q_sel

MTXQCsettings

Used for absolute quantification

x

QuantMasses

MTXQCsettings

Defined quantification masses

101.0 299.0 133.0 357.0 103.0 359.0

SE_sel

MTXQCsettings

Evaluation of MIDs if available

x

IntStd_eval

Norm_InternalStd

Evaluation of internal extraction standard

within

IntStd_fac

Norm_InternalStd

Normalisation factor based on internal extraction standard

1.090830836

area_fac

Norm_SumOfArea

Normalisation factor for sum of area normalisation

0.507591279

sum_area

Norm_SumOfArea

Sum of all peak areas of the file

17466093782

Conc_microM

Quantities

Quantities in (micromolar) only determined for extracted volumes

NA

Conc_pmio

Quantities

Quantities in (pmol)

4875.73892

IntStd_Conc

Quantities

Quantities in (pmol) after normalisation with internal standard factor

14705.47084

IntStd_Conc_microM

Quantities

Quantities in (micromolar) after normalisation with internal standard factor: only for volumes

NA

IntStd_Conc_pmio

Quantities

Quantities in (pmol/unit) after normalisation with internal standard factor

4469.747976

IntStd_sumA_Conc

Quantities

Quantities in (pmol) after normalisation with internal standard factor and sum of area normalisation

28971.0865

IntStd_sumA_Conc_pmio

Quantities

Quantities in (pmol/per unit) after normalisation with internal standard factor and sum of area normalisation

8805.801367

PeakArea

Quantities

Determined peak area based on QuantMasses

28998527

sumA_Conc

Quantities

Quantities in (pmol) after sum of area normalisation

31602.5545

sumA_Conc_microM

Quantities

Quantities in (micromolar) after sum of area normalisation

NA

sumA_Conc_pmio

Quantities

Quantities in (pmol/per unit) after sum of area normalisation

9605.639664

12.3.3 `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

12.3.4 pTop5_Calibration_Samples_lincheck.csv

Column.name

Description

Value

Lettercode

Short name of metabolite

3PGA

islinear

Evaluation of peak area in relation to calibration curve if available

NaCal

Batch_Id

Batch-Id

e18274ba

Origin

Origin of Quant1:1-value

NA

count

Number of peak areas

51

sum_lin

Sum of peak area per islinear level

51

prop

Fraction of count to sum_lin

1

12.3.5 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

intercept

Intercept of calibration curve

564.549288

slope

Slope of calibration curve

0.000194064

Frac_calcurve

Fraction of peak area in linear range of calibration curve

10

12.3.6 `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

12.4 output/inc/...

12.4.1 HeatMap_Incorporation.csv

Column.name

Description

Value

Lettercode

Lettercode name of metabolite

3PGA

Batch_Id

Batch-Id

e18274ba

Par

Parameter

NA_count

Val

Value of the parameter shown in heatmap

0.740740741

12.4.2 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

12.4.3 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

low3a_ratio

expl

2.30897367

rel_sb

expl

higher

val_score

Validation score

confident

count_score

MID evaluation

goodQ

12.4.4 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

prop

Proportion of MIDs for count_score level

1

Chapter 13

Frequently Asked Questions

13.1 What are additional quantification standards

13.2 How do I extend conversion_metabolite.csv

Chapter 14

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.

14.0.1 Autosampler settings

The table 14.0.1 summarises the defined parameter for method settings of the Gerstel MPS.

Parameter of Gerstel MPS settings

Part

Parameter

Value

System

Runtime

40 min

System

GC Cool down time

3 min

System

Cryo Timeout

15 min

MPS - Liquid Injection

Syringe

10ulALX

MPS - Liquid Injection

Sandwhich

No Sandwhich

MPS - Liquid Injection

Inj. Volume (uL)
0.8
MPS - Liquid Injection
Air Volume below (uL)
1
MPS - Liquid Injection
Inj. Speed (uL/s)
20
MPS - Liquid Injection
Fill Volume (uL)
4
MPS - Liquid Injection
Fill Strokes for Sample
6
MPS - Liquid Injection
Fill Speed (uL/s)
1
MPS - Liquid Injection
Eject Speed (uL/s)
20
MPS - Liquid Injection
Viscosity Delay (s)
1
MPS - Liquid Injection
Pre Inj. Delay (s)
0
MPS - Liquid Injection
Post Inj. Delay (s)
0
MPS - Liquid Injection
Inj. Penetration (mm)
30
MPS - Liquid Injection
Sample Tray Type
VT98
MPS - Liquid Injection

Vial Penetration (mm)

32

MPS - Rinse

Wash1 Preclean

2

MPS - Rinse

Wash1 Postclean

3

MPS - Rinse

Wash1 Preclean

2

MPS - Rinse

Wash1 Postclean

3

MPS - Rinse

Sample (4.0 uL)

0

MPS - Rinse

Rinse Fill speed (uL/s)

1

MPS - Rinse

Viscosity Delay (s)

1

MPS - Rinse

Eject Speed (uL/s)

20

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

14.0.2 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 14.2 and corresponding values in table 14.0.2.

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

Additional parameter:

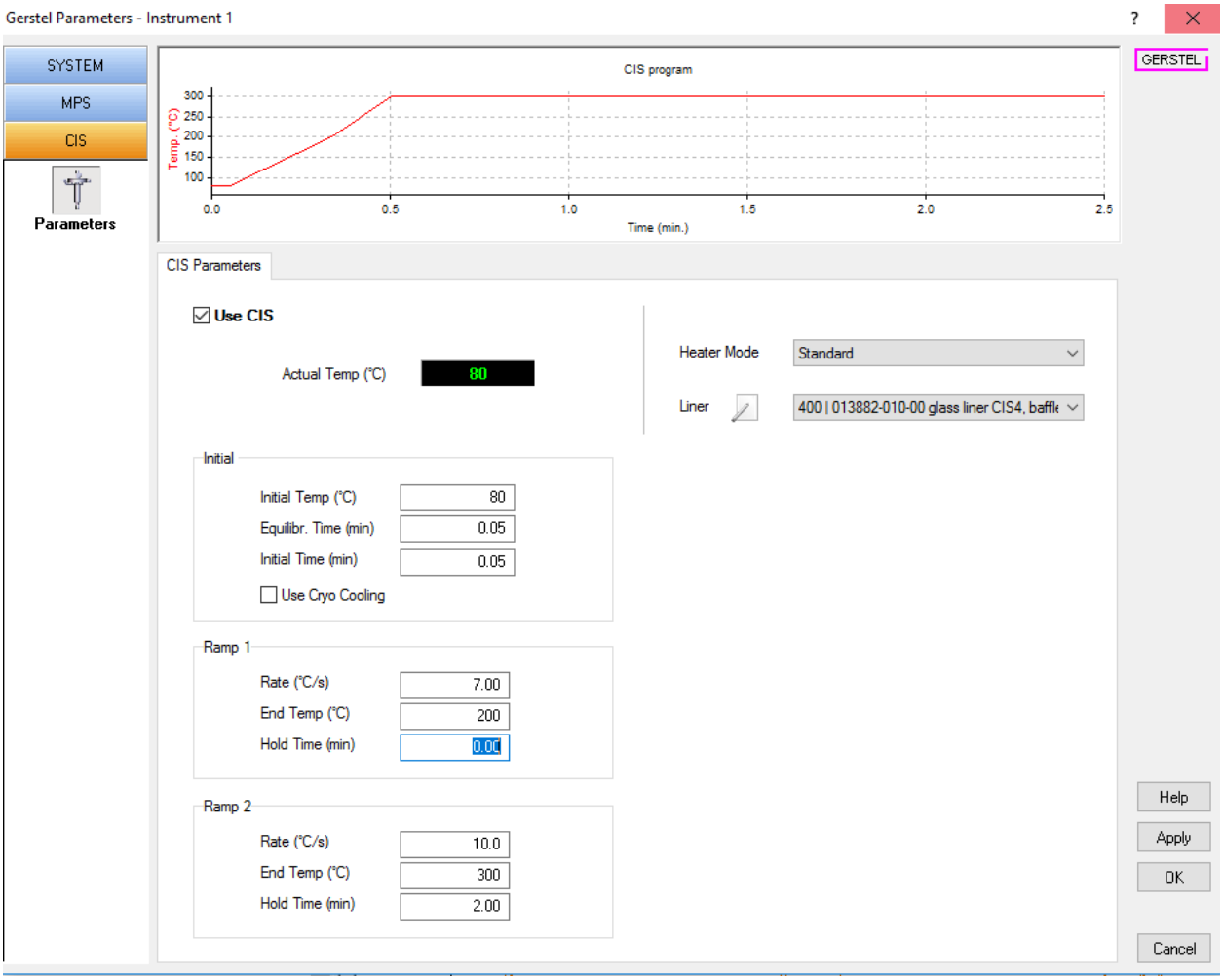

Figure 14.1: Settings temperature-regulated injection for Gerstel MPS

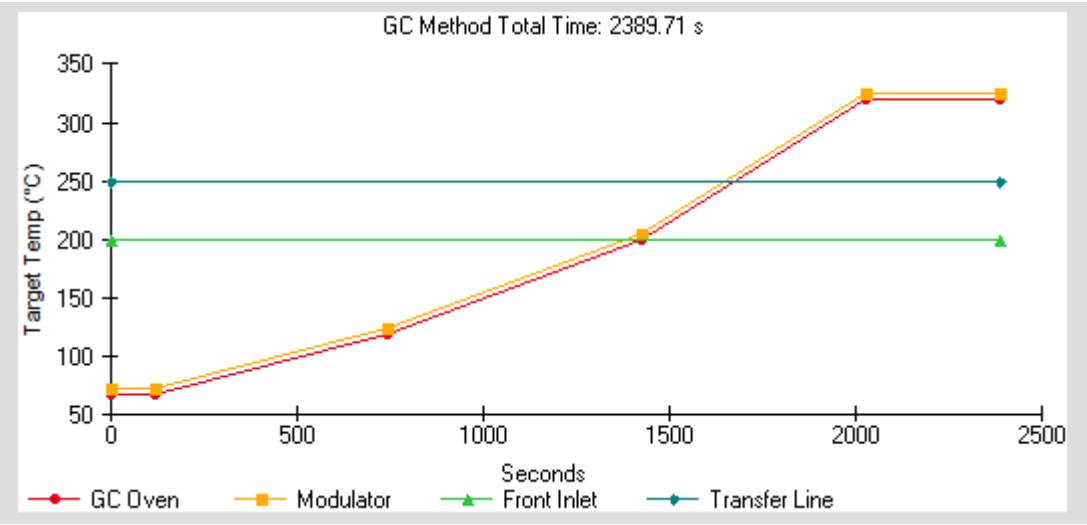

Figure 14.2: GC gradient - graphical representation. Rate in (°C/min), Target temperature in (°C), Duratin in (min).

- Carrier Gas: Helium
- Transfer line Temperature (°C): 250

Front Inlet:

- Mode: split / splitless
- Flow: 1.2 mL/min, entire run
- Septum Purge Flow: 0 mL/min
- Temperature: Initial - 200°C; duration 0 min

Oven:

- Equilibration time (s): 60
- Modulator enabled: yes
- Modulator offset (°C): 5

GC gradient profile in numbers.

Rate

Target_Temp

Duration

Initial

68

2

5

120

0

7

200

0

12

320

6

14.0.3 Mass spectrometer settings

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.