MTXQCvX2 documentation

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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 **??**). Technical relevant information are summarised in chapter **??**.

Chapter 2

Introduction

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

Chapter 3

Workflow for Maui-annotation proejcts

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 14.3 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_init

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

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.

M

10.2 Input files

10.2.1 Quantification - PeakAreas.csv[1]

In order to perform absolute quantification of

You need a file containing all extracted peak areas for each metabolite and file[2]. The header of metmax-extracted files looks like shown below (see table 1). Please, remember to delete the second header row, representing the column loads for each file before saving as csv-file. Otherwise you end up with weird imported dataframes in R. Quantification masses have to be updated while processing in ChromaToF prior the export of the data e.g., with a reference search[3] or using statistical compare. pSIRM experiments require the definition of pTop5 masses[4] instead of top5 masses in the reference in order to take into account the shift of intensities induced by the application of stable isotopes[5]

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

[1]Required for: all parameter, just not `calculation stable isotope incorporation`
[2]Tools/Options/Retention analysis, Parameter: Area
[3]See `vignette/ReferenceSearch`
[4]Extended list of quant masses considering isotope incorporation
[5]Mandatory columns: name, mass, files

MTXQCvX_part4 takes care of the formatting and correct column names of the peak areas file and saves it[6]. MTXQCvX_part4 generates also the file PeakDensities-Chroma.csv[7], in case you have selected the option to include sum of area normalisation while knitting this module.

10.2.2 Isotope incorporation - MIDs.csv[8]

In order to determine the incorporation of stable isotopes MTXQCvX2 requires as an input the mass isotopomer distributions (MIDs) for each intermediate and measurement[9]. Fragments for each intermediate have to be pre-defined in metmax at Tools/Options/metabolite masses. They can be imported[10] 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[11].

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[12]. 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[13]. Please refer for more details to `vignettes/config_mtx-files`.

10.2.3 Derivatisation efficiency - mz73.csv[14]

The extraction of intensities for the ion m/z 73 works analogous to the extraction of MIDs[15]. 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[16]. Check `inst\template_files\` for reference. Hopefully soon a new metmax button extracting specific intensities across the batch.

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

Chapter 11

Configuration of MTXQCvX2

Herein explained are the customizable tables of the MTXQCvX2 universe.

11.1 File: `conversion_metabolite.csv`

```
data = read.csv("config_mtx/conversion_metabolite.csv", TRUE, sep = ";")
colnames(data)
```

```
##  [1] "Metabolite_manual" "Metabolite"        "Metabolite_short"
##  [4] "Lettercode"        "Q1_value"          "Mass_Pos"
##  [7] "SE_sel"            "Q_sel"             "nopsirm"
## [10] "Standards"
```

11.1.1 Definition of standards

11.1.2 Adding further intermediates

11.2 File: Metabolic profile

11.3 File: `quant1-values.csv`

```
data = read.csv("config_mtx/quant1_values.csv", TRUE)
head(data)
```

```
##   Letter_Derivate Quant1_v4 Quant1_v3
## 1             2HG     57270     57270
## 2             2OG     34220     34220
## 3           3PGA      43480     43480
## 4               A      7400      7400
## 5       Adenosine     18710     18710
## 6             Ala    134700    134700
```

11.4 File: `incorporation_calc.csv`

Chapter 12

Protocols - Sample extraction

12.1 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.

12.2 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

12.3 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)

Chapter 13

Protocols - GC-ToF-MS measurement

13.1 Sample derivatisation

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 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)

13.2 GC-MS measurement

needs to be written

Chapter 14

Data processing - MAUI

14.1 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.

14.1.1 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

14.1.2 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
-

14.2 Maui notes

14.3 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.

14.3.1 `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

14.3.2 `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`

14.3.3 `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 15

Data Processing - Metmax

15.1 Resampling

15.2 1D-basic

15.3 Reference search

15.4 Export for Metmax

15.5 Data extraction with Metmax

15.5.1 Peak areas

15.5.2 MIDs

Chapter 16

Frequently Asked Questions

16.1 What are additional quantification standards

16.2 How do I extend conversion_metabolite.csv

Bibliography

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