Von Spektren zu Ergebnissen: Effiziente Analyse von massenspektrometrischen Daten mit Workflows

The OpenMS Developers

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Contents

1 General remarks

- This handout will guide you through an introductory tutorial for the OpenMS/TOPP software package [1].
- OpenMS [2] is a versatile open-source library for mass spectrometry data analysis. Based on this library, we offer a collection of command-line tools ready to be used by end users. These so-called TOPP tools (short for "The OpenMS Proteomics Pipeline") [3] can be understood as small building blocks of arbitrary complex data analysis workflows.
- In order to facilitate workflow construction, OpenMS was integrated into KNIME [4], the Konstanz Information Miner, an open-source integration platform providing a powerful and flexible workflow system combined with advanced data analytics, visualization, and report capabilities. Raw MS data as well as the results of data processing using TOPP can be visualized using TOPPView [5].
- In this hands-on tutorial session, you will become familiar with some of the basic functionalities of OpenMS/TOPP, TOPPView, and KNIME and learn how to use a selection of TOPP tools used in the tutorial workflows.
- All data referenced in this tutorial can be found in the 🗀 **Example_Data** folder that came with this tutorial.

2 Getting started

Before we get started we will install OpenMS and KNIME using the installers provided on the USB stick. Please choose the directory that matches your operating system and execute the installer. Note that these steps are not necessary if you use one of our laptops.

For example for Windows you call

- the OpenMS installer: 🗀 **Windows / OpenMS-2.0_Win64_setup.exe**
- the KNIME installer: 🗀 **Windows / OpenMS-2.0-prerequisites-installer.exe** and 🗀 **Windows / KNIME Full 3.1.1 Installer (64bit).exe**

on Mac you call

- the OpenMS installer: 🗀 **Mac / OpenMS-2.0.0_setup.dmg**
- the KNIME installer: 🗀 **Mac / knime-full_3.1.1.macosx.cocoa.x86_64.dmg**

and follow the instructions.

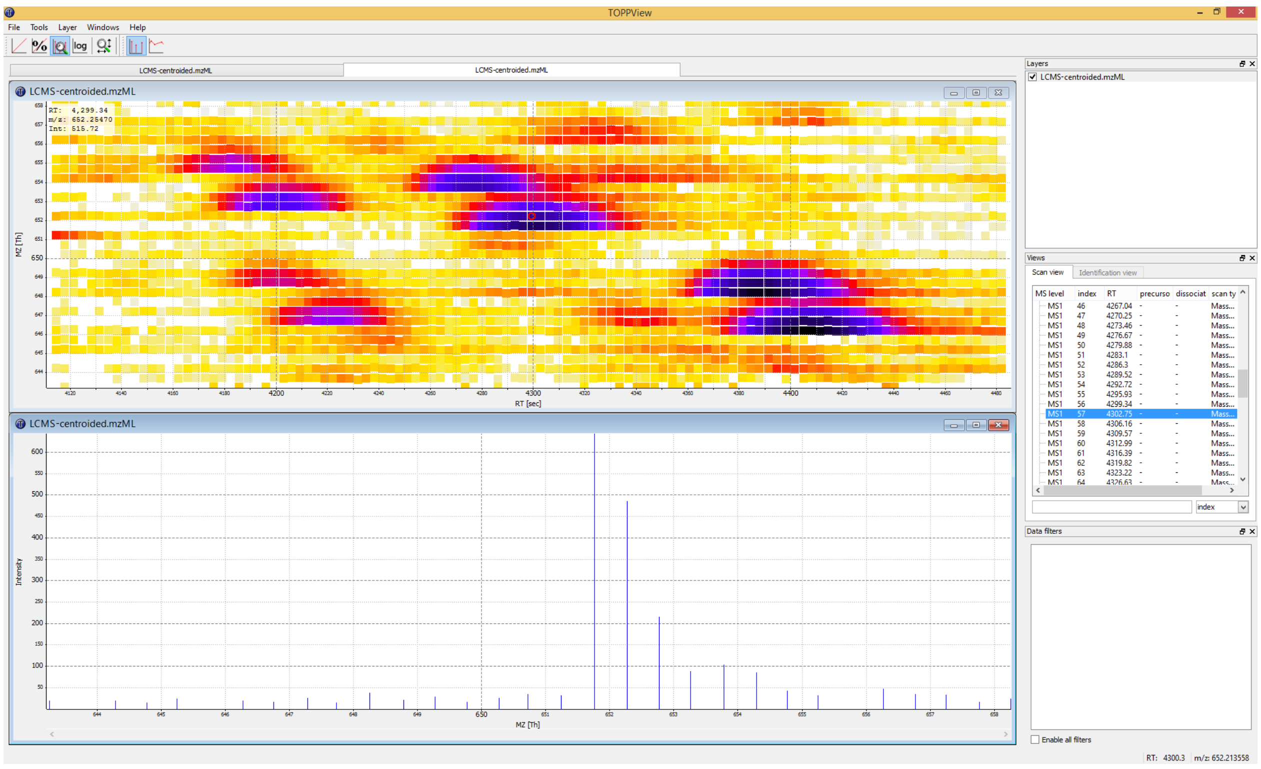
2.1 Data conversion

Each MS instrument vendor has one or more formats for storing the acquired data. Converting these data into an open format (preferably mzML) is the very first step when you want to work with open-source mass spectrometry software. A freely available conversion tool is ProteoWizard. The OpenMS installation package for Windows automatically installs ProteoWizard, so you do not need to download and install it separately.

Please note that due to restrictions from the instrument vendors, file format conversion for most formats is only possible on Windows systems, so exporting from the acquisition PC connected to the instrument is usually the most convenient option. All files used in this tutorial have already been converted to mzML by us, so you do not need to do it yourself.

2.2 Data visualization using **TOPPView**

Visualizing the data is the first step in quality control, an essential tool in understanding the data, and of course an essential step in pipeline development. OpenMS provides a



```
RT:  4,299.34
m/z: 652.25470
Int: 515.72
```

MS level	index	RT	precurso	dissociat	scan ty
MS1	46	4267.04	-	-	Mass...
MS1	47	4270.25	-	-	Mass...
MS1	48	4273.46	-	-	Mass...
MS1	49	4276.67	-	-	Mass...
MS1	50	4279.88	-	-	Mass...
MS1	51	4283.1	-	-	Mass...
MS1	52	4286.3	-	-	Mass...
MS1	53	4289.52	-	-	Mass...
MS1	54	4292.72	-	-	Mass...
MS1	55	4295.93	-	-	Mass...
MS1	56	4299.34	-	-	Mass...
MS1	57	4302.75	-	-	Mass...
MS1	58	4306.16	-	-	Mass...
MS1	59	4309.57	-	-	Mass...
MS1	60	4312.99	-	-	Mass...
MS1	61	4316.39	-	-	Mass...
MS1	62	4319.82	-	-	Mass...
MS1	63	4323.22	-	-	Mass...
MS1	64	4326.63	-	-	Mass...

Figure 1: TOPPView, the graphical application for viewing mass spectra and analysis results. Top window shows a small region of a peak map. In this 2D representation of the measured spectra, signals of eluting peptides are colored according to the raw peak intensities. The lower window displays an extracted spectrum (=scan) from the peak map. On the right side, the list of spectra can be browsed.

convenient viewer for some of the data: **TOPPView**.

We will guide you through some of the basic features of **TOPPView**. Please familiarize yourself with the key controls and visualization methods. We will make use of these later throughout the tutorial. Let's start with a first look at one of the files of our tutorial data set:

- Start **TOPPView** (see Start-Menu or Applications on MacOS)
- Go to File › Open File, navigate to the directory where you copied the contents of the USB stick to, and select 🗀 **Example_Data › Introduction › datasets › small › velos005614.mzM**
. This file contains a reduced LC-MS map (only a selected RT and m/z range was extracted using the TOPP tool **FileFilter**) of a label-free measurement of the human

platelet proteome recorded on an Orbitrap velos. The other two mzML files contain technical replicates of this experiment. First, we want to obtain a global view on the whole LC-MS map - the default option Map view 2D is the correct one and we can click the Ok button.

- Play around.
- Three basic modes allow you to interact with the displayed data: scrolling, zooming and measuring:
  - Scroll mode
    - Is activated by default (though each loaded spectra file is displayed zoomed out first, so you do not need to scroll).
    - Allows you to browse your data by moving around in RT and m/z range.
    - When zoomed in, to scroll the spectra map, click-drag on the current view.
    - Arrow keys can be used to scroll the view as well.
  - Zoom mode
    - Zooming into the data: either mark an area in the current view with your mouse while holding the left mouse button plus the ctrl key to zoom to this area or use your mouse wheel to zoom in and out.
    - All previous zoom levels are stored in a zoom history. The zoom history can be traversed using ctrl + + or ctrl + - or the mouse wheel (scroll up and down).
    - Pressing the Backspace key zooms out to show the full LC-MS map (and also resets the zoom history).
  - Measure mode
    - It is activated using the ⇧ key.
    - Press the left mouse button down while a peak is selected and drag the mouse to another peak to measure the distance between peaks.
    - This mode is implemented in the 1D and 2D mode only.
- Right click on your 2D map and select Switch to 3D view and examine your data in 3D mode

- Go back to the 2D view. In 2D mode, visualize your data in different normalization modes, use linear, percentage and log-view (icons on the upper left tool bar).

  > Note: On Apple OS X, due to a bug in one of the external libraries used by OpenMS, you will see a small window of the 3D mode when switching to 2D. Close the 3D tab in order to get rid of it.

- In **TOPPView** you can also execute TOPP tools. Go to Tools > Apply tool (whole layer) and choose a TOPP tool (e.g., FileInfo) and inspect the results.

2.3 Introduction to KNIME / OpenMS

Using OpenMS in combination with KNIME you can create, edit, open, save, and run workflows combining TOPP tools with the powerful data analysis capabilities of KNIME. Workflows can be created conveniently in a graphical user interface. The parameters of all involved tools can be edited within the application and are also saved as part of the workflow. Furthermore, KNIME interactively performs validity checks during the workflow editing process, in order to make it more difficult to create an invalid workflow.

Throughout most of the parts of this tutorial you will use KNIME to create and execute workflows. This first step is to make yourself familiar with KNIME.

2.3.1 KNIME concepts

A workflow is a sequence of computational steps applied to a single or multiple input data sets to process and analyze the data. In KNIME such workflows are implemented graphically by combining so-called nodes. A node represents a single analysis step in a workflow. Nodes have input and output ports where the data enters the node or the results are provided for other nodes after processing, respectively. KNIME distinguishes between different port types, representing different types of data. The most common representation of data in KNIME are tables (similar to an excel sheet). Ports that accept tables are marked with a small triangle. For OpenMS we use a different port type, so called file ports, representing complete files. Those ports are marked by a small blue box. Filled blue boxes represent mandatory inputs and empty boxes optional inputs.

A typical OpenMS workflow in KNIME can be divided in two conceptually different parts:

- Nodes for signal and data processing, filtering and data reduction. Here, files are passed between nodes. Execution times of the individual steps are longer as the main computational steps are performed.
- Downstream statistical analysis and visualization. Here, tables are passed between nodes.

Between file-based processing and table-based analysis a conversion node typically performs the conversion from OpenMS results into KNIME tables.

Nodes can have three different states, indicated by the small traffic light below the node.

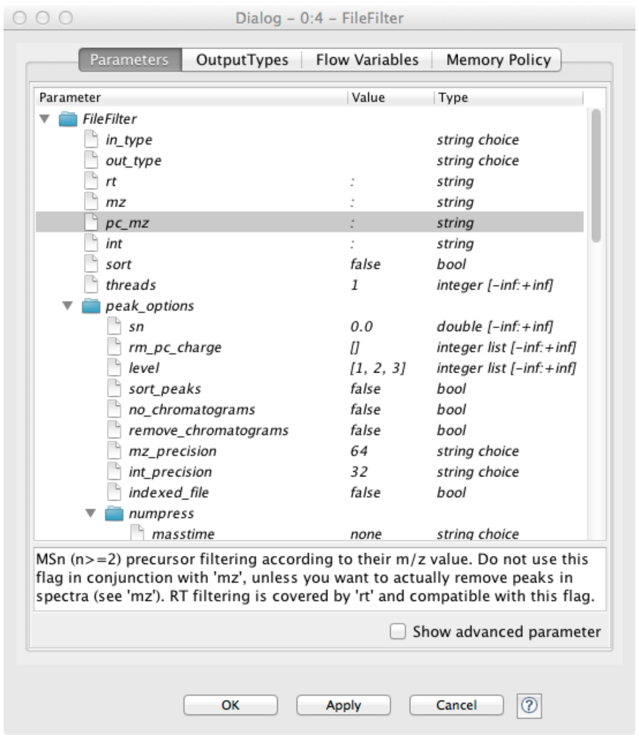
- Inactive, failed, and not yet fully configured nodes are marked red.
- Configured but not yet executed nodes are marked yellow.
- Successfully executed nodes are marked green.

If the node execution failed the node will switch to the red state.

Most nodes will be configured as soon as all input ports are connected. For some nodes additional parameters have to be provided that cannot be either guessed from the data or filled with sensible defaults. In this case, of if you want to customize the default configuration, you can open the configuration dialog of a node with a double-click on the node. For OpenMS you will see a configuration dialog like the one shown in Figure 2.

> Note: OpenMS distinguishes between normal parameters and advanced parameters. Advanced parameters are by default hidden from the users since they should only rarely be customized. In case you want to have a look at the parameters or need to customize them in one of the tutorials you can show them by clicking on the checkbox `Show advanced parameter` in the lower part of the dialog.

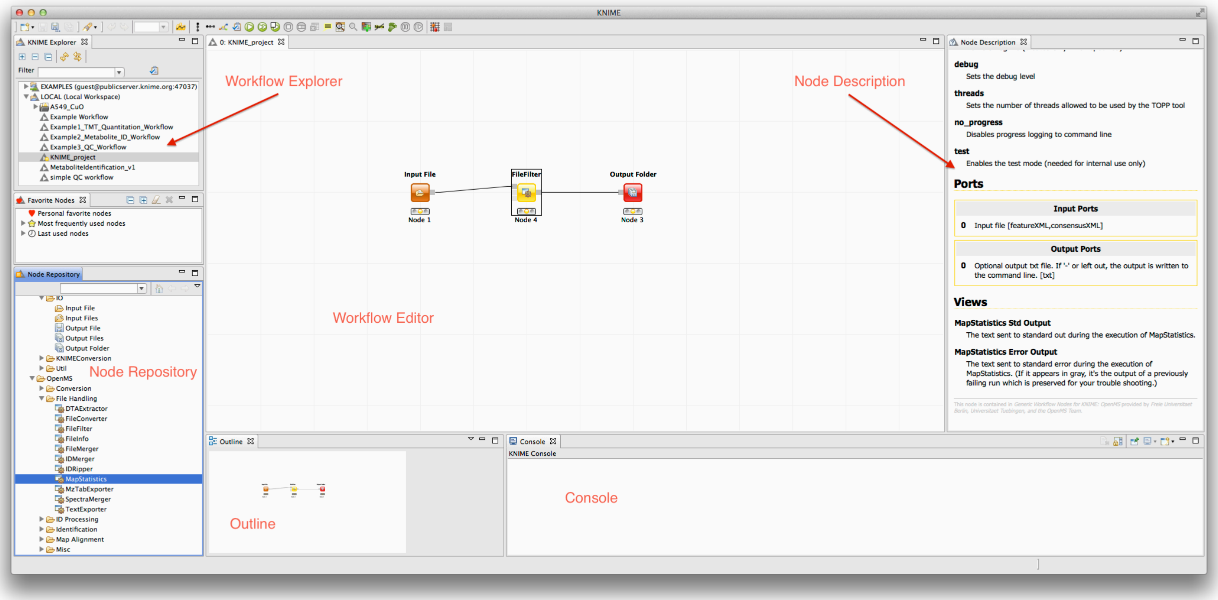
The dialog shows the individual parameters, their current value and type, and, in the lower part of the dialog, the documentation for the currently selected parameter.



Parameter	Value	Type
FileFilter		
in_type		string choice
out_type		string choice
rt	:	string
mz	:	string
pc_mz	:	string
int	:	string
sort	false	bool
threads	1	integer [-inf:+inf]
peak_options		
sn	0.0	double [-inf:+inf]
rm_pc_charge	[]	integer list [-inf:+inf]
level	[1, 2, 3]	integer list [-inf:+inf]
sort_peaks	false	bool
no_chromatograms	false	bool
remove_chromatograms	false	bool
mz_precision	64	string choice
int_precision	32	string choice
indexed_file	false	bool
numpress		
masstime	none	string choice

MSn (n>=2) precursor filtering according to their m/z value. Do not use this flag in conjunction with 'mz', unless you want to actually remove peaks in spectra (see 'mz'). RT filtering is covered by 'rt' and compatible with this flag.

Figure 2: Node configuration dialog of an OpenMS node.



debug
Sets the debug level

threads
Sets the number of threads allowed to be used by the TOPP tool

no_progress
Disables progress logging to command line

test
Enables the test mode (needed for internal use only)

Ports

Input Ports	
0	Input file [featureXML,consensusXML]

Output Ports	
0	Optional output txt file. If '-' or left out, the output is written to the command line. [txt]

Views

MapStatistics Std Output
The text sent to standard out during the execution of MapStatistics.

MapStatistics Error Output
The text sent to standard error during the execution of MapStatistics. (If it appears in gray, it's the output of a previously failing run which is preserved for your trouble shooting.)

This node is contained in *Generic Workflow Nodes for KNIME: OpenMS* provided by *Freie Universitaet Berlin, Universitaet Tuebingen, and the OpenMS Team.*

Figure 3: The KNIME workbench.

2.3.2 Overview of the graphical user interface

The graphical user interface (GUI) of KNIME consists of different components or so called panels that are shown in Figure 3. We will shortly introduce the individual panels and their purposes below.

Workflow Editor: The workflow editor is the central part of the KNIME GUI. Here you assemble the workflow by adding nodes from the Node Repository via ”drag & drop”. Nodes can be connected by clicking on the output port of one node and releasing the mouse at the desired input port of the next node.

Workflow Explorer: Shows a list of available workflows (also called workflow projects). You can open a workflow by double clicking it. A new workflow can be created with a right-click in the Workflow Explorer followed by selecting New KNIME Workflow... .

Node Repository: Shows all nodes that are available in your KNIME installation. Every plugin you install will provide new nodes that can be found here. The OpenMS nodes can be found in Community Nodes > OpenMS. Nodes for managing files (e.g., Input Files or Output Folders) can be found in Community Nodes > GenericKnimeNodes. You can search the node repository by typing the node name into the small text box in the upper part of the node repository.

Outline: The Outline panel contains a small overview of the complete workflow. While of limited use when working on a small workflow, this feature is very helpful as soon as the workflows get bigger.

Console: In the console panel warning and error messages are shown. This panel will provide helpful information if one of the nodes failed or shows a warning sign.

Node Description: As soon as a node is selected, the Node Description window will show the documentation of the node including documentation for all its parameters. For OpenMS nodes you will also find a link to the tool page in the online documentation.

2.3.3 Creating workflows

Workflows can easily be created by a right click in the Workflow Explorer followed by clicking on New KNIME Workflow... .

2.3.4 Sharing workflows

To be able to share a workflow with others, KNIME supports the import and export of complete workflows. To export a workflow, select it in the Workflow Explorer and select File > Export KNIME Workflow... . KNIME will export workflows as a zip file containing all the information on nodes, their connections, and their configuration. Those zip files can again be imported by selecting File > Import KNIME Workflow... .

> Note: For your convenience we added all workflows discussed in this tutorial to the 🗀 **Workflows** folder. If you want to check your own workflow by comparing it to the solution or got stuck, simply import the full workflow from the corresponding zip file.

2.3.5 Duplicating workflows

During the tutorial a lot of the workflows will be created based on the workflow from a previous task. To keep the intermediate workflows we suggest you create copies of your workflows so you can see the progress. To create a copy of your workflow follow the next steps.

- Right click on the workflow you want to create a copy of in the Workflow Explorer and select Copy.
- Right click again somewhere on the workflow explorer and select Paste.
- This will create a workflow with same name as the one you copied with a (2) appended.
- To distinguish them later on you can easily rename the workflows in the Workflow Explorer by right clicking on the workflow and selecting Rename.

  > Note: To rename a workflow it has to be closed.

2.3.6 A minimal workflow

Let us now start with the creation of our very first, very simple workflow. As a first step, we will gather some basic information about the data set before starting the actual development of a data analysis workflow.

- Create a new workflow.
- Add an **Input File** node and an **Output Folder** node (to be found in Community Nodes › GenericKnimeNodes › IO and a **FileInfo** node (to be found in the category Community Nodes › OpenMS › File Handling) to the workflow.
- Connect the **Input File** node to the **FileInfo** node, and the first output port of the **FileInfo** node to the **Output Folder** node.

  > Note: In case you are unsure about which node port to use, hovering the cursor over the port in question will display the port name and what kind of input it expects.

  The complete workflow is shown in Figure 4. FileInfo can produce two different kinds of output files.

- All nodes are still marked red, since we are missing an actual input file. Double-click the Input File node and select Browse. In the file system browser select 🗀 **Example_Data ▸ Introduction ▸ datasets ▸ tiny ▸ velos005614.mzML** and click Open. Afterwards close the dialog by clicking Ok.

  > Note: Make sure to use the "tiny" version this time, not "small", for the sake of faster workflow execution.

- The **Input File** node and the **FileInfo** node should now have switched to yellow, but the **Output Folder** node is still red. Double-click on the **Output Folder** node and click on Browse to select an output directory for the generated data.
- Great! Your first workflow is now ready to be run. Press ⇧ + F7 to execute the complete workflow. You can also right click on any node of your workflow and select Execute from the context menu.

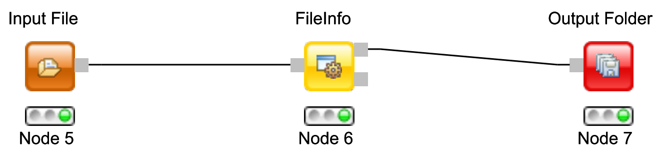


Figure 4: A minimal workflow calling FileInfo on a single file.

- The traffic lights tell you about the current status of all nodes in your workflow. Currently running tools show either a progress in percent or a moving blue bar, nodes waiting for data show the small word "queued", and successfully executed ones become green. If something goes wrong (e.g., a tool crashes), the light will become red.

- In order to inspect the results, you can just right-click the **Output Folder** node and select View: Open the output folder. You can then open the text file and inspect its contents. You will find some basic information of the data contained in the mzML file, e.g., the total number of spectra and peaks, the RT and m/z range, and how many MS1 and MS2 spectra the file contains.

Workflows are typically constructed to process a large number of files automatically. As a simple example, consider you would like to gather this information for more than one file. We will now modify the workflow to compute the same information on three different files and then write the output files to a folder.

- We start from the previous workflow.

- First we need to replace our single input file with multiple files. Therefore we add the **Input Files** node from the category Community Nodes › GenericKnimeNodes › IO.

- To select the files we double-click on the **Input Files** node and click on Add. In the filesystem browser we select all three files from the directory 🗀 **Example_Data ▸ Introduction ▸ datasets ▸ tiny**. And close the dialog with Ok.

- We now add two more nodes: the **ZipLoopStart** and the **ZipLoopEnd** node from the category Community Nodes › GenericKnimeNodes › Flow.

- Afterwards we connect the **Input Files** node to the first port of the **ZipLoopStart** node, the first port of the **ZipLoopStart** node to the **FileInfo** node, the first output

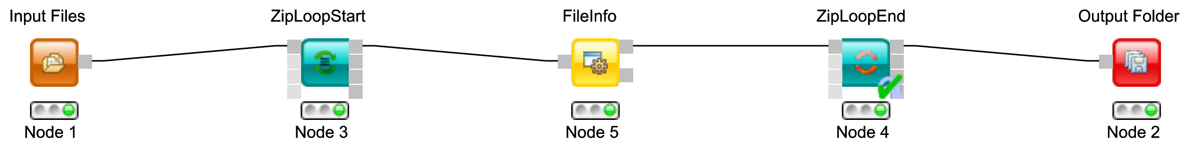


Figure 5: A minimal workflow calling FileInfo on multiple files in a loop.

port of the **FileInfo** node to the first input port of the **ZipLoopEnd** node, and the first output port of the **ZipLoopEnd** node to the **Output Folder** node (NOT to the **Output File**). The complete workflow is shown in Figure 5

- The workflow is already complete. Simply execute the workflow and inspect the output as before.

In case you had trouble to understand what ZipLoopStart and ZipLoopEnd do - here is a brief explanation:

- The **Input Files** node passes a list of files to the **ZipLoopStart** node.
- The **ZipLoopStart** node takes the files as input, but passes the single files sequentially (that is: one after the other) to the next node.
- The **ZipLoopEnd** collects the single files that arrive at its input port. After all files have been processed, the collected files are passed again as file list to the next node that follows.

2.3.7 Advanced topic: Meta nodes

Workflows can get rather complex and may contain dozens or even hundreds of nodes. KNIME provides a simple way to improve handling and clarity of large workflows:

Meta Nodes allow to bundle several nodes into a single **Meta Node**.

Task

☑ Select multiple nodes (e.g. all nodes of the ZipLoop including the start and end node). To select a set of nodes, draw a rectangle around them with the left mouse button or hold Ctrl to add/remove single nodes from the selection. Open the context menu (right-click on a node in the selection) and select Collapse into Meta Node. Enter a caption for the **Meta Node**. The previously selected nodes are now contained in the **Meta Node**. Double clicking on the **Meta Node** will display the contained nodes in a new tab window.

Task

Undo the packaging. First select the **Meta Node**, open the context menu (right-click) and select Expand Meta Node.

2.3.8 Advanced topic: R integration

KNIME provides a large number of nodes for a wide range of statistical analysis, machine learning, data processing and visualization. Still, more recent statistical analysis methods, specialized visualizations or cutting edge algorithms may not be covered in KNIME. In order to expand its capabilities beyond the readily available nodes, external scripting languages can be integrated. In this tutorial, we primarily use scripts of the powerful statistical computing language R. Note that this part is considered advanced and might be difficult to follow if you are not familiar with R. In this case you might skip this part.

R View (Table) allows to seamlessly include R scripts into KNIME. We will demonstrate on a minimal example how such a script is integrated.

Task

First we need some example data in KNIME, which we will generate using the **Data Generator** node. You can keep the default settings and execute the node. The table contains 4 columns, each containing random coordinates and one column containing a cluster number (Cluster_0 to Cluster_3). Now place a **R View (Table)** node into the workflow and connect the upper output port of the **Data Generator** node to the input of the **R View (Table)** node. Right-click and configure the node.

If you get an error message like "Execute failed: R_HOME does not contain a folder with name 'bin'.": please change the R settings in the preferences. To do so open File » Preferences » KNIME » R and enter the path to your

R installation (the folder that contains the bin directory).

If R is correctly recognized we can start writing an R script. Consider that we are interested in plotting the first and second coordinates and color them according to their cluster number. In R this can be done in a single line.

In the **R View (Table)** text editor, enter the following code:

```
plot(x=knime.in$Universe_0_0, y=knime.in$Universe_0_1, main="Plotting column ↩
    Universe_0_0 vs. Universe_0_1", col=knime.in$"Cluster Membership")
```

Explanation: The table provided as input to the **R View (Table)** node is available as R **data.frame** with name **knime.in**. Columns (also listed on the left side of the R View window) can be accessed in the usual R way by first specifying the **data.frame** name and then the column name (e.g. **knime.in$Universe_0_0**). **plot** is the plotting function we use to generate the image. We tell it to use the data in column **Universe_0_0** of the dataframe object **knime.in** (denoted as **knime.in$Universe_0_1**) as x-coordinate and the other column **knime.in$Universe_0_1** as y-coordinate in the plot. **main** is simply the main title of the plot and **col** the column that is used to determine the color (in this case it is the **Cluster Membership** column).

Now press the Eval script and Show plot buttons.

Note: Note that we needed to put some extra quotes around **Cluster Membership**. If we omit those, R would interpret the column name only up to the first space (**knime.in$Cluster**) which is not present in the table and leads to an error. Quotes are regularly needed if column names contain spaces, tabs or other special characters like $ itself.

3 Label-free quantification

3.1 Introduction

In this chapter, we will build a workflow with OpenMS / KNIME to quantify a label-free experiment. Label-free quantification is a method aiming to compare the relative amounts of proteins or peptides in two or more samples. We will start from the minimal workflow of the last chapter and, step-by-step, build a label-free quantitation workflow.

3.2 Peptide Identification

As a start, we will extend the minimal workflow so that it performs a peptide identification using the OMSSA [6] search engine. Since OpenMS version 1.10, OMSSA is included in the OpenMS installation, so you do not need to download and install it yourself.

- Let's start by replacing the input files in our **Input Files** node by the three mzML files in 🗀 **Example_Data ▸ Labelfree ▸ datasets ▸ lfq_spikein_dilution_1-3.mzML**. This is a reduced toy dataset where each of the three runs contains a constant background of S. pyogenes peptides as well as human spike-in peptides in different concentrations. [7]
- Instead of FileInfo, we want to perform OMSSA identification, so we simply replace the **FileInfo** node with the **OMSSAAdapter** node Community Nodes > OpenMS > Identification, and we are almost done. Just make sure you have connected the **ZipLoopStart** node with the **in** port of the **OMSSAAdapter** node.
- OMSSA, like most mass spectrometry identification engines, relies on searching the input spectra against sequence databases. Thus, we need to introduce a search database input. As we want to use the same search database for all of our input files, we can just add a single **Input File** node to the workflow and connect it directly with the **OMSSAAdapter database** port. KNIME will automatically reuse this Input node each time a new ZipLoop iteration is started. In order to specify the database, select 🗀 **Example_Data ▸ Labelfree ▸ databases ▸ s_pyo_sf370_potato_human_target_decoy_with_contaminants.fasta**, and we have a very basic peptide identification workflow.

> Note: You might also want to save your new identification workflow under a different name. Have a look at Section 2.3.5 for information on how to create copies of workflows.

- The result of a single OMSSA run is basically a number of peptide-spectrum-matches (PSM) with a score each, and these will be stored in an idXML file. Now we can run the pipeline and after execution is finished, we can have a first look at the results: just open the input files folder with a file browser and from there open an mzML file in **TOPPView**.
- Here, you can annotate this spectrum data file with the peptide identification results. Choose Tools > Annotate with identification from the menu and select the idXML file that **OMSSAAdapter** generated (it is located within the output directory that you specified when starting the pipeline).
- On the right, select the tab Identification view. Using this view, you can see all identified peptides and browse the corresponding MS2 spectra.

> Note: Opening the output file of **OMSSAAdapter** (the idXML file) directly is also possible, but the direct visualization of an idXML file is less useful.

- The search results stored in the idXML file can also be read back into a KNIME table for inspection and subsequent analyses: Add a **TextExporter** Community Nodes > OpenMS > File Handling node to your workflow and connect the output port of your **OMSSAAdapter** (the same port your **ZipLoopEnd** is connected to) to its input port. This tool will convert the idXML file to a more human-readable text file which can also be read into a KNIME table using the **IDTextReader** node. Add an **IDTextReader** node Community Nodes > OpenMS > Conversion after **TextExporter** and execute it. Now you can right-click **IDTextReader** and select ID Table to browse your peptide identifications.
- From here, you can use all the tools KNIME offers for analyzing the data in this table. As a simple example, you could add a **Histogram** Data Views node after **IDTextReader**, double-click it, select peptide_charge as binning column, hit OK, and execute it.

Right-clicking and selecting View: Histogram view will open a plot showing the charge state distribution of your identifications.

In the next step, we will tweak the parameters of OMSSA to better reflect the instrument's accuracy. Also, we will extend our pipeline with a false discovery rate (FDR) filter to retain only those identifications that will yield an FDR of < 1 %.

- Open the configuration dialog of **OMSSAAdapter**. The dataset was recorded using an LTQ Orbitrap XL mass spectrometer, so we can set the precursor mass tolerance to a smaller value, say 10 ppm. Set precursor_mass_tolerance to 10 and precursor_mass_tolerance_unit_ppm to true.

  > Note: Whenever you change the configuration of a node, the node as well as all its successors will be reset to the Configured state.

- Set max_precursor_charge to 5, in order to also search for peptides with charges up to 5.
- Add Carbamidomethyl (C) as fixed modification and Oxidation (M) as variable modification.

  > Note: To add a modification click on the empty value field in the configuration dialog to open the list editor dialog. In the new dialog click Add. Then select the newly added modification to open the drop down list where you can select the correct modification.

- A common step in analyis is to search not only against a regular protein database, but to also search against a decoy database for FDR estimation. The fasta file we used before already contains such a decoy database. For OpenMS to know which OMSSA PSM came from which part of the file (i.e. target versus decoy), we have to index the results. Therefore extend the workflow with a **PeptideIndexer** node Community Nodes > OpenMS > ID Processing. This node needs the idXML as input as well as the database file.

> Note: You can direct the files of an **Input File** node to more than just one destination port.

- The decoys in the database are prefixed with “REV_”, so we have to set decoy_string to REV_ and prefix to true in the configuration dialog of **PeptideIndexer**.
- Now we can go for the FDR estimation, which the **FalseDiscoveryRate** node will calculate for us Community Nodes > OpenMS > ID Processing. As we have a combined search database and thus only one idXML per mzML we will only use the in port of the **FalseDiscoveryRate** node.
- In order to set the FDR level to 1%, we need an **IDFilter** node from Community Nodes > OpenMS > ID Processing. Configuring its parameter score $\rightarrow$ pep to 0.01 will do the trick. The FDR calculations (embedded in the idXML) from the **FalseDiscoveryRate** node will go into the in port of the **IDFilter** node.
- Execute your workflow and inspect the results using **IDTextReader** like you did before. How many peptides did you identify at this FDR threshold?

  > Note: The finished identification workflow is now sufficiently complex that we might want to encapsulate it in a Meta node. For this, select all nodes inside the ZipLoop (including the **Input File** node) and right-click to select Collapse into Meta node and name it ID. Meta nodes are useful when you construct even larger workflows and want to keep an overview.

3.3 Quantification

Now that we have successfully constructed a peptide identification pipeline, we can add quantification capabilities to our workflow.

- Add a **FeatureFinderCentroided** node Community Nodes > OpenMS > Quantitation which gets input from the first output port of the **ZipLoopStart** node. Also, add an **IDMapper** node Community Nodes > OpenMS > ID Processing which gets input from the **FeatureFinderCentroic** node and the ID Meta node (or **IDFilter** node if you haven’t used the Meta node). The output of the **IDMapper** is then connected to the **ZipLoopEnd** node.

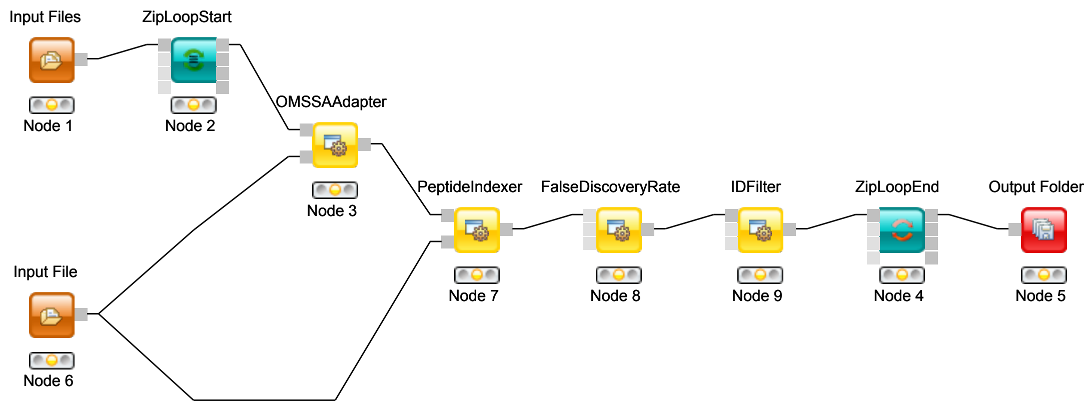


Figure 6: OMSSA ID pipeline including FDR filtering.

- **FeatureFinderCentroided** finds and quantifies peptide ion signals contained in the MS1 data. It reduces the entire signal, i.e., all peaks explained by one and the same peptide ion signal, to a single peak at the maximum of the chromatographic elution profile of the monoisotopic mass trace of this peptide ion and assigns an overall intensity.

- **FeatureFinderCentroided** produces a featureXML file as output, containing only quantitative information of so-far unidentified peptide signals. In order to annotate these with the corresponding ID information, we need the **IDMapper** node.

- Run your pipeline and inspect the results of the **IDMapper** node in TOPPView.

- In order to assess how well the feature finding worked, you can project the features contained in the featureXML file on the raw data contained in the mzML file. In TOPPView choose File › Open file and select the mzML file corresponding to your featureXML file in 🗀 **Example_Data › Labelfree › datasets**. In the dialog that pops up, select Open in › New layer. Zoom in until you see boxes (found features) around the peptide signals in the raw data.

  > Note: The RT range is very narrow. Thus, select the full RT range and zoom only into the m/z dimension by holding down CTRL (CMD on Mac) and repeatedly dragging a narrow box from the very left to the very right.

- You can see which features were annotated with a peptide identification by first selecting the featureXML file in the **Layers** window on the upper right side and then clicking on the icon with the letters A, B and C on the upper icon bar. Now, click on the small triangle next to that icon and select **Peptide identification**.

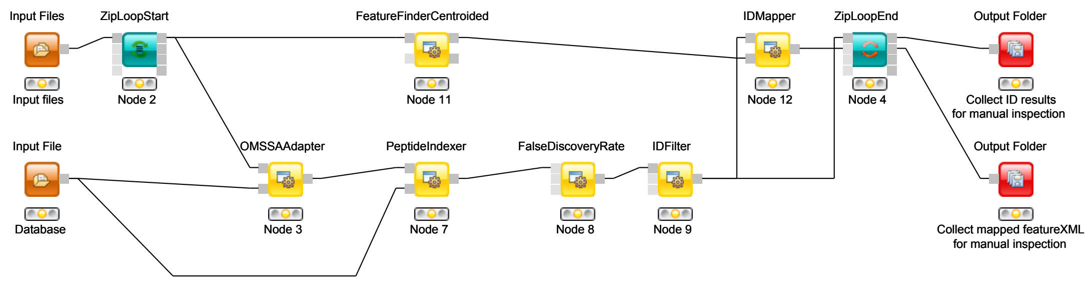


Figure 7: Extended workflow featuring peptide identification and quantification.

3.4 Combining quantitative information across several label-free experiments

So far, we successfully performed peptide identification as well as quantification on individual LC-MS runs. For differential label-free analyses, however, we need to identify and quantify corresponding signals in different experiments and link them together to compare their intensities. Thus, we will now run our pipeline on all three available input files and extend it a bit further, so that it is able to find and link features across several runs.

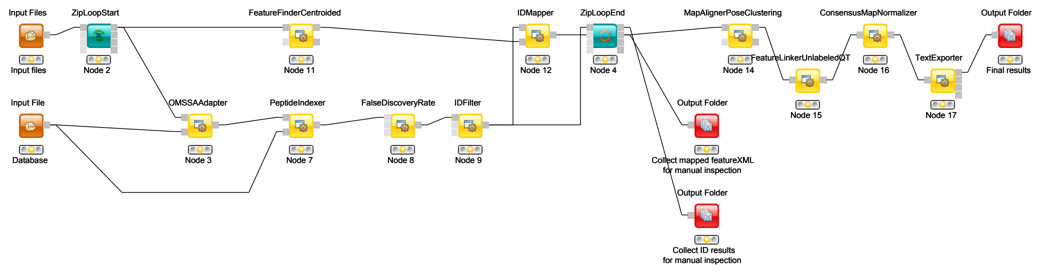


Figure 8: Complete identification and label-free quantification workflow.

- To find features across several maps, we first have to align them to correct for retention time shifts between the different label-free measurements. With the

MapAlignerPoseClustering Community Nodes » OpenMS » Map Alignment, we can align corresponding peptide signals to each other as closely as possible by applying a transformation in the RT dimension.

> Note: **MapAlignerPoseClustering** consumes several featureXML files and its output should still be several featureXML files containing the same features, but with the transformed RT values. In its configuration dialog, make sure that OutputTypes is set to featureXML.

- With the **FeatureLinkerUnlabeledQT** node Community Nodes » OpenMS » Map Alignment, we can then perform the actual linking of corresponding features. Its output is a consensusXML file containing linked groups of corresponding features across the different experiments.
- Since the overall intensities can vary a lot between different measurements (for example, because the amount of injected analytes was different), we apply the **ConsensusMapNormalizer** Community Nodes » OpenMS » Map Alignment as a last processing step. Configure its parameters with setting algorithm_type to **median**. It will then normalize the maps in such a way that the median intensity of all input maps is equal.
- Finally, we export the resulting normalized consensusXML file to a csv format using **TextExporter**. Connect its out port to a new **Output Folder** node.

> Note: You can specify the desired column separation character in the parameter settings (by default, it is set to “ ” (a space)). The output file of **TextExporter** can also be opened with external tools, e.g., Microsoft Excel, for downstream statistical analyses.

3.4.1 Basic data analysis in KNIME

For downstream analysis of the quantification results within the KNIME environment, you can use the **ConsensusTextReader** node Community Nodes » OpenMS » Conversion instead of the **Output Folder** node to convert the output into a KNIME table (indicated by a triangle as output port). After running the node you can view the KNIME table by right clicking on the **ConsensusTextReader** and selecting Consensus Table. Every row in this table corresponds to a so-called consensus feature, i.e., a peptide signal quantified across several runs. The first couple of columns describe the consensus feature as a whole (average RT and m/z across the maps, charge, etc.). The remaining columns describe the exact positions and intensities of the quantified features separately for all input samples (e.g., intensity_0 is the intensity of the feature in the first input file). The last 11 columns contain information on peptide identification.

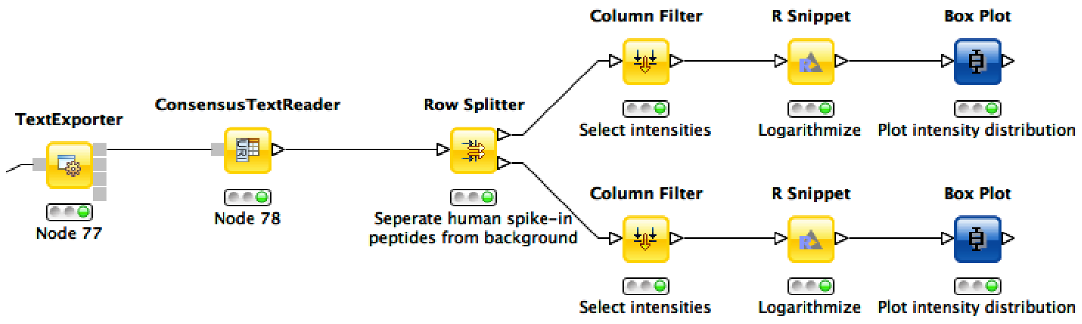


Figure 9: Simple KNIME data analysis example for LFQ.

- Now, let's say we want to plot the log intensity distributions of the human spike-in peptides for all input files. In addition, we will plot the intensity distributions of the background peptides.
- As shown in Fig. 9, add a **Row Splitter** node Data Manipulation › Row › Filter after **ConsensusTextR**
  Double-click it to configure. The human spike-in peptides have accessions starting with "hum". Thus, set the column to test to accessions, select pattern matching as matching criterion, enter hum* into the corresponding text field, and check the contains wild cards box. Press OK and execute the node.
- **Row Splitter** produces two output tables: the first one contains all rows from the input table matching the filter criterion, and the second table contains all other rows. You can inspect the tables by right-clicking and selecting Filtered and Filtered Out. The former table should now contain only peptides with a human accession, whereas the latter should contain all remaining peptides (including unidentified ones).

- Now, since we only want to plot intensities, we can add a **Column Filter** node Data Manipulation > Column > Filter, connect its input port to the Filtered output port of the **Row Filter**, and open its configuration dialog. We could either manually select the columns we want to keep, or, more elegantly, select Wildcard/Regex Selection and enter intensity_? as the pattern. KNIME will interactively show you which columns your pattern applies to while you're typing.

- Since we want to plot log intensities, we will now compute the log of all intensity values in our table. The easiest way to do this in KNIME is a small piece of R code. Add an **R Snippet** node R after **Column Filter** and double-click to configure. In the R Script text editor, enter the following code:

```r
x <- knime.in        # store copy of input table in x
x[x == 0] <- NA      # replace all zeros by NA (= missing value)
x <- log10(x)        # compute log of all values
knime.out <- x       # write result to output table
```

- Now we are ready to plot! Add a **Box Plot** node Data Views after the **R Snippet** node, execute it, and open its view. If everything went well, you should see a significant fold change of your human peptide intensities across the three runs.

- In order to verify that the concentration of background peptides is constant in all three runs, you can just copy and paste the three nodes after **Row Splitter** and connect the duplicated **Column Filter** to the second output port (Filtered Out) of **Row Splitter**, as shown in Fig. 9. Execute and open the view of your second **Box Plot**.

- That's it! You have constructed an entire identification and label-free quantification workflow including a simple data analysis using KNIME!

> Note: For further inspiration you might want to take a look at the more advanced KNIME data analysis examples in the metabolomics tutorial.

4 Metabolomics

4.1 Introduction

Quantitation and identification of chemical compounds are basic tasks in metabolomic studies. In this tutorial session we construct a UPLC-MS based, label-free quantitation and identification workflow. Following quantitation and identification we then perform statistical downstream analysis to detect quantitation values that differ significantly between two conditions. This approach can, for example, be used to detect biomarkers. Here, we use two spike-in conditions of a dilution series (0.5 mg/l and 10.0 mg/l, male blood background, measured in triplicates) comprising seven isotopically labeled compounds. The goal of this tutorial is to detect and quantify these differential spike-in compounds against the complex background.

4.2 Quantifying metabolites across several experiments

For the metabolite quantification we choose an approach similar to the one used for peptides, but this time based on the OpenMS **FeatureFinderMetabo** method. This feature finder again collects peak picked data into individual mass traces. The reason why we need a different feature finder for metabolites lies in the step after trace detection: the aggregation of isotopic traces belonging to the same compound ion into the same feature. Compared to peptides with their averagine model, small molecules have very different isotopic distributions. To group small molecule mass traces correctly, an aggregation model tailored to small molecules is thus needed.

- Create a new workflow called for instance ”Metabolomics”.
- Add a **Input Files** node and configure it with all mzML files from 🗀 **Example_Data ▸ Metabolomics ▸ datasets**.
- Add a **ZipLoopStart** node and connect the **Input Files** node to the first port of the **ZipLoopStart** node.
- Add a **FeatureFinderMetabo** node (from Community Nodes ❯ OpenMS ❯ Quantitation and connect the first output port of the **ZipLoopStart** to the **FeatureFinderMetabo**.

- For an optimal result adjust the following settings. Please note that some of these are advanced parameters.

parameter	value
algorithm → common → chrom_fwhm	8.0
algorithm → mtd → trace_termination_criterion	sample_rate
algorithm → mtd → min_trace_length	3.0
algorithm → mtd → max_trace_length	600.0
algorithm → epd → width_filtering	off

- Add a **ZipLoopEnd** node and connect the output of the **FeatureFinderMetabo** to the first port of the **ZipLoopEnd** node.

To facilitate the collection of features corresponding to the same compound ion across different samples, an alignment of the samples' feature maps along retention time is often helpful. In addition to local, small-scale elution differences, one can often see constant retention time shifts across large sections between samples. We can use linear transformations to correct for these large scale retention differences. This brings the majority of corresponding compound ions close to each other. Finding the correct corresponding ions is then faster and easier, as we don't have to search as far around individual features.

- After the **ZipLoopEnd** node add a **MapAlignerPoseClustering** node (Community Nodes > OpenMS > Map Alignment), set its Output Type to featureXML, and adjust the following settings

parameter	value
algorithm → max_num_peaks_considered	-1
algorithm → superimposer → mz_pair_max_distance	0.005
algorithm → superimposer → num_used_points	10000
algorithm → pairfinder → distance_RT → max_difference	20.0
algorithm → pairfinder → distance_MZ → max_difference	20.0
algorithm → pairfinder → distance_MZ → unit	ppm

The next step after retention time correction is the grouping of corresponding features in multiple samples. In contrast to the previous alignment, we assume no linear relations of features across samples. The used method is tolerant against local swaps in elution order.

- After the **MapAlignerPoseClustering** add a **FeatureLinkerUnlabeledQT** (Community Nodes > OpenMS > Map Alignment) and adjust the following settings

parameter	value
algorithm → distance_RT → max_difference	40.0
algorithm → distance_MZ → max_difference	20.0
algorithm → distance_MZ → unit	ppm

- After the **FeatureLinkerUnlabeledQT** add a **TextExporter** node (Community Nodes > OpenMS > File Handling).
- Add an **Output Folder** node and configure it with an output directory where you want to store the resulting files.
- Run the pipeline and inspect the output.

You should find a single, tab-separated file containing the information on where metabolites were found and with which intensities. You can also add **Output Folder** nodes at different stages of the workflow and inspect the intermediate results (e.g., identified metabolite features for each input map). The complete workflow can be seen in Figure 10. In the following section we will try to identify those metabolites.

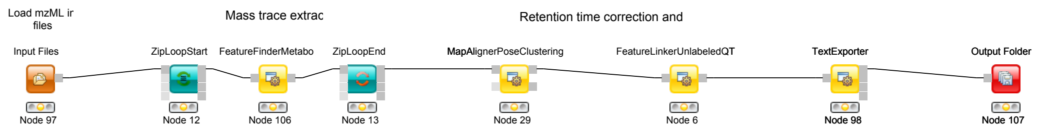


Figure 10: Label-free quantification workflow for metabolites

4.3 Identifying metabolites in LC-MS/MS samples

At the current state we found several metabolites in the individual maps but so far don't know what they are. To identify metabolites OpenMS provides multiple tools, including search by mass: the **AccurateMassSearch** node searches observed masses against the Human Metabolome Database (HMDB)[8, 9, 10]. We start with the workflow from the previous section (see Figure 10).

- Add a **FileConverter** node and connect the output of the **FeatureLinkerUnlabeledQT** to the incoming port.
- Open the Configure dialog of the **FileConverter** and select the tab "OutputTypes". In the drop down list for FileConverter.1.out select "featureXML".
- Add an **AccurateMassSearch** node and connect the output of the **FileConverter** to the first port of the **AccurateMassSearch**.
- Add four **Input File** nodes and configure them with the following files
  - 🗀 **Example_Data ▸ Metabolomics ▸ databases ▸ PositiveAdducts.tsv**
    This file specifies the list of adducts that are considered in the positive mode. Each line contains the formula and charge of an adduct separated by a semi-colon (e.g. M+H;1+). The mass of the adduct is calculated automatically.
  - 🗀 **Example_Data ▸ Metabolomics ▸ databases ▸ NegativeAdducts.tsv**
    This file specifies the list of adducts that are considered in the negative mode analogous to the positive mode.
  - 🗀 **Example_Data ▸ Metabolomics ▸ databases ▸ HMDBMappingFile.tsv**
    This file contains information from a metabolite database in this case from HMDB. It has three (or more) tab-separated columns: mass, formula, and identifier(s). This allows for an efficient search by mass.
  - 🗀 **Example_Data ▸ Metabolomics ▸ databases ▸ HMDB2StructMapping.tsv**
    This file contains additional information about the identifiers in the mapping file. It has four tab-separated columns that contain the identifier, name, SMILES, and INCHI. These will be included in the result file. The identifiers in this file must match the identifiers in the HMDBMappingFile.tsv.

- In the same order as they are given above connect them to the remaining input ports of the **AccurateMassSearch** node.
- Add an **Output Folder** node and connect the first output port of the **AccurateMassSearch** node to the **Output Folder**.

The result of the **AccurateMassSearch** node is in the mzTab format [11] so you can easily open it in a text editor or import it into Excel or KNIME, which we will do in the next section. The complete workflow from this section is shown in Figure 11.

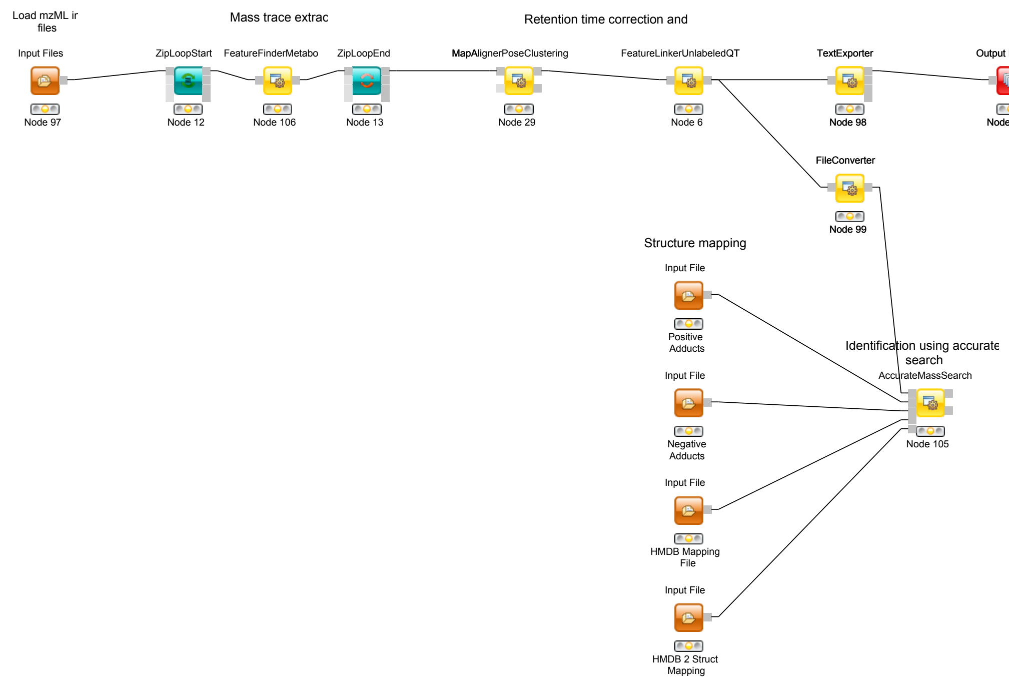


Figure 11: Label-free quantification and identification workflow for metabolites

4.4 Convert your data into a KNIME table

The result from the **TextExporter** node as well as the result from the **AccurateMassSearch** node are files while standard KNIME nodes display and processes only KNIME tables. To convert these files into KNIME tables we need two different nodes. For the **AccurateMassSearch** results we use the **MzTabReader** node (Community Nodes > OpenMS > Conversion > mzTab), for the result of the **TextExporter** we use the **ConsensusTextReader** (Community Nodes > OpenMS > Conversion).

When executed, both nodes will import the OpenMS files and provide access to the data as KNIME tables. You can now easily combine both tables using the **Joiner** node (Data Manipulation > Column > Split & Combine) and configuring it to match the m/z and retention time values of the respective tables. The full workflow is shown in Figure 12.

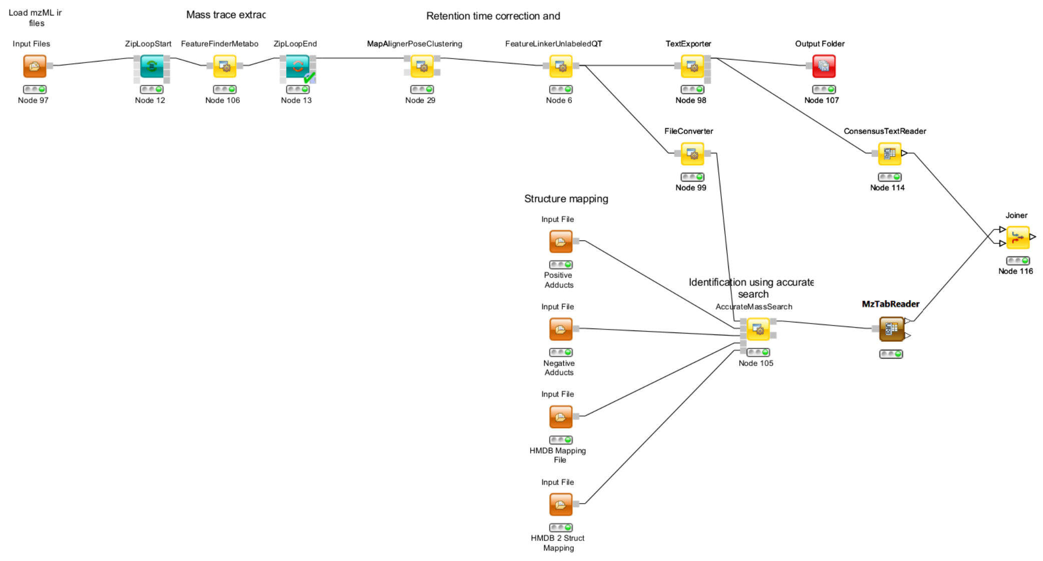


Figure 12: Label-free quantification and identification workflow for metabolites that loads the results into KNIME and joins the tables.

4.4.1 Bonus task: Visualizing data

Now that you have your data in KNIME you should try to get a feeling for the capabilities of KNIME.

Task

☑ Check out the **Molecule Type Cast** node (Chemistry > Translators) together with subsequent cheminformatics nodes (e.g. **RDKit From Molecule** (Community Nodes > RDKit > Converters)) to render the structural formula contained in the result table.

Task

✓ Have a look at the **Column Filter** node to reduce the table to the interesting columns, e.g., only the Ids, chemical formula, and intensities.

Task

✓ Try to compute and visualize the m/z and retention time error of the different elements of the consensus features.

4.5 Downstream data analysis and reporting

In this part of the metabolomics session we take a look at more advanced downstream analysis and the use of the statistical programming language R. As laid out in the introduction we try to detect a set of spike-in compounds against a complex blood background. As there are many ways to perform this type of analysis we provide a complete workflow.

Task

✓ Import the workflow from **Workflows ▸ metabolite_ID.zip** in KNIME: File ▸ Import KNIME Workflow...

The section below will guide you in your understanding of the different parts of the workflow. Once you understood the workflow you should play around and be creative. Maybe create a novel visualization in KNIME or R? Do some more elaborate statistical analysis? Feel free to experiment and show us your results if you like. Note that some basic R knowledge is required to fully understand the processing in **R Snippet** nodes.

4.5.1 Data preparation ID

This part is analogous to what you did for the simple metabolomics pipeline.

4.5.2 Data preparation Quant

The first part is identical to what you did for the simple metabolomics pipeline. Additionally, we convert zero intensities into NA values and remove all rows that contain at least one NA value from the analysis. We do this using a very simple **R Snippet** and subsequent **Missing Value filter** node.

Task

✓ Inspect the **R Snippet** by double-clicking on it. The KNIME table that is passed to an **R Snippet** node is available in R as a data.frame named knime.in. The result of this node will be read from the data.frame knime.out after the script finishes. Try to understand and evaluate parts of the script (Eval Selection). In this dialog you can also print intermediary results using for example the R command head() or cat() to the Console pane.

4.5.3 Statistical analysis

After we linked features across all maps, we want to identify features that are significantly deregulated between the two conditions. We will first scale and normalize the data, then perform a t-test, and finally correct the obtained p-values for multiple testing using Benjamini-Hochberg. All of these steps will be carried out in individual **R Snippet** nodes.

- Double-click on the first **R Snippet** node labeled ”log scaling” to open the **R Snippet** dialog. In the middle you will see a short R script that performs the log scaling. To perform the log scaling we use a so-called regular expression (grepl) to select all columns containing the intensities in the six maps and take the log_2 logarithm.
- The output of the log scaling node is also used to draw a boxplot that can be used to examine the structure of the data. Since we only want to plot the intensities in the different maps (and not m/z or rt) we first use a **Column Filter** node to keep only the columns that contain the intensities. We connect the resulting table to a **Box Plot** node which draws one box for every column in the input table. Right-click and select View: Box Plot.
- The median normalization is performed in a similar way to the log scaling. First we calculate the median intensity for each intensity column, then we subtract the median from every intensity.

- Open the **Box Plot** connected to the normalization node and compare it to the box plot connected to the log scaling node to examine the effect of the median normalization.
- To perform the t-test we defined the two groups we want to compare. Then we call the t-test for every consensus feature unless it has missing values. Finally we save the p-values and fold-changes in two new columns named p-value and FC.
- The **Numeric Row Splitter** is used to filter less interesting parts of the data. In this case we only keep columns where the fold-change is $\geq$ 2.
- We adjust the p-values for multiple testing using Benjamini-Hochberg and keep all consensus features with a q-value $\leq$ 0.01 (i.e. we target a false-discovery rate of 1%).

4.5.4 Interactive visualization

KNIME supports multiple nodes for interactive visualization with interrelated output. The nodes used in this part of the workflow exemplify this concept. They further demonstrate how figures with data dependent customization can be easily realized using basic KNIME nodes. Several simple operations are concatenated in order to enable an interactive volcano plot.

- We first log-transform fold changes and p-values in the **R Snippet** node. We then append columns noting interesting features (concerning fold change and p-value).
- With this information, we can use various Manager nodes (Data Views > Property) to emphasize interesting data points. The configuration dialogs allow us to select columns to change color, shape or size of data points dependent on the column values.
- The **Scatter Plot** node (Data Views) enables interactive visualization of the logarithmized values as a volcano plot: the log-transformed values can be chosen in the 'Column Selection' tab of the plot view. Data points can be selected in the plot and HiLited via the menu option. HiLiteing transfers to all other interactive nodes connected to the same data table. In our case, selection and HiLiteing will also occur in the **Interactive Table** node (Data Views).

- Output of the interactive table can then be filtered via the HiLite menu tab. For example, we could restrict shown rows to points HiLited in the volcano plot.

Task

✓ Inspect the nodes of this section. Customize your visualization and possibly try to visualize other aspects of your data.

4.5.5 Advanced visualization

R Dependencies: This section requires that the R packages ggplot2 and ggbiplot are both installed. ggplot2 is part of the KNIME R Statistics Integration (Windows Binaries) which should already be installed via the full KNIME installer, ggbiplot however is not. In case that you use an R installation where one or both of them are not yet installed, add an **R Snippet** node and double-click to configure. In the R Script text editor, enter the following code:

```
#Include the next line if you also have to install ggplot2:
install.packages("ggplot2")
#Include the following lines to install ggbiplot:
install.packages("devtools")
library(devtools)
install_github("vqv/ggbiplot")
```

Press Eval script to execute the script.

Even though the basic capabilities for (interactive) plots in KNIME are valuable for initial data exploration, professional looking depiction of analysis results often relies on dedicated plotting libraries. The statistics language R supports the addition of a large variety of packages, including packages providing extensive plotting capabilities. This part of the workflow shows how to use R nodes in KNIME to visualize more advanced figures. Specifically, we make use of different plotting packages to realize heatmaps.

- The used **RView (Table)** nodes combine the possibility to write R snippet code with visualization capabilities inside KNIME. Resulting images can be looked at in the output RView, or saved via the **Image Port Writer** node.

- The heatmap nodes make use of the gplots libary, which is by default part of the R Windows binaries for the KNIME 3.1.1 full installation. We again use regular expressions to extract all measured intensity columns for plotting. For clarity, feature names are only shown in the heatmap after filtering by fold changes.

4.5.6 Data preparation for Reporting

Following the identification, quantification and statistical analysis our data is merged and formatted for reporting. First we want to discard our normalized and logarithmized intensity values in favor of the original ones. To this end we first remove the intensity columns (**Column Filter**) and add the original intensities back (**Joiner**). Note that we use an Inner Join [1]. Combining ID and Quantification table into a single table is again achieved using a **Joiner** node.

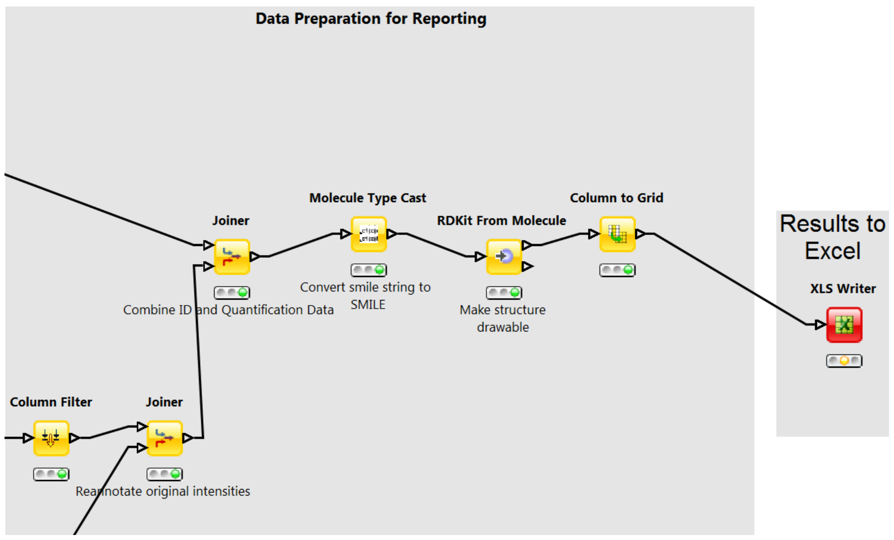


Figure 13: Data preparation for reporting

Question

What happens if we use an Left Outer Join, Right Outer Join or Full Outer Join instead of the Inner Join?

[1] Inner Join is a technical term that describes how database tables are merged.

Task

✓ Inspect the output of the join operation after the Molecule Type Cast and RDKit molecular structure generation.

While all relevant information is now contained in our table the presentation could be improved. Currently, we have several rows corresponding to a single consensus feature (=linked feature) but with different, alternative identifications. It would be more convenient to have only one row for each consensus feature with all accurate mass identifications added as additional columns. To this end, we use the **Column to Grid** node that flattens several rows with the same consensus number into a single one. Note that we have to specify the maximum number of columns in the grid so we set this to a large value (e.g. 100). We finally export the data to an Excel file (**XLS Writer**).

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