Mascot DIA LFQ tutorial

Version: 2026-09-21
Author: Matrix Science Ltd

Requirements

Mascot Server: version 3.2

Mascot Distiller: versions 3.0

Supported instrument types:

Thermo: .raw files from any instrument using the Thermo Xcalibur™ acquisition software including the Orbitrap Astral

Sciex: .wiff files from QTOF systems (e.g. ZenoTOF 7600), QTRAP systems (e.g. QTRAP 6500+), Triple Quad systems (e.g. Triple Quad 7500), TripleTOF systems (e.g. TripleTOF 6500+), X-Series TOF (e.g. X500B QTOF)

Contents

Mascot DIA LFQ tutorial 1

Requirements 1

Introduction 2

Requirements 2

1. Download the Thermo Orbitrap QE HF-X raw files from PRIDE 2

2. Add the Human, Yeast and *E.coli* UniProt proteomes to your Mascot Server 3

3. Ensure you have a suitable label-free method setup for DIA data 3

4. Configure Mascot Distiller to connect to your Mascot Server 3

5. Create a new multifile DIA project in Mascot Distiller 4

6. Set the project default search settings 6

7. Run peak-picking and search the data 8

8. Set up the LFQ samples 10

9. Review the quantitation results 12

Introduction

This tutorial is intended as an introduction to processing, searching and quantifying label-free DIA datasets with Mascot DIA. Mascot DIA consists of Mascot Distiller 3.0 and Mascot Server 3.2. The example will use a subset of Thermo Orbitrap QE HF-X raw files from PRIDE project PXD028735, but the principles apply to processing data from Sciex instruments as well.

PRIDE project PXD28735 contains a reference dataset processed on multiple different instrument types and in DDA and DIA acquisition modes as outlined in the associated publication (*et al.* [1]). The dataset consists of mixtures of human, yeast and *E.coli* proteins in known concentrations to simulate two different conditions. Each condition has 3 simulated biological replicates and each of those has 3 technical replicates.

The expected log2 ratios for each of species between the two samples are:

A/B	Expected Log2 ratio
Human	0.00
Yeast	1.00
E.coli	-2.00

In the tutorial, we'll take two of the files to reprocess, search and quantify to demonstrate the Mascot DIA workflow.

When processing the data, Mascot Distiller will take the isolation windows used directly from the raw files. There is no requirement for the isolation windows used to be fixed, variable or overlapping – all these schemes are supported.

Requirements

Mascot Server: version 3.2

Mascot Daemon: version 3.2

Mascot Distiller: version 3.0

1. Download the Thermo Orbitrap QE HF-X raw files from PRIDE

Download the following files from the PRIDE repository accession PXD028735 to your local workstation with Mascot Distiller 3.0 installed:

LFQ_Orbitrap_AIF_Condition_A_Sample_Alpha_02.raw
LFQ_Orbitrap_AIF_Condition_B_Sample_Alpha_02.raw

2. Add the Human, Yeast and *E.coli* UniProt proteomes to your Mascot Server

You can find instructions on how to add UniProt proteomes to your Mascot Server here. The required proteome ids are as follows:

Human	UP000005640
Yeast	UP000002311
E.coli (strain K12)	UP000000625

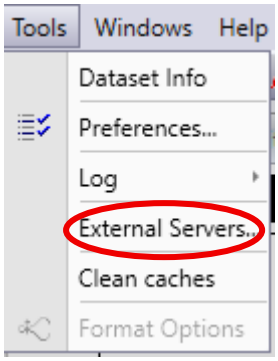
Or you can enable the predefined proteomes UP2311_S_cerevisiae, UP5640_H_sapiens, UP625_E_coli_K12 in Database Manager on Mascot.

3. Ensure you have a suitable label-free method setup for DIA data

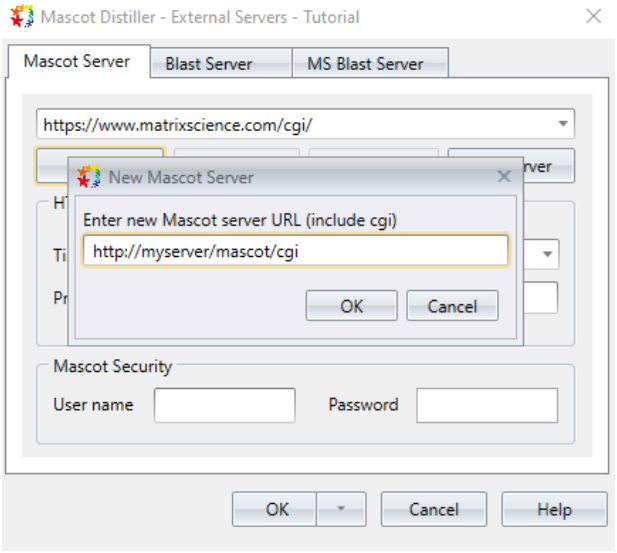
If your Mascot Server 3.2 installation is a fresh installation, it should have a supplied quantitation method called "DIA Label-free [MD]" which will be suitable. This has a reduced number of scans and lower time alignment offset compared with the standard "Label-free [MD]" method. If the method is not present, please contact us and we will send you the required method and instructions on how to install it on your Mascot Server.

4. Configure Mascot Distiller to connect to your Mascot Server

If this is the first time you've used Mascot Distiller, by default it will be connecting to our public website. If you've not already configured Mascot Distiller to use your Mascot Server, open the Mascot Distiller GUI and open Tools->External Servers…

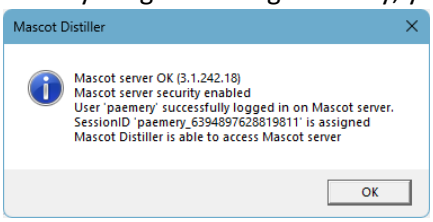


Click “Add URL” on the Mascot Server tab of the dialog that opens and enter the URL of your Mascot Server including the cgi directory as shown below:



Click “OK” on the “New Mascot Server” dialog and then “Test Server” on the main Mascot Server tab. If you have Mascot Security enabled, add your user name and password and the click “Test Server” button.

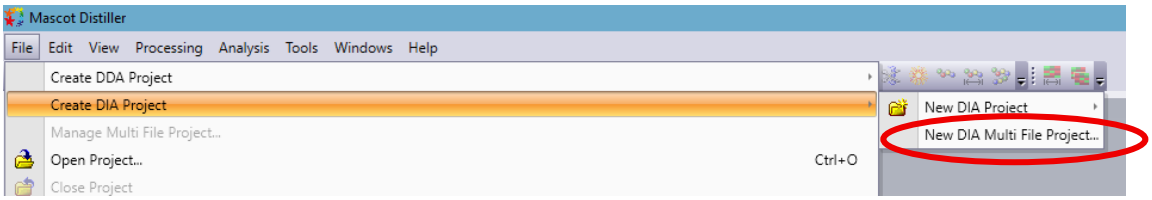
If everything is working correctly, you should see a message like this:



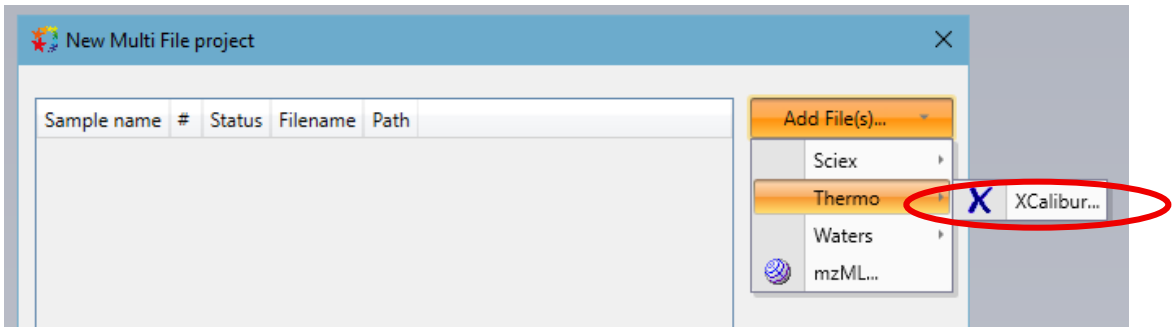
If everything is working correctly, click on the “OK” button of the “External Servers” dialog. This will set the default Mascot Server for all newly created Mascot Distiller project files to your newly added Mascot Server.

5. Create a new multifile DIA project in Mascot Distiller

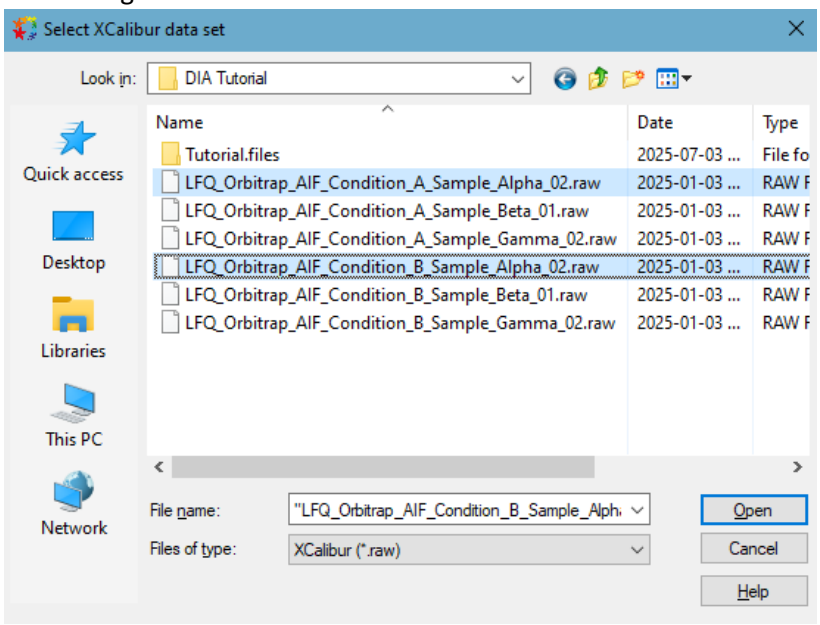
Open Mascot Distiller and open File->Create DIA Project->New DIA Multi File Project



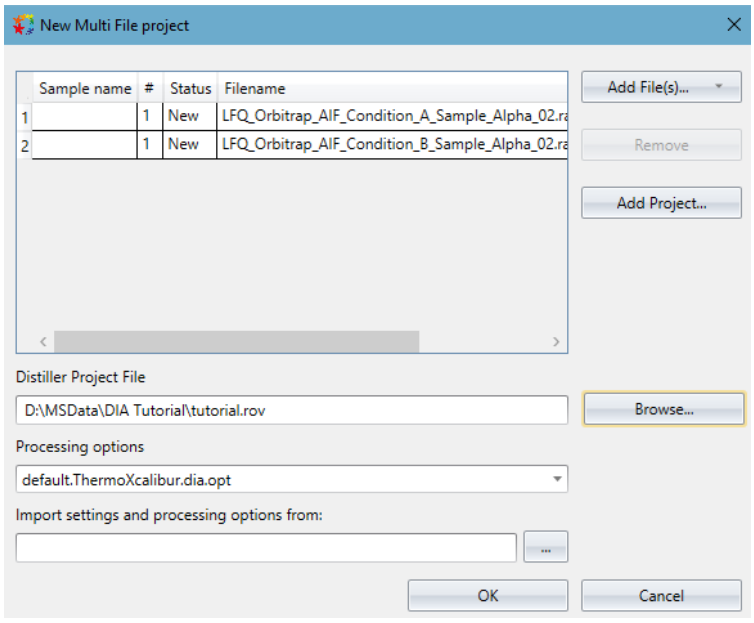
In the dialog that opens, click on ‘Add File(s)…’->Thermo->Xcalibur



Then navigate to and select the two files downloaded from the PRIDE repository, then click “Open”:



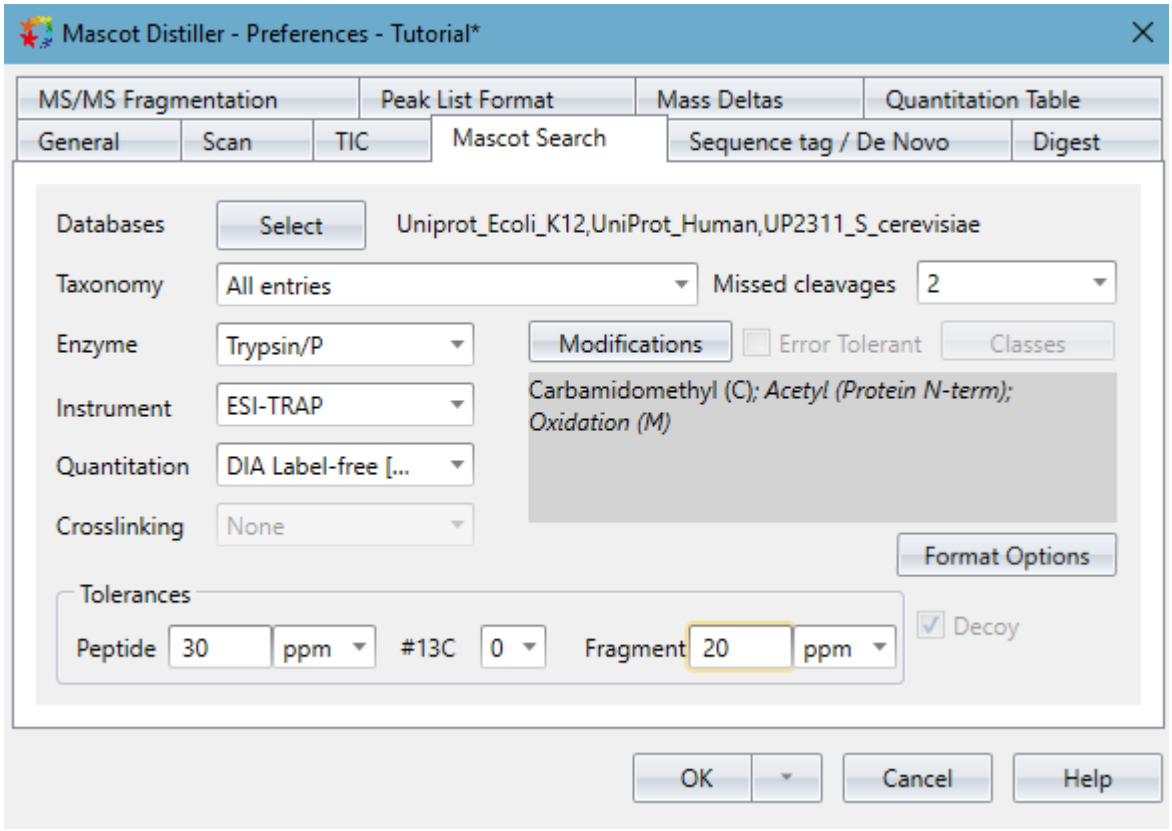
The table in the “New Multi File project” dialog will now populate with the details of the two selected files:



Processing options can be changed here from the drop-down menu. You can change these later, but it will save a few clicks if you select the right ones here. For these data, the default.ThermoXcalibur.dia.opt files are fine so we can keep those. Change the name and path of the master .rov project file using the "Browse…" button if you need to, then click "OK" to create the project.

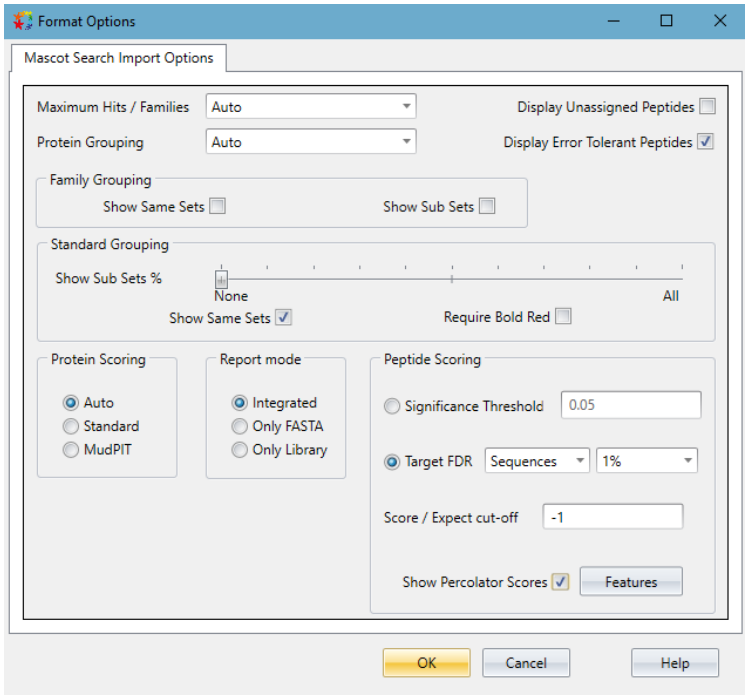
6. Set the project default search settings

Open Tools->Preferences and select the "Mascot Search" tab. Select the following default search settings:

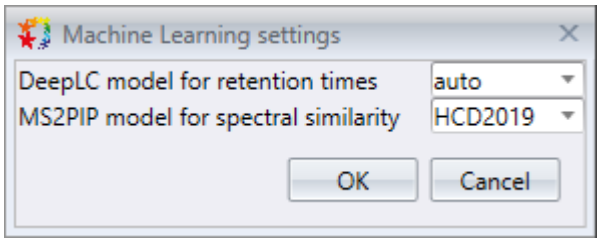


Then click on "Format Options" and check "Show Percolator Scores".

The “Features” button will become enabled:



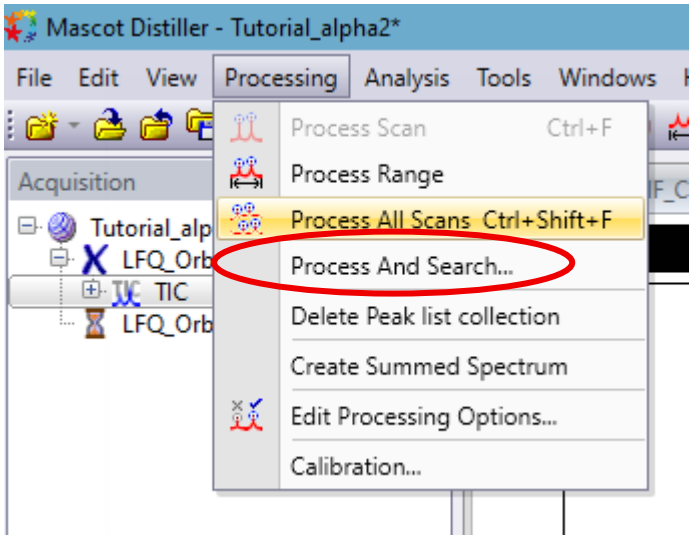
Click on the “Features” button and in the dialog that opens, enable DeepLC by selecting auto from the drop-down, and also a MS2Pip model to use in rescoring the search results. For these data, the HCD2019 model is a good choice.



Close the dialogs by clicking “OK” on each.

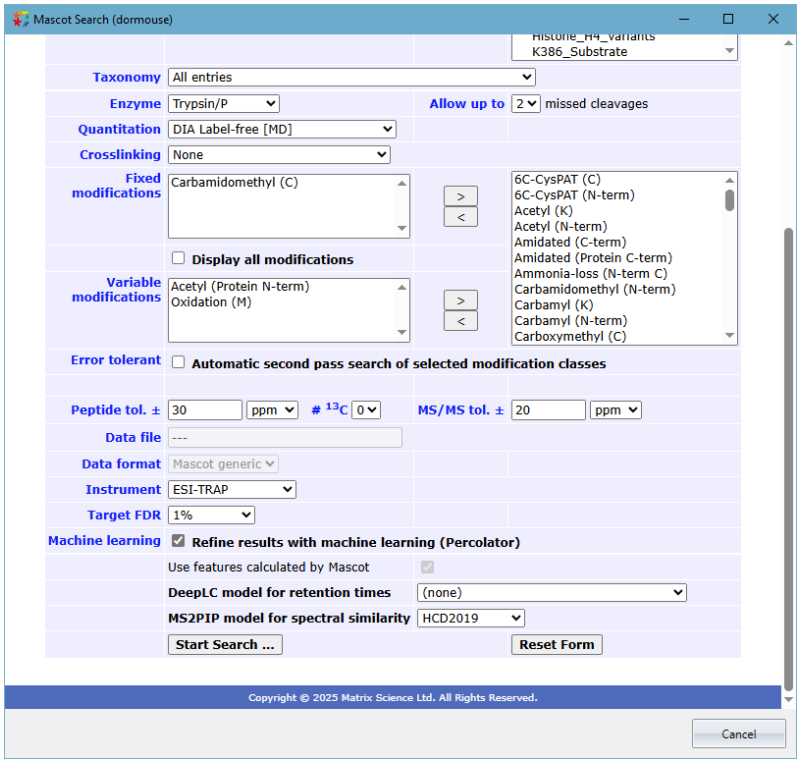
7. Run peak-picking and search the data

In Mascot Distiller, open Processing->Process and Search:

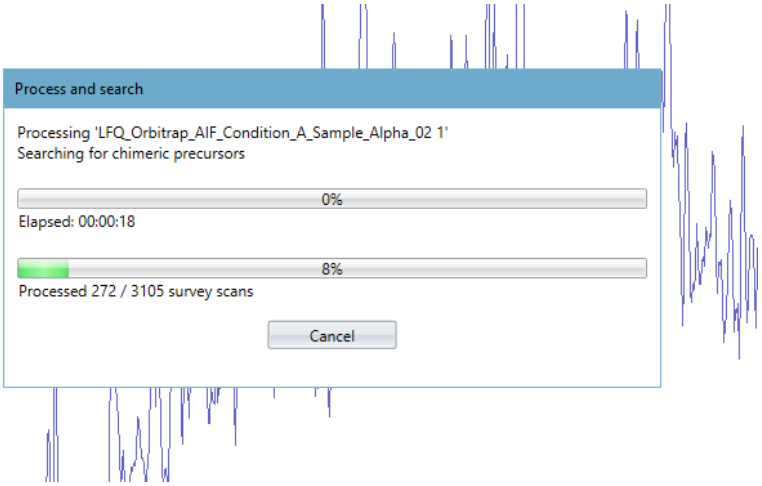


You'll see a warning dialog about higher charge states. Because these data have centroided MS/MS scans and the default.ThermoXcalibur.dia.opt method does not uncentroid them, we won't have charge states for the fragment ions we can ignore this message and click "OK" to close the dialog.

The Mascot Search form will open. This will be populated with the settings you entered in step 6, but this is a chance to change and from the default if you need to. Scroll down to the bottom of the form and click "Start Search …"

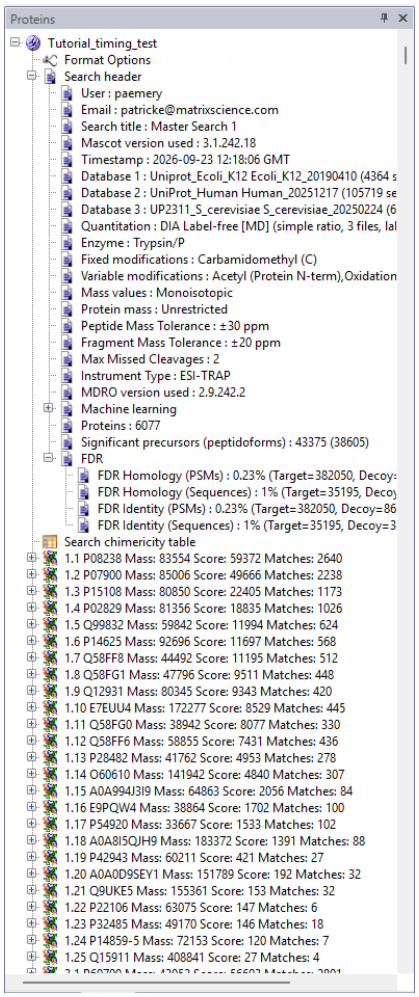


Mascot Distiller will now process the raw data files to peaklists working in series. As peak picking is completed it will queue the generated peaklists files for searching using the specified setting on the associated Mascot Server.



Results will be automatically refined and imported back into Mascot Distiller and the results merged. Because this is a multi-file project with more than one raw file, Mascot Distiller will autosave as it progresses through each step, so once you've clicked "Start Search…" you can leave Mascot Distiller until the process has completed.

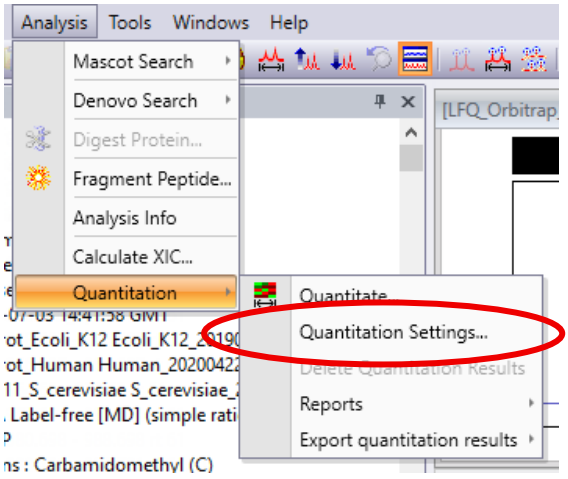
Once all the peak picking and database search tasks have completed, the merged search results will be imported into Mascot Distiller. A new "Proteins" tab will be added to the explorer trees panel on the left of the GUI showing the results:



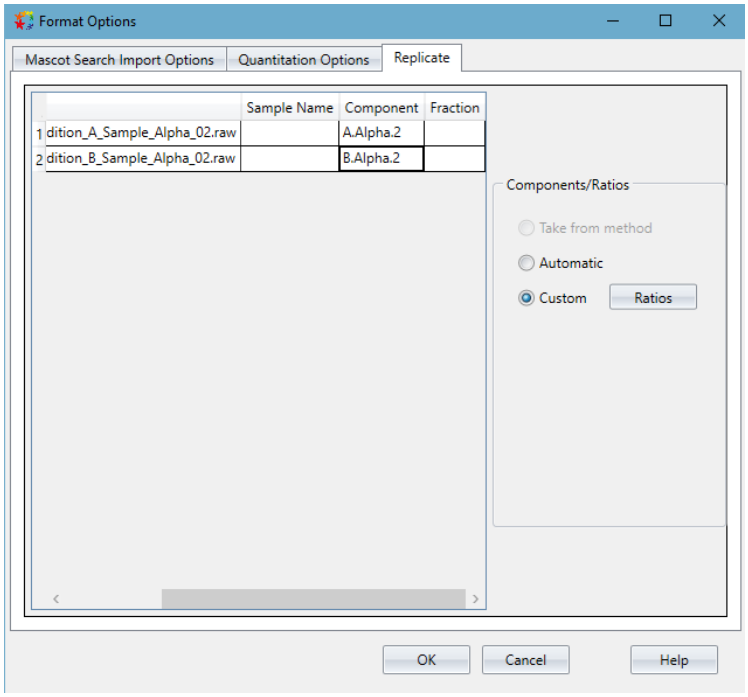
You can expand individual protein hits to view the peptide matches. Right click over a node to bring up a context menu which allows you to carry out specific activities – for example, right click over a protein hit node brings up a context menu which will allow you to view the protein view report from the Mascot Server. Left clicking on a peptide match node will display the sequence ladder over the raw spectrum.

8. Set up the LFQ samples

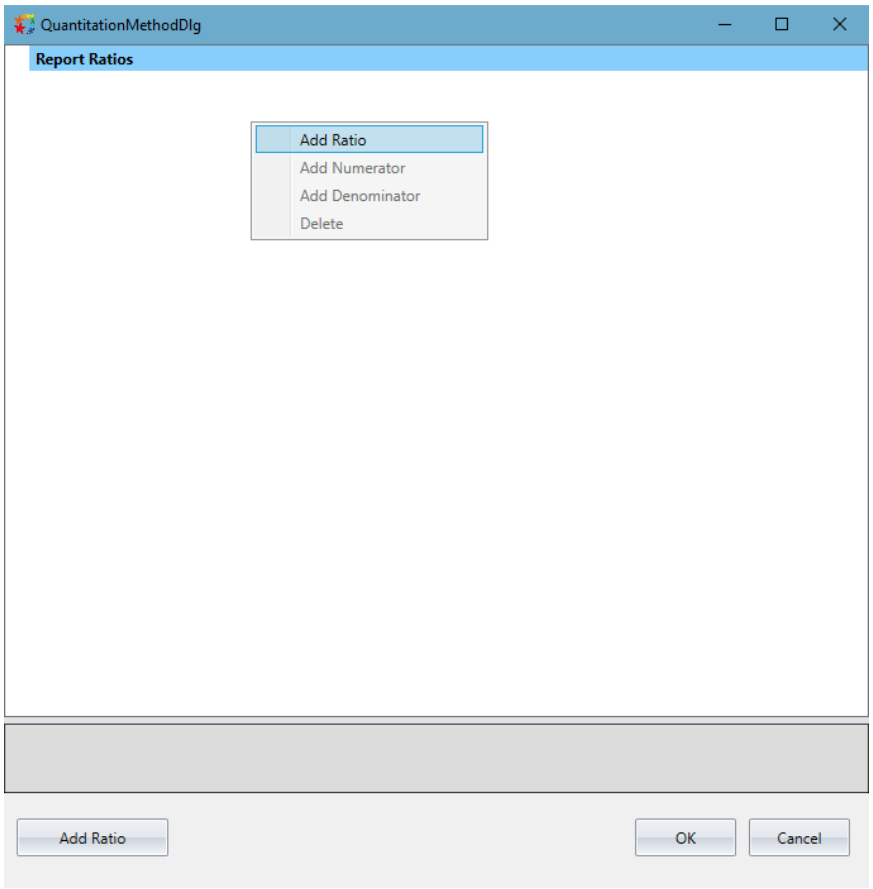
Left click on the "Format Options" node at the top of the proteins tree or Analysis->Quantitation->Quantitation settings



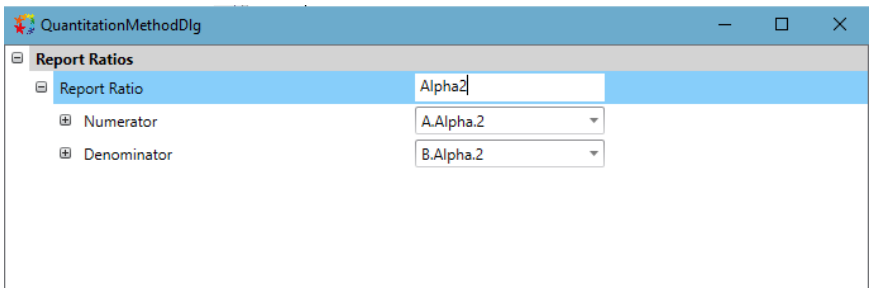
On the dialog that opens, because this dataset is comprised of protein digests from different species mixed at different target ratios, ensure that normalisation is disabled on the "Quantitation Options" tab. Select the "Replicate" tab. By default, each file will have been given a Component name. The first file will have been selected as the "Ref" or reference component and the other files will have been names C1, C2, C3 etc. Mascot Distiller will have automatically added ratios of each of the components over the "Ref" channel. However, in this case we want to give the files more meaningful names and setup custom components called A.Alpha.2 and A.Beta.2 respectively:



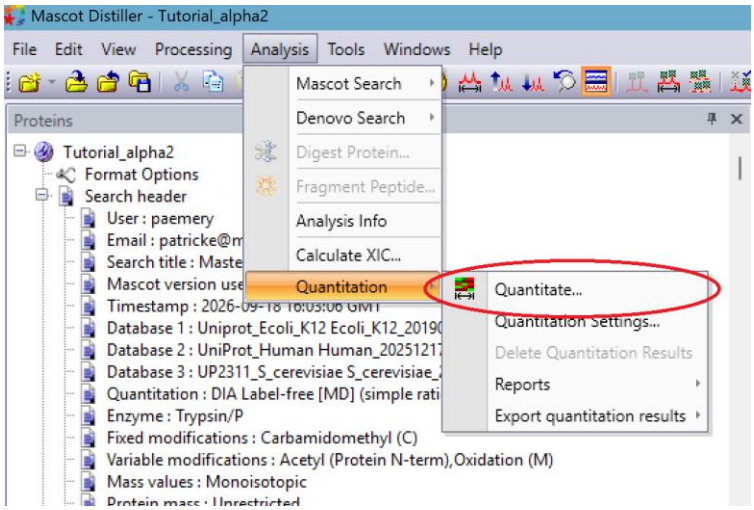
The Sample Name column is filled from the raw file if it has a suitable field set. These data aren't fractionated so you can ignore the "Fraction" column. Ensure the "Custom" ratio button in the "Components/Ratios" frame and click on the "Ratios" button.



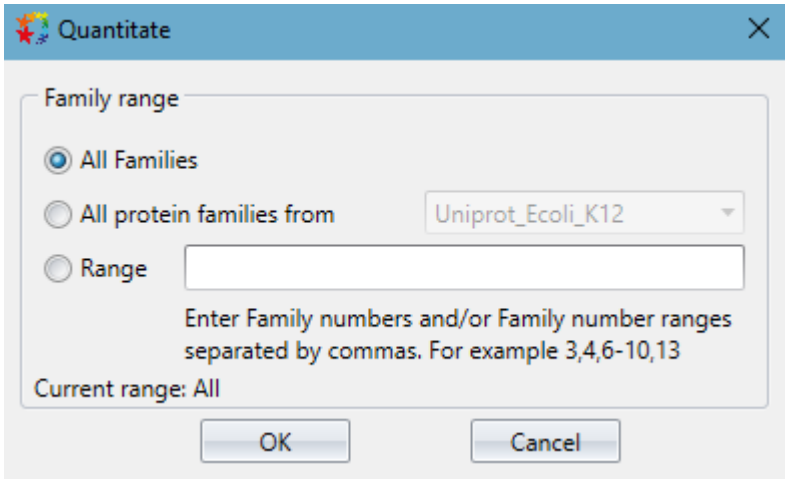
Right click anywhere over the main frame to bring up a context menu as shown above. Click on "Add Ratio" and the select the numerator and denominator for a condition A to condition B pair and give the ratio a descriptive name.



Once you've set up all three ratios, click "OK" on this and the parent dialog and then open "Analysis->Quantitation->Quantitate…":



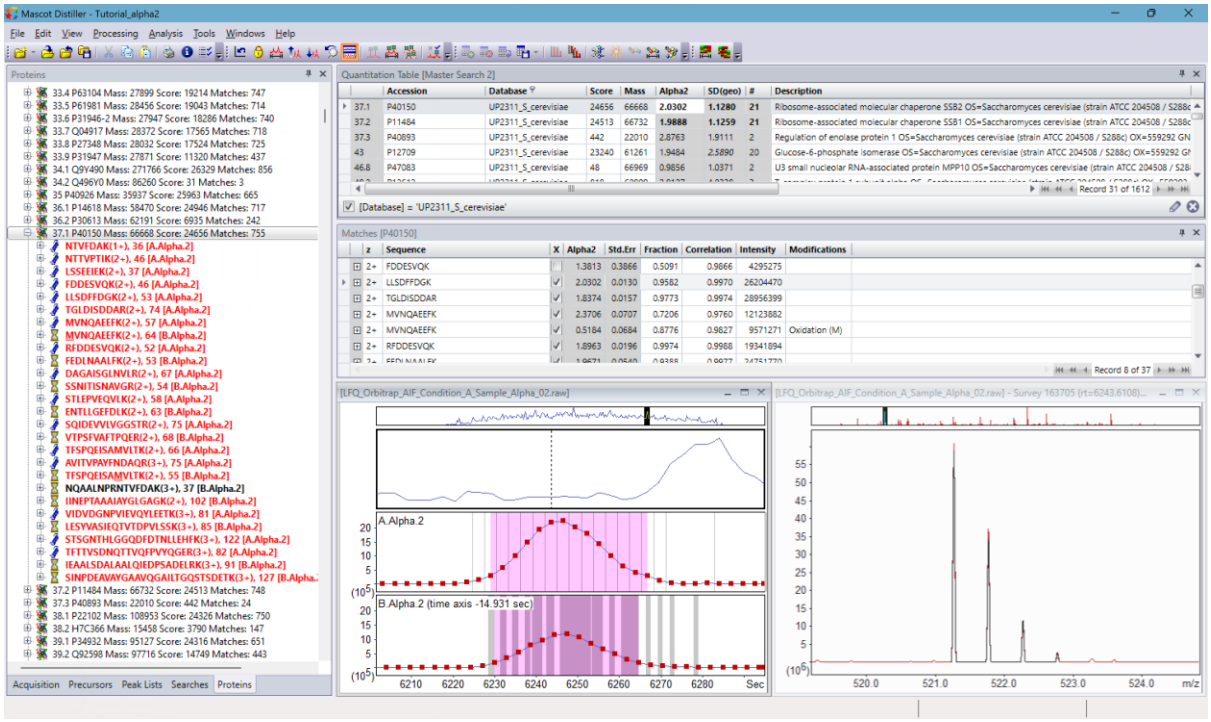
This opens a dialog where we can choose whether to quantitate the protein and peptide hits from all protein families, all protein families with matches from a specified database or a range or protein hits:



Leave this on the default of "All Families" and click "OK" to start Quantitation. Again, because this is a multi-file project, Mascot Distiller will autosave as quantitation progresses so you can leave it running in the background until it completes.

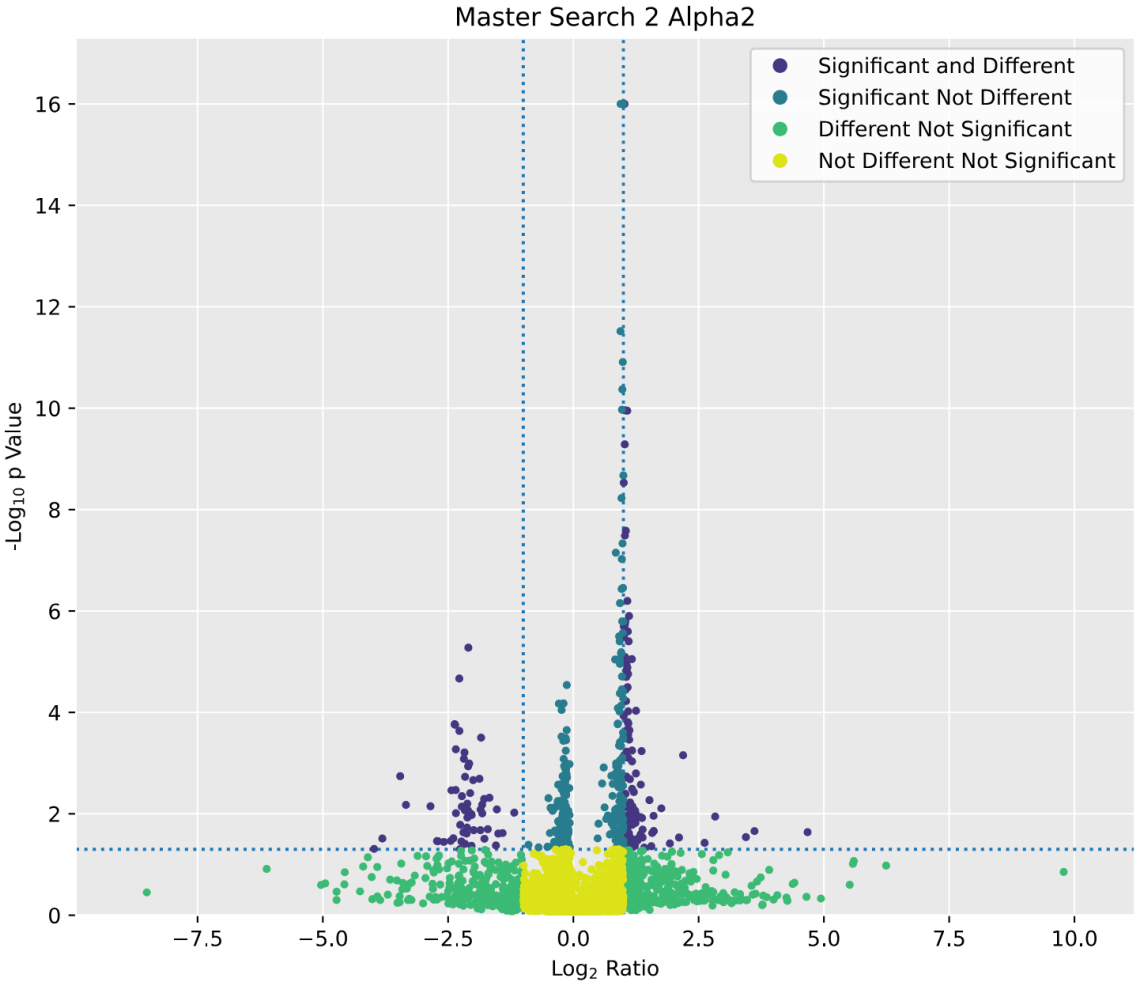
9. Review the quantitation results

Once Mascot Distiller has finished running quantitation, a new table is populated in the main pane of the application window with a table of protein quantitation results. Click on a protein hit row in that table and a child table will be populated with the individual peptide quantitation results. Click on a peptide quantitation row and the application will display the XICs for peptide. In the example below, the table has been filtered for matches to yeast proteins.



The grey bars on the peptide XICs show where we have significant peptide spectrum matches (PSMs) to the selected peptide from the Mascot database searches – in this instance you can see that we have significant PSMs across the entire XIC range for both samples.

Mascot Distiller comes with a number of built it reports, such as a volcano plot report, a PCA report and many others, which you'll find under Analysis->Quantitation->Reports. The screenshot below is an example volcano plot for this dataset, which shows protein ratios clustered at the expected log2 ratios of -2, 0 and +1:



There are also a number of export formats. The most flexible is the “Stats package export” which generates several files for protein and peptide matches and quantification results in a tab separate file format ideal for importing into packages such as R or Perseus from the Max Planck Institute. You find that under “Analysis->Quantitation->Export quantitation results->Save Stats Package Export…”